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# RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES Including

12

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY,  
INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY,  
PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

ADA 028361

ANNUAL PROGRESS REPORT  
1 July 1974 - 30 June 1975

VOLUME I

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REGISTERED  
AUG 11 1976  
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WALTER REED ARMY INSTITUTE OF RESEARCH  
WALTER REED ARMY MEDICAL CENTER  
WASHINGTON, D.C. 20012

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19. PSYCHIATRY  
SURGERY  
VETERINARY MEDICINE

ACCESSION FOR	
RTIS	Public Section <input checked="" type="checkbox"/>
DIC	Ref. Section <input type="checkbox"/>
UNANNOUNCED	<input type="checkbox"/>
JUSTIFICATION	<input type="checkbox"/>
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DISTRIBUTION AVAILABILITY CODES	
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RCS MEDDII - 288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

INCLUDING

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY,  
INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY,  
PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

(Projects, tasks, and work units  
are listed in Table of Contents)

Annual Progress Report  
1 July 1974 - 30 June 1975

Volume I

Walter Reed Army Institute of Research  
Walter Reed Army Medical Center  
Washington, D. C. 20012

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The findings in this report are not to be construed as an official Department of  
the Army position unless so designated by other authorized documents.

## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Researches, National Academy of Sciences - National Research Council.

## SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of investigators are given at the beginning of each report.

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PROJECT 3A161101A91C  
IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00  
In-House Laboratory Independent Research

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF JOURNAL <sup>b</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)30.50	
				DA OB 6494	75 06 30		
3. DATE PREVIOUSLY REPORTED	4. KIND OF SUMMARY	5. SUMMARY EXTENT	6. WORK SECURITY	7. REGRADING <sup>c</sup>	8a. DISSEM INSTR <sup>d</sup>	8b. SPECIFIC DATA CONTRACTOR ACCESS <sup>e</sup>	8. LEVEL OF DISSEM <sup>f</sup>
74 07 01	H. Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODE <sup>g</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	61101A	3A16J101A91C	00	104			
11. TITLE (Proceed with Security Classification Code) <sup>h</sup>							
(U) Basic Studies of Drug Antagonists							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
002000 Bi. logy							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 07		75 06		DA		C. In-House	
17. CONTRACT/GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDENCE		b. FUNDS (In thousands)	
b. NUMBER: NA				FISCAL YEAR		74	
c. TYPE:				CURRENT		0.1	
d. KIND OF AWARD:				75		1.75	
e. CUM. AMT.						58	
21. RESPONSIBLE S&T ORGANIZATION				22. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Division of Medicinal Chemistry			
				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Andromed (see Method))			
NAME: Buescher, COL E. L.				NAME: Kinnamon, Kenneth E., LTC			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2292			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
23. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Loizeaux, Peter S., LTC			
				NAME:			
24. KEYWORDS (Proceed with Security Classification Code) (U) Antagonists; (U) Drug Abuse; (U) Drug Addiction; (U) Amphetamines; (U) Drug Dependence							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRAM (Publish individual paragraphs identified by number. Proceed last of each with Security Classification Code.)							
23. (U) To develop a test system for evaluating the ability of drugs to antagonize amphetamine toxicity and/or dependence; to identify and combat amphetamine toxicosis in military personnel.							
23. (U) Development of an animal model test system capable of rapidly screening large numbers of chemical compounds for their agonistic/antagonistic effect on amphetamines.							
25. (U) 74 07 - 75 06 Two test systems have been developed using the laboratory mouse. A mortality test measures the ability of compounds to prevent or delay amphetamine induced death. An activity test measures by electronic means the ability of compounds to modify total activity. To adopt the activity test system several electronic activity meters with automatic printing counters are required. Both tests are objective in nature and are considered satisfactory for pilot studies. However, in order to be effective for mass screening of drugs, both systems require constant, controlled environmental conditions which are not currently available. The sensitivity of the amphetamine intoxicated animal magnifies the effect of test system variables which, although of only minor significance in other types of drug evaluation, assume great importance in amphetamine testing. The testing environment must be isolated from personnel, radiation, and electrical interference; soundproof and maintained at constant temperature, humidity, and light cycle. Because of the difficulty of establishing physical facilities and constant baseline data, the planned screening program will be terminated in favor of higher priority drug screens. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

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DD FORM 1498  
1 MAR 66

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 104 Basic studies of drug antagonists

Investigators.

Principal: LTC Kenneth E. Kinnamon, VC

Associates: LTC Peter S. Loizeaux, VC; Ms. Marie M. Grenan

The primary work unit objective has been achieved. Tests with end-points based upon mortality and activity have been developed. These are capable of rapidly screening large numbers of drugs for their ability to antagonize amphetamine. Both systems use the laboratory mouse as test animal.

The mortality test measures the ability of drugs to prevent or delay amphetamine induced death. In the Walter Reed strain of mouse 10 mg/kg of amphetamine subcutaneously will produce 60% mortality in 2 to 12 hours and 80% mortality in 48 hours. An increase in amphetamine dose will increase mortality but also produce a more variable death pattern and require a longer time for the animals to expire. Test animals are given 10 mg/kg of amphetamine followed in 5 minutes by the test drug administered intraperitoneally. Mortality is recorded at periodic intervals up to 48 hours and compared with amphetamine controls. Direct observation of the mice for the first 4 to 6 hours of the test provides information regarding modification of amphetamine toxicosis such as salivation, piloerection, mydriasis, and ptosis; and the modification of characteristic behavior patterns such as circling, fighting, trembling, and a rapid reverse movement are observed. Such observation can provide valuable ancillary information, however, quantification is largely subjective and imposes severe restrictions upon the number of tests which can be performed. If observations are limited strictly to mortality, the screen is capable of rapidly examining a large number of compounds. Approximately 150 drugs were evaluated in the mortality test. The majority were known modifiers of amphetamine induced behavior, such as tranquilizers and barbiturates, to determine if a valid test could be established. In addition the basically substituted alkyl-aryl-ethers were identified as a potentially rewarding class of chemicals for future investigations.

Activity monitoring, if performed by electronic means, has also been found to be a valid, objective test for identifying amphetamine antagonists. The influence of external factors on amphetamine intoxicated animals is readily apparent in the activity test and the difficulty of establishing stable environmental controls quickly became apparent. To adopt the test several electronic activity

meters with automatic printing counters are required. Additionally it would be desirable to select equipment with an increased number of sensors in a different spatial configuration than found on the equipment used to develop the test.

Both test systems are considered satisfactory for pilot studies. However, in order to be effective for mass screening of drugs, both systems require constant, controlled environmental conditions which are not currently available. The sensitivity of the amphetamine intoxicated animal magnifies the effect of test system variables which, although of only minor significance in other types of drug evaluation, assume great importance in amphetamine testing. The test environment must be isolated from personnel, radiation, and electrical interference; soundproof and maintained at constant temperature, humidity, and light-cycle. Because of the difficulty of establishing the proper physical facilities and constant baseline data, the planned screening program will be terminated in favor of higher priority drug screens.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL DD-DR&F(AR)8.1F		
3. DATE PREV SUMRY		4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8A. ORG'R INSTR'N	8B. SPECIFIC DATA- CONTRACTOR ACCESS	8C. LEVEL OF SUM A. WORK UNIT
74 07 01		D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. CODES <sup>6</sup>		PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		61101A	3A161101A9TC		00	114		
B. CONTRIBUTING								
C. CONTRIBUTING								
11. TITLE (Precede with Security Classification Code) <sup>7</sup>								
(U) Neurophysiological Control of Antibody Response								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>8</sup>								
002400 Bioengineering 016200 Stress Physiology								
13. START DATE			14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 04			CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)
A. DATES/EFFECTIVE: NA				B. EXPIRATION:		C. PREVIOUS		
D. NUMBER:				E. AMOUNT:		F. FISCAL YEAR		
G. TYPE:				H. CUM. AMT.		75		3
I. KIND OF AWARD:						76		.2
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research				
ADDRESS: Washington, DC 20012				ADDRESS: Division of Neuropsychiatry Washington, DC 20012				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DODR if U.S. Academic Institution)				
NAME: Buescher, COL E. L.				NAME: Spector, N. H., Ph.D.				
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3457				
23. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]				
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS				
				NAME: Koob, G. F., CPT DA				
				NAME: Martin, G. E., Ph.D.				
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Neurophysiology; (U) Neuropsychiatry; (U) Disease; (U) Trauma; (U) Stress; (U) Malaria; (U) Virus; (U) Interferon								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23. (U) The principal objectives of this research are (a) to study the role of the brain in the body's defenses against disease, and (b) to apply this knowledge to the prevention and cure of illness, such as malaria, a disease of military importance.								
24. (U) The disciplines and research techniques of neurophysiology, immunology, and parasitology are used.								
25. (U) 74 07 - 75 06 Experiments with brain lesions and malarial infection were continued. Brain lesioned rats and mice were infected with malarial parasites, or were injected with egg albumin with pertussis vaccine adjuvant, or with sheep red blood cells. Brain lesions and even "sham" surgery alone altered the immunological responses as measured by titers of parasitized red blood cells, interferon, and various types of antibodies. These experiments are being conducted in collaboration with the Division of Communicable Diseases and Immunology, WRAIR. Similar experiments utilizing diencephalic-lesioned mice infected with Newcastle's Disease Virus are in progress in collaboration with Dr. Samuel Baron's laboratory at the National Institutes of Health. Such research contributes to our knowledge of central nervous system mechanisms of resistance to infectious disease. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.								

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1 MAR 68

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 114 Neurophysiological control of antibody response

Investigators.

Principal: N. H. Spector, Ph.D.

Associate: LTC Carter L. Diggs, MC; CPT George F. Koob, MSC;  
MAJ Larry K. Martin, MSC; Gregory E. Martin, Ph.D.

DESCRIPTION.

This research was undertaken to study the role of diencephalic "centers," particularly in the hypothalamus, in the control and regulation of antibody production, immune responses and other mechanisms in the body's defenses against disease and chemical invasion. Particular emphasis was placed upon the study of malarial and virus infections and immunological responses to various foreign proteins.

The resources of the Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research and of the National Institutes of Health were utilized in several collaborative experiments.

PROGRESS.

Anterior Hypothalamic Lesions: Effects on Antibodies to Ovalbumin.

In our previous work, hypothalamic lesions failed to produce significant changes in circulating antibody (Ab) titers or parasitemia levels induced by malaria when the lesion groups were compared to sham-penetrated animals (Spector *et al*, 1974). Therefore, an attempt was made to repeat one experiment (Tyrey and Nalbandov, 1972) among a number of others in which Ab titers were reported to have been lower in immunized lesioned animals.

Following procedures similar to those of Tyrey and Nalbandov, we innoculated male adult rats with an intraperitoneal injection of ovalbumin and pertussis vaccine. The animals were divided into six groups; those with (a) large bilateral anterior hypothalamic and preoptic (AH-PO) lesions, (b) medium-sized bilateral AH-PO lesions, (c) small bilateral AH-PO lesions, (d) sham operations, including bilateral penetration of electrodes to AH-PO region, (e) sham operations without rupture of the meninges, and (f) no operation.

Taken as a whole, the mean Ab titer (modification of hemagglutin-

ation method of Bing et al, 1967) for all lesioned animals was slightly lower ( $\sim 1 \log_2$  unit) than that of the two (e,f) control groups. However, no consistent relationship was observed between size of lesion and consequent Ab level. Penetration alone (no lesions) resulted in an average Ab titer  $4 \log_2$  units lower than the mean Ab titer of the unoperated controls, and also lower than the means of 2 of the lesioned groups.

At this stage in our (and other's) research, results of lesion experiments must be viewed with caution, several different kinds of controls are essential in such experiments, and much more experimental work is needed before we can begin to understand the complex relationships among the hypothalamus, the "limbic system", hormones, and immune responses.

These experiments were done and are continuing with the collaboration of L. T. Cannon and C. L. Diggs of the Department of Immunology at the Walter Reed Army Institute of Research.

#### Hypothalamic Influences Upon Interferon and Antibody Responses To Viral Injections.

Although a fair-sized literature has been accumulating dealing with the effects of central nervous systems lesions upon immune responses, there have been no prior attempts to examine effects of brain lesions upon interferon production and antibody (Ab) titers in response to viral injection.

In collaboration with Samuel Baron and his staff at the National Institute of Allergy and Infectious Diseases, we have continued our studies of reactions to Newcastle's Disease Virus (NDV). Electrolytic lesions were placed in 3 different hypothalamic sites in NIH male Swiss mice. Subsequent to recovery from surgery each of these and 3 groups of control mice were inoculated with approximately  $10^{8.3}$  Herts strain of NDV. The controls consisted of: (a) mice with no operation; (b) mice with operations in which the skull was exposed, but not penetrated; and, (c) mice with electrodes lowered into the side of the hypothalamic lesions, but with no electric current applied, and therefore, no gross lesions.

Interferon titers were measured at 4, 6, and 24 hours after inoculation in all animals, and Ab levels to NDV were determined at 14 days post-inoculation.

There was a positive and statistically significant correlation between interferon titers and eventual Ab levels in individual animals.

### Hypothalamic Lesions and Antibody Responses to Sheep Red Blood Cells.

After recovery from NDV injections, the six experimental groups of NIH strain Swiss mice as well as one "naive" group were inoculated with sheep red blood cells (SRBC's). Subsequently they were examined, at various times, for titers of antibodies to SRBC's. This study was intended to supplement our research on the effects of hypothalamic lesions on Ab responses in the rat to other foreign proteins (e.g. ovalbumin).

Our examination of histological material prepared from the brains of these animals is now complete and further analysis of the data from these and from the NDV studies is in progress.

### Malaria: Twenty-four Hour Continuous Body Temperature Recordings.

A post-parasitemia hyperthermia in the rat has been reported following inoculation with red blood cells parasitized with malaria (Spector, et al, 1975). Since the previous data was based on temperatures recorded once daily, we examined the core temperature of the rat on a continuous 24 hour per day basis throughout the malaria parasitemia. Thus the aberrations in body temperature during a malarial infection and its aftermath could be examined more completely.

Twenty-five male albino rats of the Walter Reed Strain weighing between 350-500 gms were used. A temperature sensor designed in our laboratory was implanted intraperitoneally. Access wires from the thermistor were run under the skin and fastened to the rat's skull. A connector from the skull leading to a multi-channel swivel slip-ring assembly and thence to a chart recorder, permitted the constant monitoring of temperature in the animal. Control temperatures were monitored for at least 7 days after surgery before the parasitized red blood cells were injected intraperitoneally. Then a blood sample was taken every other day to determine the level of parasitemia remaining in each animal.

Although initially some elements of the temperature recording circuits proved to be unstable in the early phases of these experiments, we have collected continuous body temperature records in several animals for up to 50 days following surgery. The data on the circadian and ultradian rhythms of the rat's temperature will be analysed in detail after more such data from additional subjects is accumulated. However, it is already apparent that, in the rat, there were no dramatic short-term peaks of "spiking" malarial fever (as in man) that were missed by the previous technique of recording rectal temperature once a day.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 114 Neurophysiological control of antibody response

Investigators.

Principal: N. H. Spector, Ph.D.

Associate: LTC Carter L. Diggs, MC; CPT George F. Koob, MSC;  
MAJ Larry K. Martin, MSC; Gregory E. Martin, Ph.D.

Literature Cited.

References:

1. Bing, D. H., Weyand, J. G. M., and Stravitsky, A. B.: Hemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. Proc. Soc. Exp. Bio. Med. 124: 1166, 1967.
2. Spector, N. H., Martin, L. K., Diggs, C. L., and Koob, G. F.: Hypothalamic lesions: effects upon malaria and antibody production in rats. Proc. XXVI International Cong. of Physiol. Sci. 11: 393, 1974. (Abstract).
3. Spector, N. H., Martin, L. K., Diggs, C. L., and Koob, G. F.: Malaria: effects upon body temperature in rats with and without hypothalamic lesions. Fed. Proc., 1975. (Abstract).
4. Tyrey, L. and Nalbanbov, A. V.: Influence of anterior hypothalamic lesions on circulating antibody titers in the rat. Amer. J. Physiol. 222: 179, 1972.

Publications.

1. Spector, N. H., Cannon, L. T., Diggs, C. L., Morrison, J. E., and Koob, G. F.: Hypothalamic lesions: effects on immunological responses. Proc. Amer. Physiol. Soc., 1975. (Abstract).

2. Spector, N. H., Martin, L. K., Diggs, C. L., and Koob, G. F.: Hypothalamic lesions: effects upon malaria and antibody production in rats. Proc. XXVI International Cong. of Physiol. Sci. 11: 393, 1974. (Abstract).

3. Spector, N. H., Martin, L. K., Diggs, C. L. and Koob, G. F.: Malaria: effects upon body temperature in rats with and without hypothalamic lesions. Fed. Proc. 1975. (Abstract).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA OB 6506	75 07 01	DD DR&E(A)1&JA	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8A. ORG'S INSTR <sup>h</sup>	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES <sup>g</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		61101A	3A161101A91C	00	115		
B. CONTRIBUTING							
C. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>f</sup>							
(U) Behavioral Scheduling in Psychosomatic Disease							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>h</sup>							
002500 Clinical Medicine 013400 Psychology 012900 Physiology 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES <sup>i</sup> /EFFECTIVE: NA				PRECEDING		1	
B. NUMBER <sup>j</sup>				FISCAL YEAR		55	
C. TYPE				CURRENT		2	
D. KIND OF AWARD				76		69	
E. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: Sodetz, MAJ F. J.			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Hegge, F. W., Ph.D.			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Psychosomatic Disease; (U) Autonomic Dysfunction; (U) Operant Conditioning; (U) Stress							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Precede individual paragraphs identified by number. Precede rest of each with Security Classification Code.)							
23 (U) This work unit is to systematically define functional relationships between behavioral variables and the pathogenesis of psychosomatic disorders, e.g., hypertension and duodenal ulcer, and autonomic dysfunction to identify potentially effective measures for prevention and treatment of this class of disorders in military personnel.							
24 (U) Empirically-deprived behavior principles are applied in studies of non-human primates to describe the functional relationship between behavioral variables, such as reinforcement, punishment, conditioned reinforcers and conditioned aversive stimuli, and activity in a variety of physiological systems implicated in psychosomatic disorders. The focus is on arrangement of consequences for behavior and the parallel control such consequences maintain over physiologic variables. Techniques include the development of effective means of chronically monitoring physiologic status of the organisms as well as the continuous updating of appropriate operant technology as a prerequisite for the exploration of potentially productive methods of treatment.							
25 (U) 74 07 - 75 06 Non-human primates, prepared to permit direct observation of the gastric and duodenal wall, were exposed to increments in the aversiveness in performance demands required of them and monitored for the development of chronic peptic ulcer. All lesions produced by this procedure were self-limited, usually occurring in the early phase of exposure to the task. This study appears to rule out shock frequency, shock intensity, escape learning, avoidance learning, and punishment of avoidance responding as sufficient conditions for the production of behaviorally-induced chronic pathology of the gastro-intestinal system. For a technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 74 - 30 June 75.							

\* Available to contractors upon originator's approval.

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 115 Behavioral scheduling in psychosomatic disease

Investigators.

Principal: MAJ Frank J. Sodetz, MSC

Associate: MAJ Benjamin H. Natelson, MC;  
Frederick W. Hegge, Ph.D.

Description.

The modification of autonomic function by perturbations in the environment has been well recognized for many years. However, only recently has the concept been extended to control by conditioned stimuli, that is, stimuli which acquire their controlling properties by virtue of repeated pairing with stimuli already effective in eliciting changes in autonomic activity. While appealing as a potential factor in the etiology of certain psychosomatic disorders, pathological responses to conditioned stimuli are known to be of limited duration occurring only in response to, and in the presence of, eliciting stimuli. It remained difficult to conceptualize long-term pathogenic changes in autonomic function resulting from brief exposures to such stimuli. In situations in which stressful stimuli were known to persist for extended periods, there was usually evidence of adaption to stress. However, within the past four years, evidence has begun to accumulate that the autonomic nervous system responds not only to eliciting stimuli antecedent to responses, but also to stimuli presented following changes in autonomic activity. It has been determined that autonomic activity can be brought under the control of environmental stimuli presented contingent upon a change in autonomic activity. This is precisely the arrangement of response and consequences that produces stable alterations in more conventional behaviors. To date, little has been done to systematically explore the control of autonomic function by manipulating the consequences of alterations in autonomic activity. The purpose of this work unit is to take advantage of these recent findings to examine the relationship of various arrangements of stimuli and responses to determine their role in the production and maintenance of the kind of chronic change in autonomic function thought to be significant in the etiology of psychosomatic disease and to develop models for the study of this class of disorders.

Progress.

Analysis of the behavioral variables contributing to the development of gastro-intestinal ulcers. This study was designed to use the principles developed from behavioral research to pursue development of a sub-human primate model for psychosomatic peptic ulcer disease. To do this, unpredictability of shock presentation and conflict, i.e., punishing previously appropriate responses, were manipulated in a series of sequential experiments during which each monkey's gastric-duodenal mucosa was examined biweekly using a fiberoptic gastroscope.

In a previous report of this study was observed that acute superficial gastric lesions developed upon exposure of rhesus monkeys to the early acquisition phase of several variations of traditional escape and avoidance learning paradigms. It was possible to confirm two of the lesions histologically at the termination of the study. The study demonstrated a clear relationship between environmental variables and the production of gastric pathology. Whenever lesions were observed, their appearance coincided with planned changes in the behavioral demands made upon the organism. However, because all of the observed lesions were acute, the study did not produce definitive evidence of the means by which environmental demands might participate in the development of chronic ulcer disease. This outcome was anticipated in view of the difficulties others have encountered in attempting to replicate the initial observations of Brady and others that chronic ulcer disease could be produced in monkeys by purely behavioral means. The importance of the present study, is that it reduces the number of potentially relevant variables. On the basis of the study concluded this year, it can be said that escape learning, avoidance learning, shock-intensity, and punishment of avoidance responding are, in of themselves, insufficient to produce chronic gastric pathology. Furthermore, because the appearance of acute gastric pathology was well correlated with changes in performance demands being made upon the organism, it is clear that any subsequent work should focus on the physiological consequences of exposure to new performance demands and to the conditions that facilitate or impair acquisition of behaviors under aversive control. The data suggest rather strongly that if the conditions requisite to the production of chronic gastric pathology exist at any time, it must be during the acquisition phase of learning prior to the time that performance becomes stable. Judging from the time of onset and duration of acute gastric erosions, gastric pathology could not be anticipated in an organism which has successfully adapted its behavior to meet new environmental demands.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA OB 6508	75 07 01	DD-DR&E(A)836	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8. DISSEM INSTR <sup>6</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>7</sup>	10. LEVEL OF SUM A. WORK UNIT
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>8</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		61101A	3A161101A91C	001	116		
B. CONTRIBUTING							
C. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>9</sup>							
(U) Autoregulation of autonomic response							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>10</sup>							
012900 Physiology 013400 Psychology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				B. PREVIOUS		C. FUNDS (In thousands)	
EXPIRATION:				FISCAL YEAR		D. FUNDS	
C. TYPE:				75		2.5	
E. KIND OF AWARD:				76		41	
F. AMOUNT:				2		80	
G. CUM. AMT.				2		41	
18. RESPONSIBLE DOD ORGANIZATION				19. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: Hegge, F. W. Ph.D.			
TELEPHONE: 202-576-3551				TELEPHONE: 427-5521			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Redmond, MAJ D. P.			
				NAME: Garcia, MAJ J.			
22. KEYWORDS (Precede EACH with Security Classification Code) (U)Autonomic Dysfunction; (U)Psychosomatic Disease; (U)Human Volunteer; (U)Conditioning; (U)Non-Invasive Monitoring; (U)Cardiovascular Function							
23. (U) Refinement of behavioral techniques for outpatient management of psychosomatic dysfunctions in military personnel through extension of operant and respondent conditioning principles. Nonpharmacologic management of wound related intractable pain syndromes.							
24. (U) Knowledge of operant and respondent conditioning principles, autonomic nervous system functioning, and sophisticated non-invasive bioinstrumentation are applied to normal and target populations to effect clinically relevant changes in biological functions. Procedures developed are extended, simplified, and standardized to facilitate application on an outpatient basis.							
25. (U) 74 07 - 75 06 Work on the development and integration of non-invasive cardiovascular monitoring techniques has continued. Calibration procedures for miniature infrared photoplethysmographs have been implemented. The role of electrode separation in partitioning arterial and venous components of tetrapolar impedance plethysmography signals has begun. After intensive study, little evidence of sympathetic nervous system activity asymmetries related to causal state has been established. For technical progress, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 74 - 30 JUN 75.							

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1 MAR 68

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 116 Autoregulation of autonomic response

Investigators.

Principal: Frederick W Hegge, Ph.D.

Associate: MAJ Daniel r. Redmond, MC; LTC Albert J. Tahmoush, MC; CPT John G. Varni, MSC; CPT John R. Jennings, MSC.

Description

The applicability of operant and respondent conditioning principles to clinical syndromes involving autonomic dysfunction, such as pain states, hypertensive cardiovascular disease, and cardiac arrhythmia is systematically investigated. Such research leads to an understanding of the pathogenesis of and techniques for intervention in the clinical course of psychosomatic diseases. Transcutaneous stimulation techniques (TST) have been found clinically effective in the treatment of pain (1). Current work involves laboratory assessment of counter-stimulation techniques, such as TST as well as investigation of autonomic involvement in causalgia. Continuing technical developments include sophisticated and non-invasive techniques for monitoring cardiovascular variables that will allow detailed assay of conditioning methods in terms of both mechanisms and efficacy.

Progress

1. Causalgia: A Study of Sympathetic Activity in Affected Subjects

Indirect estimates of local sympathetic nervous system activity were obtained on six patients with causalgia of at least 18 months duration and six age and sex-matched normal subjects. Measurements of skin conductance (SC), and blood volume pulse amplitude (BVP) were performed on the affected and the homologous non-affected extremities. Skin conductance was determined by the constant voltage method. Blood volume pulse amplitude was obtained with a photoelectric plethysmograph developed for this study (2). Measurements were performed in a controlled environmental chamber on two consecutive days in two 30-minute periods following an AB-BA design.

A within-group analysis of variance revealed no significant asymmetry in either BVP or SC between affected versus non-affected extremity in either the causalgia or the normal subject group. A between-group analysis of variance revealed no significant difference in the magnitudes of the two measures between patients with causalgia and normal subjects. In addition, a dependent t-test was performed for each measure comparing affected versus non-affected extremities.

Two patients had no significant asymmetry of either SC or BVP, one patient had an asymmetry of BVP (greater amplitude in the affected extremity), and three patients had a significant asymmetry of SC (in two patients the magnitude was greater in the affected extremity and in one patient it was greater in the non-affected extremity). Three normal subjects had no significant asymmetry of either SC or BVP, one normal subject had an asymmetry of BVP (greater amplitude in the "non-affected" extremity), one subject had an asymmetry of both BVP and SC (greater amplitude of BVP in "non-affected" and of SC in "affected" extremity), and one subject had an asymmetry of SC only (greater amplitude in "affected" extremity). It is therefore clear that asymmetries in these two measures do occur in both patients with causalgia and in normal controls. However, the sudomotor and vasomotor abnormalities are not consistent within either group, and the magnitudes of these measures are not significantly different in the two groups.

It has been suggested by Melzack (3) that the pain of causalgia is mediated through increased efferent sympathetic activity in the affected extremity manifested as increased sweating (+ SC) and vasoconstriction (+ BVP amplitude). Bonica (4) has suggested that causalgia be redefined as a reflex sympathetic dystrophy. These suggestions have been based on clinical observations and the efficacy of local sympathectomy for the treatment of causalgia. This study fails to support either of these suggestions. Our results suggest that causalgia is not associated with either a local or generalized increase in sympathetic efferent activity (5).

## 2. TST Effects on Pain Estimates and Skin Conductance in Normal Volunteers.

The effects of transcutaneous stimulation on the pain estimates of normal subjects stimulated by radiant heat was examined in a double-blind study. The experiment consisted of bilateral magnitude estimation before, during and after a stimulation condition. The stimulation conditions were: a) TST applied over the median nerve; b) TST applied over the lateral portion of the upper arm; c) a placebo condition in which stimulation was alleged but not actually delivered; or d) an explicit control condition. Skin temperature near the pain site and palmar skin conductance reactions were recorded in addition to the pain judgments. Pain was induced with a Hardy-Wolff dolorimeter and TST was administered with a Medtronics Transcutaneous Stimulator.

The results failed to demonstrate significant differential pain decrements attributable to the locus or the presence of actual nerve stimulation. During the stimulation period, the placebo and both actual stimulation conditions produced equal pain decrements relative to the control and to pre-stimulation judgments. Pain decrements due to the after-effects of TST were observed in the placebo and upper-arm-stimulated subjects while median-nerve-stimulated subjects failed

to differ from the controls. Skin temperature changes generally mirrored pain changes and all pain changes disappeared when skin temperature variations were controlled statistically. In combination with our previous work, these results raise doubts concerning the direct physiological efficacy of TST.

### 3. Technical Progress -- Monitoring of Cardiovascular Responses

Continuous research effort has been directed at the development of techniques for non-invasive measurement and recording of human cardiovascular system behavior with the aim of simultaneous, heartbeat-by-heartbeat monitoring of numerous inter-related physiological variables. Such an approach is appropriate to a multi-dimensional view of the system which permits greater understanding of the complexity of mechanisms and specificity of cardiovascular interactions with neural and cognitive factors.

The development of an experimental chamber equipped for such monitoring is complete. Transducers and peripheral electronics provide continuous recording of analog signals on paper, microfilm, and analog tape. Ten recording channels are currently applied to the following sources of data: 1) Time-base, with resolution exceeding one millisecond; 2) electrocardiogram; 3) phonocardiogram (120-500 Hz filtrate); 4) vibrocardiogram (less than 30 Hz filtrate); 5) carotid pulse waveform (accelerometric); 6) radial pulse waveform (pneumatic tonometer); 7) digital pulse waveform (Photoplethysmographic); 8) sphygmomanometric pressure (deflation phase of intermittent (q 30 sec) blood pressure measurement cycles) 9) doppler-shift "sounds", analogous to Korotkoff sounds, associated with cuff deflation; and 10) wrist articular motion artifact, obtained from strain-gauge in proximity to pulse tonometer. Two additional channels are immediately available for recording of other data which may be optional or undergoing development, e.g., impedance plethysmograph, respiration, and event marking. Versatility is enhanced by facilities for mixing or multiplexing of present analog channels.

The first five channels provide time/event references for the determination of systolic time intervals for each cardiac cycle. These measures have been empirically related to directly measured parameters such as ejection fraction and stroke volume (6). Pulse wave data allow recording of differential amplitude and time-derivative changes, as well as the velocity of propagation of the pulse, parameters which are functions of stroke/volume/work factors and vascular compliance (7). In addition to intermittent systolic and diastolic pressure recording, the sphygmomanometric/Doppler-shift device provides propagation velocity data, as a function of pressure, over successive beats and allows an approximate replication of the brachial artery pressure curve (8). Taken in aggregate, these data provide a cluster of indirect indices of cardiovascular system behavior far more inclusive than heretofore applied to psychophysiological research.

Using the monitoring system outlined above, a brief experiment, utilizing three normal male volunteers, was completed to provide qualitative information regarding the sensitivity and stability of the measurements. Analog data were recorded for 1½ hours, during which each subject was studied 1) at rest, 2) during two cold-pressor tests, and 3) during two 15-second Valsalva maneuvers. Data, recorded on paper, were coarsely sampled by pencil-and-ruler methods with attention to systolic time intervals, pulse waveforms and propagation time. This analysis demonstrated a general stability of signals, that was subject to motion artifact. Carotid pulse pickup was most sensitive in this regard, and equipment modifications to minimize this problem have been made. Systolic interval measures, especially ejection time, propagation and contour of pulses were all sensitive to the dynamic changes anticipated for the two stimulus conditions, showing systematic alteration and recovery directionally appropriate to the specific condition. Assessment of sensitivity to smaller changes in more subtle circumstances awaits a more quantitative analysis.

The volume of data obtained in a single experiment precludes quantitative analysis by hand methods. For a single heartbeat, the raw data include at least 30 distinct "words" of information to be recorded and analyzed separately. Consequently, the second major phase of development has consisted of design and construction of on-line processing equipment to decrease analysis time to real time. This objective is also made desirable by the need for rapid access to ongoing physiological data in projected studies of the effects of acute psychological stimuli, including the investigation of autococontrol phenomena.

Circuits to detect and mark "points of interest" on analog channels are complete. Such points are referenced to the time-base and to the "R" wave of each heart beat, providing immediate measurement of all the relevant time intervals. Interval data are temporarily stored, in digital format, to the nearest millisecond, as each interval completes itself, and updated on each successive heartbeat. In equipment in early stages of construction, amplitude data (e.g., pulse wave contour, 1st and 2nd derivatives) will be tracked, analog-to-digital converted, and placed into temporary storage at the appropriate instant. Permanent storage of processed data on digital tape is provided by interfacing circuitry now being installed. This total facility, approaching completion, will thus allow rapid processing, and statistical analysis of data, by computer on both on-line and off-line bases.

Initial studies will be designed to further assess the technology, in quantitative terms, and in terms of the empirical meaning of such data per se. The development of on-line processing capability will lead to application in a broad array of psychological models. The versatility of the technology also lends itself to rapid application of new methods of monitoring, such as complex analyses of telemetric or "field recorded" data, further development of the photoplethysmographic and impedance plethysmographic technology, and other novel transducing devices which are under investigation within this department.

#### 4. Technical Progress - Photoplethysmography.

The use of an LED-phototransistor plethysmograph for the indirect monitoring of changes in peripheral circulation has been described (1). This device has been found useful for monitoring changes in superficial arterial pulses as well as changes in cutaneous vascular beds.

During the last year, our attention has been focused on further determining the characteristics of this transducer and its possible application for the measurement of tissue blood volume and blood flow. A series of studies were performed to examine the output response of the phototransistor to linear light intensity changes, temperature changes, and light history effects. The radiation pattern of the LED was also examined. It was found that the phototransistor output was linear in a wide range which encompassed the DC-coupled plethysmographic signal recorded from a normal group of subjects. The DC-signal reflected from a ceramic tile was found to increase by 4 mV for each 1°C increase in temperature. Storing the device in a light or dark environment had no effect on transducer output. These results indicate that the LED-transistor plethysmograph may faithfully measure the DC-coupled tissue blood volume signal and not be sensitive to extraneous variables affecting other devices. The radiation pattern of the LED was found to approximate a point source. Since optical modeling of cutaneous tissue usually assumes a point light source, this finding is of great importance.

Work continues on the development of a calibration system for plethysmography. It is commercially possible to calibrate a "standard" plethysmograph in terms of absolute light intensity response as indicated by voltage output. Other plethysmographs can be compared to this device by examining the response to a constant input. A system approach to modeling and calibration is being undertaken. By use of an opto-mechanical calibrator designed and built in our laboratory, a standardized sinusoidal optical signal can be presented to the plethysmograph. In this way a transfer function can be determined for the plethysmograph as a system, including amplifiers, recording equipment, light source and receiver. The inclusion of the plethysmograph light source within the calibration procedure is possible through use of a reflective calibrator. Sinusoidal intensity changes are achieved by imparting a sinusoidal movement to the reflecting surface. Application of the inverse square law gives

$$I(t) = \frac{I_0 x_0^2}{x_0^2 + 2Ax_0 \sin \omega t + A^2 \sin^2 \omega t},$$

where  $I(t)$  = light intensity sensed by receiver;  $x$  = distance from reflector to source(receiver);  $A$  = amplitude of sinusoidal motion;  $x_0$  = mean value of  $x$ ,  $I_0 = I(t)$  when  $x = x_0$ ;  $\omega$  = angular frequency;  $t$  = time. By keeping  $A$  much smaller than  $x$ , we can reduce this

expression to

$$\frac{I(t)}{I_0} = \frac{1}{1 + \frac{2A \sin(\omega t)}{X_0} + \frac{A^2 \sin^2(\omega t)}{X_0^2}} \approx \frac{1}{1 + \frac{2A \sin(\omega t)}{X_0}},$$

neglecting higher order terms.

$$\text{Further, } \frac{I(t)}{I_0} = \frac{1 - \frac{2A \sin(\omega t)}{X_0}}{1 - \frac{4A^2 \sin^2(\omega t)}{X_0^2}} \approx 1 - \frac{2A \sin(\omega t)}{X_0}$$

Thus, for  $A \ll X_0$ ,  $\frac{d}{dt} \{ I(t) \} \approx K \cos(\omega t)$ ,

$$\text{where } K = - \frac{2A \omega I_0}{X_0}$$

A reliable estimation of blood flow from the plethysmograph signal can not be made at this time. An examination of the optical properties of cutaneous tissue is planned, and the derivation of a model permitting estimation of blood flow from changes in scattered light is projected for the next year.

As its name indicates, a plethysmograph records volume or "fullness" rather than motion or flow. Researchers have occasionally erred in confusing changes in blood volume with net blood flow. Unfortunately, any compliant system will show pulsatile volume changes in response to pulsatile pressure changes -- even when the throughput, or net flow is zero. Thus, to deduce information about net blood flow, additional data are needed. In an attempt to increase our available data, we have made plans to build and test a bi-chromatic optical plethysmograph. By using the known absorption characteristics of oxygenated and reduced hemoglobin it may be possible to relate changes in  $O_2$  utilization rate to changes in net blood flow.

##### 5. Technical Progress - Impedance Plethysmography

In developing the tetrapolar electrode configuration for impedance measurement, Lord Kelvin performed a valuable piece of groundwork for

impedance plethysmography. The four-electrode system has become the method of choice in this field due to its capacity for separating complex electrode and transcutaneous impedance from the internal tissue impedance whose measurement is sought.

Our laboratory has addressed itself to two fundamental issues which are important to electrical impedance plethysmography. The first issue was the development of a stable electrode for monitoring cardiovascular regulation. Two promising electrode materials have been tested. A silver-impregnated Silastic cloth developed for NASA produced excellent results while new. Unfortunately, the performance declined rapidly with repeated cycles of use. More promising was an inexpensive foil tape used in conjunction with Beckman electrode paste. The resulting impedance at the driving electrodes has remained well within the limits imposed by our measurement system. Impedance amplitude and phase spectra derived from blood pulse waveforms indicated that the optimal interelectrode spacing was two inches between driving and sensing electrodes. This placement afforded maximal separation between the two major components of the waveform, that have tentatively been identified as separate arterial and venous components. The identification is based on the study of waveform changes that accompany various maneuvers that include limb positioning, respiratory variations, and application of pressure cuffs. Further confirmation of this technique as a non-invasive means of separating peripheral arterial and venous pulse components will enhance the study of cardiovascular auto-regulation.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 116 Autoregulation of autonomic response

Literature Cited.

References:

1. Meyer, G. A., & Fields, H. L.: Causalgia treated by selected large fiber stimulation of peripheral nerves. *Brain*, 95: 163-168, 1973.
2. Lee, A. L., Tahmoush, A. J., & Jennings, J. R.: An LED-Transistor Photoplethysmograph. *IEEE Trans. Biomed. Eng.* 22: 248-250, 1975.
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4. Bonica, J. J.: Causalgia and other reflex sympathetic dystrophies. *Postgrad. Med.* 53: 143-152, 1973.
5. Tahmoush, A. J., Jennings, J. R., & Plett, A. B.: Measurements of skin conductance and peripheral vascular activity in patients with causalgia. *Psychosomat. Med.* 37: 96, 1975.
6. Weissler, A. R., Peeler, R., & Rochill, W.: Relationships between LVET, SV, and HR in cardiovascular disease. *Am. Heart. J.* 62: 367, 1961.
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8. Rodbard, S., Rubinstein, H. M., & Rosenblum, S.: Arrival time and calibrated contour of the pulse wave determined indirectly from recordings of arterial compression sounds. *Am. Heart J.* 53: 205, 1957.

Publications:

1. Lee, A. L., Tahmoush, A. J., & Jennings, J. R.: An LED-Transistor Photoplethysmograph. *IEEE Trans. Biomed. Eng.* 22: 248-250, 1975.
2. Tahmoush, A. J., Jennings, J. R., & Plett, A.B.: Measurements of skin conductance and peripheral vascular activity in patients with causalgia. *Psychosomat. Med.* 37: 96, 1975. (Abstract)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA OB 6512	75 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8. DRG'S INSTN <sup>6</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS <sup>7</sup>	10. LEVEL OF SUM <sup>8</sup>
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>9</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		61101A	3A161101A91C	00	120		
b. CONTRIBUTING							
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>11</sup>							
(U) Antigenic Components of the Cell Wall of Neisseria meningitidis							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>12</sup>							
010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE EFFECTIVE: NA				PRECESSION		b. FUNDS (in thousands)	
b. NUMBER:				75		3	
c. TYPE:				FISCAL YEAR		120	
d. KIND OF AWARD:				76		4	
e. AMOUNT:						264	
f. CUM. AMT.							
20. RESPONSIBLE S&T ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3758			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: DA			
23. KEYWORDS (Precede with Security Classification Code) (U) Cell wall; (U) Protein; (U) Lipopolysaccharide; (U) Polysaccharide; (U) Antibodies; (U) Meningitis; (U) Human Volunteers							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To analyze the cell surface structure of the meningococcus with emphasis on extracting and purifying the dominant antigens. The goal is to develop candidate vaccines for this disease of military importance.							
24 (U) Protein, lipopolysaccharide, and polysaccharide antigens will be characterized biophysically and immunochemically. Antibody responses of animals and humans will be measured by a variety of serologic methods. Antibodies will be purified by immunoabsorbent techniques. Cellular immune responses will be measured by in vitro techniques.							
25 (U) 74 07 - 75 06 Cell wall protein antigens from meningococci of serogroups B and C have been prepared as vaccines and shown to be safe and immunogenic in animals. Two of these protein vaccines have been tested in five human volunteers and shown to be safe. Greater than four-fold rises in bactericidal antibody activity against groups B and C organisms occurred in the volunteers. Rises in bactericidal activity were detected against all group B strains tested indicating group specificity in contrast to rabbit responses which demonstrated type specificity. Analysis of 102 organisms typed by the radiobactericidal system indicated that a protein determinant was a marker for epidemic potential in terms of absence of bactericidal antibody directed against it. The immunoepidemiology of groups B and C is similar, while that of group Y is different. A solid phase radioimmunoassay has been developed to investigate protein antigens and quantitate antibody responses to them. A hemagglutination inhibition system has identified eight different lipopolysaccharide (LPS) types of the meningococcus. An LPS Farr test has also demonstrated serologic differences. Lymphocytes participate in antibody dependent cellular cytotoxicity against the meningococcus. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.							

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DD FORM 1 MAR 66 1498

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Project 3A161101A91C In-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 120 Antigenic components of the cell wall of Neisseria meningitidis

Investigators

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I. Meningococcal vaccine studies on humans.

A. Experience with meningococcal polysaccharides vaccines.

Group A and C meningococcal polysaccharide vaccines have been shown to be safe and effective in preventing group A and C disease [1,2]. Group C vaccines are currently used routinely in military recruits.

In January of 1975 a study was conducted at Fort Ord to determine what effect, if any, administration of both group A and C meningococcal polysaccharide vaccines together would have on the antibody response to each of the components. For this study, groups of 50 men were immunized subcutaneously with 50 micrograms each of group A and C vaccine in two sites in the same arm or 50 micrograms of either group A or C vaccine alone. The men receiving the group A vaccine alone were immunized with the group C vaccine at two weeks to protect them from the possibility of group C meningococcal disease. Sera were obtained prior to and at two, four and six weeks post-vaccination. The serologic methods used in this study were the indirect hemagglutination test (IHA), the radio-active antigen binding assay (RABA) and the serum bactericidal test (SBT). The antibody studies completed at present are shown in Table 1.

Analysis of the IHA data indicates there was no effect on the immunogenicity of either component of the vaccine when the two were given together. When the geometric mean change in titer (2 wk less 0 wk) obtained with the A-RABA were examined by the t test, the mean change in titer between A and C together (56.5 ng antigen bound) and A alone (32.1 ng antigen bound) were found to be significantly different only at the 10 percent level. This probably indicates no effect when the polysaccharides are administered together. Further conclusions will have to wait until the antibody analyses are complete.

Acknowledgement: The following non-WRAIR personnel participated in the above studies: LTC Creed D. Smith, Chief, Bacteriology Dept.,

Table 1. Change in geometric mean titers of antibody after vaccination with group A and C polysaccharide from *Neisseria meningitidis* when administered either together or alone.

	IHA		RABA	SBT	
	A	C			
	Change in titer % with rise	Change in titer % with rise	Change in titer (ng antigen bound)	% with rise	Change in titer % with rise
A and C together Lot A-440 and C-9	4.28	4.44	56.5	1:222	91.7
A alone Lot A-440	4.32		32.1		81.6
C-two weeks after A alone, Lot C-9		4.56			93.6
C alone Lot C-9		5.0			98.0

Sixth USA Medical Laboratory, Ft. Baker, CA; COL H. A. Leighton, Chief, Preventive Medicine Activities, Ft. Ord, CA; Members of the Sixth USA Medical Laboratory Respiratory Disease Investigation Team; Mr. Angus Hull, Sixth USA Medical Laboratory, Ft. Baker, CA.

In a separate study the duration of antibody responses after vaccination with the group C vaccine was investigated. Persistence of serum antibodies after vaccination with group C polysaccharide was followed in 23 volunteers. Each subject had received a single 50 microgram dose of vaccine subcutaneously or intradermally one to five years earlier. The serologic methods used were the indirect hemagglutination test, radioactive antigen binding assay and the serum bactericidal test. Hemagglutinating antibody titers rose significantly in 22 of 23 volunteers within two to four weeks of injection; mean titers fell two to three-fold within the first year and then remained stable for four years. The serum bactericidal test showed antibody responses in 86 percent of the subjects with antibody persistence comparable to that found in the hemagglutination test. Titers of radioactive antigen binding antibodies increased significantly in all 22 subjects tested and after two to four years the titers remained at 30 percent or more of the peak concentration. The prolonged duration of antibody responses to polysaccharides in humans suggests that immunity will also persist.

B. Studies of a meningococcal cell wall protein vaccine.

The failure of the group B polysaccharide (sss) to induce antibody responses in humans [3] has led to studies of the epidemiologically important protein serotype antigen, CII-B2 (vide Section II) as a potential vaccine. Such a protein antigen might be prepared from either a group B or group C strain and be expected to cross react.

During the past two years, two lots of protein vaccine were prepared and tested. They differed from those previously used in several respects: (1) a group C strain (138I) was used; (2) milder procedures were used in their preparation; (3) analysis by polyacrylamide gel electrophoresis showed fewer protein bands and lack of degraded proteins.

Vaccine lots 138I-0 and 138I-M-1 were derived from isolated outer membrane of the group C strain 138I, which is the serotype II reference strain. Lot 138I-0 is the purified protein fraction of the membrane and lot 138I-M-1 contains both the protein and group specific polysaccharide components of the membrane at a ratio of 2:1. LPS was specifically removed from these two lots by Sephadex G-100 chromatography.

The results of the testing of these two vaccine lots in animals for immunogenicity and toxicity were summarized in the WRAIR Annual Report 1974, Work Unit 120. Briefly, rabbits immunized with the two

trial vaccine lots 138I-0 and 138I-M-1 produced bactericidal and hemagglutinating antibodies with geometric mean titers of 1:1096 and 1:2048 respectively. The antisera were capable of opsonizing type II (group B and C) organisms for phagocytosis and the phagocytic killing was inhibited by purified cell wall proteins from the homologous strains and from a type II group B strain. Both protein vaccines protected mice from lethal challenge with a group B strain 99M(B). These two vaccine lots were found to be nontoxic in mice and guinea pigs and nonpyrogenic in rabbits at the same levels as used for the capsular polysaccharide vaccines.

During the past year upon approval of the Army Investigational Drug Review Board these two protein vaccine lots were tested in five human volunteers. Two individuals (K.Y. and G.L.) received lot 138I-0 and three individuals (W.Z., J.S., and A.C.) lot 138I-M-1. Three injections of 50 µg protein each were given subcutaneously at 0, 5, and 9 weeks. Serum samples and throat cultures were taken at 0, 2, 5, 7, 9, 11, 13, and 17 weeks. An intradermal skin test, performed one hour prior to the first injection using one µg of vaccine, produced no immediate reactions, and delayed reactions of only slight erythema persisting for about 24 hrs. The vaccinations appeared to be well tolerated. A slight tenderness in the area of the injections was reported by only one volunteer.

Two individuals had consistently positive throat cultures. The isolates from one (W.Z.) were consistently group B and from the other, nongroupable. The remaining three individuals had consistently negative throat cultures.

Pre- and post-immunization sera from the five volunteers were tested in several serological assays for rises in antibody titer. The sera were tested in passive hemagglutination assays (Table 2) for antibody rises to five different antigens: 138I(C) outer membrane protein (OMP = vaccine lot 138I-0), 99M(B) OMP, 138I(C) LPS, group C polysaccharide (C<sub>SSS</sub>) and group B polysaccharide (B<sub>SSS</sub>). Only two individuals (K.Y. and W.Z.) showed a four-fold or greater increase in antibody titer against the vaccine OMP. Three individuals, however, had a four-fold or greater rise against OMP from the group B strain 99M(B), which shares the type II protein with 138I(C). No significant rises were detected against the LPS from the vaccine strain. This was expected since LPS was specifically removed during preparation of the vaccine. All three volunteers who received the vaccine lot 138I-M-1 which contained both protein and C<sub>SSS</sub> had a rise in antibody titer against the C<sub>SSS</sub>, while one volunteer (K.Y.) who received the purified protein (lot 138I-0) also developed a four-fold rise against C<sub>SSS</sub> at two weeks. Two individuals (W.Z. and G.L.) showed a four to eight-fold rise against the B<sub>SSS</sub>. One of these (W.Z.), however, was a group B carrier during the study.

Table 2. HA antibody response of human volunteers to trial meningococcal protein vaccine.  
 HA Titer\*  
 vs.

Volunteer	Vaccine	Serum (week)	99M(B) Protein	138I(C) Protein	138I(C) LPC	C <sub>ss</sub>	B <sub>ss</sub>
K.Y.	138I-0	0	4	0	4	4	16
		2	64	8	8	16	16
		7	128	8	8	8	16
		11	128	4	8	8	32
G.L.	138I-0	0	32	16	32	8	8
		2	64	2	32	16	32
		7	32	2	32	16	32
		11	16	2	16	8	32
J.S.	138I-M-1	0	2	0	8	4	4
		2	64	0	16	128	8
		7	32	2	16	128	8
		11	64	0	16	128	8
W.Z.	138I-M-1	0	1024	0	4	4	8
		2	1024	256	8	16	64
		7	1024	256	4	32	16
		11	1024	256	4	32	32
A.C.	138I-M-1	0	4	0	8	8	8
		2	64	2	16	512	16
		7	64	2	16	512	16
		11	128	2	16	512	16

\*Reciprocal of highest dilution causing agglutination; 0 indicates less than 1:2.

The sera were also tested for rises in bactericidal activity against two group C strains (the vaccine strain 138I and a type I strain 60E) and five group B strains, including three that were type II (99M, B16B6 and M986) and two nontype II strains (M1080 and M136) (Table 3). Two types of bactericidal assays were used: 1) The radioactive bactericidal test (RBCT) in which about  $10^7$  organisms per tube are used and killing is monitored by release of radioactivity from  $C^{14}$ -Sodium Acetate labeled organisms [4], and 2) the standard bactericidal test (SBT) in which about  $3 \times 10^3$  organisms per tube are used and killing is monitored by plating on solid medium and counting colonies.

Using the RBCT, rises in bactericidal activity against the group C strains were only detected for those individuals who received vaccine lot 138I-M-1 which contained both protein and  $C_{SSS}$ . Using the more sensitive SBT, significant rises against the vaccine strain were detected in all five volunteers. With one exception (W.Z.) no bactericidal activity against the group C strains was detected in the pre-vaccination sera.

With either RBCT or the SBT four of five volunteers showed four-fold or greater rises in bactericidal activity to four or five of the group B strains tested. The other individual (G.L.) showed at least a two-fold rise against four of the five group B strains. Four of the five volunteers had prevaccination bactericidal activity against two or more of the five strains. Prevaccination bactericidal titers ranged from less than 1:40 to 1:160. There was no apparent type specificity to the antibody response. The rises against nontype II strains were as frequent and large as against the type II strain. This result was unexpected since rabbits had responded to the vaccine with highly type-specific antibodies.

The overall antibody response to the vaccine was satisfactory in that bactericidal antibodies were produced against both group C and B strains, which had not previously been achieved by vaccination. The antibody response was complicated, however, by the fact that the vaccine from a group C organism stimulated an antibody response against all group B strains tested, rather than just those that shared the type II protein.

Experiments were carried out to determine the nature of the antigens on the group B and C strains against which the bactericidal antibodies were directed. The bactericidal activity of several postvaccination sera was inhibited or absorbed with a variety of different antigens. The results were compared to similar results obtained with human convalescent sera from patients with systemic group B meningococcal disease and with hyperimmune rabbit serum made against the vaccine and against whole viable organisms. These studies are summarized in Table 4.

The bactericidal activity of rabbit antisera against the protein vaccine was found to have the same specificity as that of antisera

Table 3. Bactericidal antibody response of human volunteers to trial meningococcal protein vaccine.

Volunteer	Vaccine	Serum (week)	Bactericidal Titer*																
			138I(C)		60E(C)		99M(B)		B16B6(B)		M1080(B)		M986(B)		M136(B)				
			RBCT	SBT	RBCT	SBT	RBCT	SBT	RBCT	SBT	RBCT	SBT	RBCT	SBT	RBCT	SBT			
K.Y.	138I-0	0	0	0	0	0	160	0	0	0	160	0	160	0	160	0	40		
		2	0	640	0	0	0	640	160	0	640	160	0	640	160	0	640	640	
		7	0	160	0	0	640	160	160	0	640	160	160	160	160	160	640	640	640
		11	0	160	0	0	640	160	160	0	640	160	40	160	160	160	640	640	640
		13	0	0	0	0	640	320	320	640	640	640	320	320	320	320	640	640	640
G.L.	138I-0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		2	0	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40
		7	0	160	0	0	40	0	0	0	0	0	0	0	0	0	0	40	40
		11	0	160	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0
		13	0	0	0	0	40	0	0	0	0	0	0	0	0	0	0	0	40
J.S.	138I-M-1	0	0	0	0	0	160	0	0	0	40	0	0	0	0	0	0	0	
		2	320	640	160	0	160	160	160	160	160	160	160	160	160	160	160	160	160
		7	160	640	0	0	160	160	160	160	160	160	160	160	160	160	160	160	160
		11	160	640	0	0	160	160	160	160	160	160	160	160	160	160	160	160	160
		13	0	0	0	0	160	160	160	160	160	160	160	160	160	160	160	160	160
W.Z.	138I-M-1	0	0	40	0	0	80	40	0	0	0	0	0	0	0	0	0	0	
		2	40	640	0	0	640	640	640	640	640	640	640	640	640	640	640	640	640
		7	20	640	0	0	640	160	160	160	160	160	160	160	160	160	160	160	160
		11	40	640	0	0	640	160	160	160	160	160	160	160	160	160	160	160	160
		13	0	0	0	0	640	320	320	640	640	640	320	320	320	320	640	640	640
A.C.	138I-M-1	0	0	0	0	0	160	0	0	0	160	0	160	0	160	0	160	160	
		2	320	2560	640	0	640	640	640	640	640	640	640	640	640	640	640	640	640
		7	320	2560	640	0	640	640	640	640	640	640	640	640	640	640	640	640	640
		11	320	2560	640	0	640	640	640	640	640	640	640	640	640	640	640	640	640
		13	0	0	640	640	640	640	640	640	640	640	640	640	640	640	640	640	640

\* Reciprocal of highest dilution causing >25% of maximum release of radioactivity (radioactive bactericidal test - RBCT) or >25% killing (standard bactericidal test = SBT); 0 indicates 20 or less.

Table 4. Specificity of bactericidal antibodies against group B *N. meningitidis* in human and rabbit sera.

Treatment of Serum	Bactericidal Activity of Indicated System					
	Human vs. CSF isolate	Human vs. 1381(C)	Human vs. 99M(B)	Rabbit vs. Immunizing Strain	Rabbit vs. 99M(B)	Rabbit vs. 99M(B)
None	+	+	+	+	+	+
<u>Absorbed with:</u>						
Group B (same type) (diff type)	-	+	-	-	-	-
Group C (same type) (diff type)	+	+	-	+	+	+
SRBC	+	+	+	+	+	+
SRBC - LPS	+	+	+	+	+	+
SRBC - Bsss	+	+	+	+	+	+
Sepharose-Protein	+			- <sup>a</sup>		-
<u>Inhibited with:</u>						
OMC (same type) (diff type)	-		+	-	-	-
Crude Bsss	+		-	+	+	+
Csss	+		+	+	+	+
Bsss	+		+	+	+	+
OMC-Pronase treated	+		+	+	+	+
Protein	+		+	- <sup>a</sup>		-

<sup>a</sup> a Serum preabsorbed with group B organisms of heterologous serotype.

against whole type II organisms that had been absorbed to remove group specific antibodies. These rabbit antisera were type specific and could kill either group C or B organisms of the homologous type. The bactericidal activity against group B strains was not removed by absorption with B<sub>SSS</sub> or the homologous LPS. Outer membrane complex (OMC) or the purified OMP of the same serotype were able to inhibit the activity, while pronase treated OMC was a very poor inhibitor. The bactericidal activity of the rabbit sera was thus shown to be directed predominantly against the type specific protein antigens.

Several human group B case sera were analyzed and found to have properties similar but not identical to the rabbit sera. The bactericidal antibodies against group B strains were mostly type specific, reacting only with organisms or OMC of the same type. The antigen in the OMC which reacted with the bactericidal antibodies was pronase sensitive and purified B<sub>SSS</sub> or LPS could not inhibit the activity. The major difference between human and rabbit sera was that the type specific protein purified from the OMC was unable to react with the human antibodies, even though those antibodies appeared to be reacting with a type specific protein in the native OMC. It was concluded, therefore, that the type specific protein determinant to which humans responded following systemic disease loses its antigenic activity during purification.

The bactericidal activity against 99M(B) of two postvaccination sera (K.Y. and A.C.) was similarly analyzed and was completely removed by absorption with any of six different group B strains representing five different serotypes. Furthermore, the activity was not removed by group C strains of homologous serotype. The homologous OMC was a rather poor inhibitor compared to its ability to inhibit the bactericidal antibodies in rabbit sera and human case sera, and the inhibition it produced was neither type specific nor affected by pronase treatment. Crude preparations of group B polysaccharide, however, inhibited strongly and some, but not all, more highly purified B<sub>SSS</sub> preparations had good inhibitory activity. The bactericidal antibodies produced in response to the protein vaccines, therefore, appeared to have group specificity rather than type specificity; however, the precise nature of the antigen on group B organisms against which these bactericidal antibodies are directed is still under investigation.

When the specificity of the vaccine-induced bactericidal antibodies against group C strains, particularly the vaccine strain 138I(C), was investigated it was found that C<sub>SSS</sub> could inhibit essentially all of the activity, even in sera from volunteers who received vaccine lot 138I-0, which contained less than one percent C<sub>SSS</sub>.

Although rabbits responded to the protein vaccine with high titered, type specific bactericidal antibodies, vaccine induced human antibodies showed no detectable type specificity. The presence of type

specific bactericidal antibodies in convalescent sera from patients with systemic meningococcal disease suggests that the failure of the volunteers to make type specific bactericidal antibodies was due to alteration or loss during purification of the type-specific protein determinant to which humans are capable of responding. This determinant is apparently more labile than the type specific determinant to which rabbits respond. This conclusion is supported by the observation that the purified protein, e.g. vaccine lot 138I-0, is able to inhibit the type specific bactericidal activity of rabbit sera but not of human case sera.

The results of HA tests and solid phase radioimmunoassays (data not shown) indicate that the volunteers developed some increase (2-4 fold) in antibodies against determinants of the protein. These antibodies, however, did not appear to have bactericidal activity and it has not yet been determined whether they have type specificity or are able to opsonize bacterial cells for phagocytosis.

It is not clear how the vaccine, consisting of protein or protein and C<sub>555</sub>, and prepared from a group C strain, was able to induce increases in bactericidal antibodies with group B specificity. This group specificity, as well as the results of the inhibition studies, strongly suggest involvement of the B<sub>555</sub>. However, it may be a particular conformation of the B<sub>555</sub> or a complex of it with a second antigen. Other possibilities include: (1) The vaccine produced a non-specific stimulation of the immune system, which might be tested by looking for rises to unrelated antigens against which most people have antibodies. It may be significant in this regard that the preexisting bactericidal antibodies in a normal serum that was studied appeared to have the same pattern of inhibition as the postvaccination sera. (2) A minor component of the vaccine was sufficiently cross-reactive with the B<sub>555</sub> or something closely associated with the B<sub>555</sub> to stimulate production of antibodies against it. (3) The vaccine may have been contaminated with some B<sub>555</sub>. However, when we inhibited the B<sub>555</sub> HA test with up to 1 mg/ml of vaccine (lot 138I-M-1) no inhibition was observed with either rabbit antiserum or human antiserum (two week postvaccination serum from W.Z.). Therefore, any gross contamination of the vaccine with B<sub>555</sub> seems unlikely. (4) The group B specific bactericidal antibodies were produced in response to nasopharyngeal carriage of group B organisms. Strains of group B meningococci, however, were cultured from the throat of only one of the volunteers (W.Z.) who developed increased anti-group B bactericidal antibodies. It is clear that further studies are required to determine the mechanism of induction of group B specific antibodies.

Regardless of its immunological mechanism, the induction of bactericidal antibodies with group B specificity is certainly a desirable result with respect to development of a vaccine against this serogroup, especially since attempts to induce group B specific bactericidal antibodies with purified polysaccharide have thus far been unsuccessful.

Finally, it is clear that the rabbit is a poor model for testing the immunogenicity of meningococcal protein serotype antigens in humans. Future vaccine preparations will need to be tested for their ability to specifically react with the type specific bactericidal antibodies in human case serum.

## II. Bactericidal serotyping of meningococci.

### A. Determination of serotypes of disease-producing strains of Neisseria meningitidis.

Classically, strains of N. meningitidis have been divided into serogroups on the basis of the antigenic specificity of their capsular polysaccharides, denominated by letters, of which groups B, C, and Y currently account for 90-95 percent of all isolates from human cases in the United States. More recently, sub-capsular antigens against which protective bactericidal antibodies are directed have been utilized to divide strains of N. meningitidis into distant serotypes which are independent of their capsular polysaccharide serogroup and denominated by roman or arabic numerals [5,6]. Serotyping of meningococci of the four important serogroups (A, B, C and Y) has been accomplished using bactericidal and bactericidal inhibition assays, as well as micro-precipitin and agar gel double diffusion techniques with similar results [5,6,7]. The responsible antigens have been localized to the outer membrane of the cell envelope and at least one major serotype antigen (CII-B2) has been identified as protein in nature [8,9].

Two separate serotyping systems have evolved. One, developed by Gold [5], utilizes antisera to group C meningococci, cross-absorbed with heterologous group C organisms to remove antibody to the group specific capsular polysaccharide, and tested in a bactericidal assay. Serotypes are denominated by roman numerals and will be referred to as the Gold system or by the letter "C" and the appropriate numeral (i.e., CII). The second system, developed by Frascch and Chapman [6], utilizes either absorbed or unabsorbed antisera to group B organisms; initially in a bactericidal assay, but subsequently in microprecipitin and agar gel double diffusion systems [6,10]. Serotypes are denominated by arabic numbers and will be referred to as the Frascch system or by the letter "B" and the appropriate numeral (B2). The most prevalent serotype in each system (CII/B2) has been shown to be antigenically identical [10]. Since the antigenic specificity of serotype antisera is independent of serogroup, either system can be used to type strains of serogroups other than the prototype strains [7,10].

The Gold system has been developed extensively since his initial description of six types [5]. Both the antigenic specificity of existing types and additional serotypes have been sought [7,8,9], and further cross-absorptions carried out in an attempt to more precisely isolate specificity for each type.

Ninety-seven strains of N. meningitidis were selected for study from the collection of the Department of Bacterial Diseases. Twenty-four strains of serogroups B and Y isolated from military recruit patients at Fort Dix, NJ and Fort Leonard Wood, MO, from December 1969 through October 1971, and representing all strains of these two serogroups recovered during this time frame, were included. A sample of 42 serogroup C strains isolated from the same two Army posts over the same time period were selected to coincide temporally as nearly as possible to the time of isolation of the group B and Y strains and to nearly represent each of the 23 months of this period. There was no other selection bias. These strains will be referred to as "epidemic" strains. The remaining 31 strains of serogroups B, C, and Y, for which serotype data were available, were isolated from both civilian and military patients over a 10 year period from widely scattered geographic areas (Table 5). These strains will be referred to as "non-epidemic" strains. For completeness of comparison, serotype data from five serogroup A strains previously reported from this laboratory [7] were included in this analysis for a total of 102 strains. Strains were serotyped with the radioactive bactericidal test (RBCT [4,11]).

Of the 102 strains, 52 were group C, 28 group B, 17 group Y and 5 group A [7]. Forty-nine were isolated from patients at Fort Dix (14 B, 28 C, 7 Y) and 24 at Fort Leonard Wood (18 C, 6 Y). The remainder (5 A, 14 B, 6 C, 4Y) were of world wide origin (Table 5). Two group B and one group C strains were isolated from the nasopharynx, while the origin of one A and two B strains were obscure or lost. The remaining 96 strains were isolated from cerebrospinal fluid or blood.

Sixteen antisera against potential serotypes were investigated. Antisera against types I-VI were originally described by Gold, et al. [5]; types IIIa and b, IVa and b, and IX were variations on these original serotypes; VII was a variation of the serotype VII of Kasper, et al. [7], while VII, X, XI and XII were developed from antisera to group B strains which were thought to possess unique types.

The 16 serotypes were shared broadly by strains of all four serogroups (Table 6). The greatest diversity was seen in group B strains, which were of wide geographic and temporal origin, the least in group A. Three group B strains were killed by none of the 16 antisera. The relative preponderance of types II, III and VI in group C strains were due to the large number of antigenically similar epidemic strains of this serogroup from Fort Dix and Fort Leonard Wood (42 or 52) [5].

Since the array of antigens of neither the immunizing nor absorbing strain is precisely known, the observed broad cross-reactions between strains were not unexpected. However, the immunizing and absorbing strains for several of the antisera were initially chosen in the expectation that they would increase the specificity for a given serotype. In Table 7a, for example, are listed the cross-absorptions undertaken in an attempt to separate the broad but incompletely

Table 5. Characterization of 102 strains of *N. meningitidis*.

Origin <sup>1</sup>	Date	Status <sup>2</sup>	Serogroup				Total
			A	B	C	Y	
Dix	1964-1968	Mil, Car	-	4	3	-	7
Dix	1969-1971	Mil	-	11	25	7	43
L-W	1966-1971	Mil	-	-	18	6	24
Non-U.S. <sup>3</sup>	1964-1973	Mil, Civ	4	1	5	-	10
Knox, Sill, Polk, WR, Ord		Mil, Civ	-	7	1	3	11
Misc. U.S.		Mil, Civ, Car	1	5	-	1	7
Total			5	28	52	17	102

<sup>1</sup> Dix = Fort Dix, NJ; L-W = Fort Leonard Wood, MO; Knox = Fort Knox, KY; Sill = Fort Sill, OK; Polk = Fort Polk, LA; WR = Walter Reed Army Medical Center, Wash, DC; Ord = Fort Ord, CA.

<sup>2</sup> Mil = military recruit; Civ = civilian; Car = carrier strain.

<sup>3</sup> Germany 3C, 2A; Brazil 2C; Canada 1B; Mali 1A, Morocco 1A.

Table 6. Distribution of serotype antigens in *N. meningitidis* by serogroup (A, B, C and Y).

Serotype	Serogroup <sup>1</sup>				All strains
	A (5)	B (28)	C (52)	Y (17)	
I	0	7.1	9.6	17.6	9.8
II	20 <sup>2</sup>	53.6	86.5	52.9	68.6
III	0	75.0	84.6	76.5	77.5
IIIa	- <sup>3</sup>	36 (25)	37.5 (8)	100 (1)	38.2
IIIb	-	32 (25)	11.1 (9)	0 (1)	25.7
IV	20	21.4	19.2	52.9	25.5
IVa	-	12.0 (25)	44.4 (9)	0 (1)	20
IVb	-	61.5 (26)	77.8 (18)	83.3 (6)	70
V	80	46.4	53.8	70.6	55.9
VI	0	25.0	40.4 (47)	35.3	35.1 (97)
VII	0	0 (26)	16.7 (18)	0 (6)	6
VIII	-	15.4 (26)	16.7 (18)	33.3 (6)	18
IX	-	19.2 (26)	38.9 (18)	0 (6)	24
X	-	15.4 (13)	0 (15)	0 (6)	5.9
XI	-	16.7 (12)	0 (15)	0 (6)	6.1
XII	-	7.7 (13)	6.7 (15)	0 (6)	5.9

<sup>1</sup> ( ) = number tested. Not all strains were tested for all serotypes.

<sup>2</sup> Percentage of tested strains of each serogroup positive for a given serotype.

<sup>3</sup> - = not tested.

Table 7. Separation of serotype III and IV specificities.

a. Rationale for choosing immunizing and absorbing strains

Serotype designation	Immunizing strain	Serotype <sup>1</sup>	Absorbing strain	Serotype	Expected sero-type specificity
III	C-126E	III, IV	C-60E	I	III, IV
IIIb	C-118V	IV, III	C-118V	IV, III	III
IVb	C-118V	IV, III	C-89I	IV, VIII	III
IV	C-118V	IV, III	C-60E	I	IV, III
IVa	C-126E	III, IV	C-126E	III, IV	IV
IIIa	C-126E	III, IV	C-60E + B-190I	VIII, III, I	IV

<sup>1</sup> Major serotype listed first

b. Frequency of occurrence of serotypes by serotype - percentage of tested strains of each serotype positive for a given second serotype.

Serotype	III	IIIb	IVb	IV	IIIa	IVa
III	-	33.3	89.5	29.1	54.2	20.8
IIIb	88.9	-	55.6	22.2	44.4	11.1
IVb	97.1	23.8	-	31.4	61.9	19.0
IV	88.5	22.2	78.6	-	62.5	44.4
IIIa	100	30.8	100	38.5	-	15.4
IVa	71.4	14.3	57.1	57.1	28.6	-

Table 8. Frequency of occurrence of serogroup and serotype antigens in *N. meningitidis* by serotype.

Strains	Serogroup						Serotype					
	A	B	C	Y	I	II	III	IV	V	VI		
All	4.9 <sup>1</sup>	27.5	51	16.7	9.8	68.6	77.5	25.5	55.9	35.1		
Serotype												
I	0	20	50	30	-	30	70	60	50	20		
II	1.4	21.4	64.3	12.9	4.3	-	87.1	12.9	64.3	50.8		
III	0	26.6	55.7	16.5	8.9	77.2	-	29.1	54.4	42.5		
IV	3.8	23.1	38.5	34.6	23.1	34.6	88.5	-	38.5	28.0		
V	7	22.8	49.1	21.1	8.8	78.9	75.4	17.5	-	29.6		
VI	0	20.6	61.8	17.6	5.9	97.1	91.2	20.6	47.1	-		

<sup>1</sup> Percent of tested strains of each serotype positive for a given second serotype or serogroup.

Table 9. Patterns of association of serotypes I through VI<sup>1</sup> for 102 strains of *N. meningitidis*.

Serotype <sup>1</sup>	No. of strains <sup>2</sup>					Associated serotypes <sup>3</sup>					
	Gr A	B	C	Y	Total	I	II	III	IV	V	VI
I	0	1	1	0	2	+	-	-	-	-	-
I	0	1	1	0	2	+	-	v	-	v	-
I	0	0	1	2	3	+	-	+	+	v	v
I-II	0	0	2	1	3	+	+	+	+	v	v
II	0	1	1	0	2	-	+	-	-	-	-
II	0	12	34	4	50	-	+	+	-	v	v
II	0	1	4	3	8	-	+	+	+	v	v
II	1	1	4	1	7	-	+	-	-	+	v
III	0	3	0	0	3	-	-	+	-	-	-
IV	1	1	1	0	3	-	-	-	+	-	-
IV	0	4	3	0	7	-	-	+	+	-	-
IV	0	0	0	3	3	-	-	+	+	+	-
V	3	0	0	3	6	-	-	-	-	+	-
VI	0	0	0	0	0	-	-	-	-	-	+
Untypable	0	3	0	0	3	-	-	-	-	-	-

<sup>1</sup> After Gold, et al. [5]

<sup>2</sup> By serogroup

<sup>3</sup> + =  $\geq 20\%$  maximum <sup>14</sup>C-release by antisera against indicated serotype;

- =  $< 20\%$  maximum <sup>14</sup>C release; v = variably associated.

Table 10. Association of type III strains with other serotypes<sup>1</sup>.

Serotype <sup>1</sup>	Associated serotype	No. of strains (%)			
		Total	B <sup>2</sup>	C	Y
I & II	III-V	24 (40.0)	6 (42.9)	13 (36.1)	5 (50)
"	III-VI	18 (30.0)	1 (7.1)	12 (33.3)	5 (50)
"	III-V-VI	13 (21.7)	6 (42.9)	7 (19.4)	0
"	III	5 (8.3)	1 (7.1)	4 (11.1)	0
<b>Total</b>		<b>60 (100)</b>	<b>14 (23.3)</b>	<b>36 (60)</b>	<b>10 (16.7)</b>
I	III-V	4 (57.1)			
"	III-VI	2 (28.6)			
"	III-V-VI	0 (0)			
"	III	1 (14.3)			
<b>Total</b>		<b>7 (11.7)</b>			
II	III-V	20 (37.7)			
"	III-VI	16 (30.2)			
"	III-V-VI	13 (24.5)			
"	III	4 (7.6)			
<b>Total</b>		<b>53 (88.3)</b>			
IV	III-V-VI	12 (20.0)			

<sup>1</sup> As defined by Gold, et al. [5].

<sup>2</sup> By serogroup.

overlapping specificities of type III and IV antisera. In Table 7b it can be seen that while antisera IVa and IVb provided a modest improvement in specificity, monospecific antisera was hardly achieved. Similar failures to isolate specificities were noted for the combinations of I, V, and IX antisera.

Types VII, X, XI and XII proved more useful, particularly in distinguishing group B strains, but were of such a low frequency of occurrence, particularly in the epidemic strains from Fort Dix and Fort Leonard Wood, that they were deleted when testing the latter 50 strains from these two locations.

#### Linkage of serotypes.

All 102 strains were tested for the original six Gold serotypes. Sixteen were positive for only one type; the remainder for multiple types. To determine if multiple types occurred in the same strain independently of one another, the frequency of occurrence of a given serotype with any other serotype was compared with its frequency of occurrence among strains of all serotypes (Table 8). Using this criterion, several consistent patterns of association between serotypes were apparent (Table 9).

Types I and IV were positively associated (60 percent of type I strains were also type IV, versus 25.5 percent of all strains; and 23.1 percent vs. 9.8 percent of type IV strains were type I), and negatively associated with type II. Types III and VI were positively associated both with one another and with type II. Type V was also positively associated with type II but negatively associated with types IV and VI. Since both the combination III-VI and V were positively associated with type II, their combined occurrence was analyzed, both independently of their association with either of the negatively associated pair I or II and by association with types I or II separately (Table 10). Of 60 such strains, 40 percent were III-V, 30 percent III-VI and 21.7 percent III-V-VI; only 8.3 percent of type III strains associated with either type I or II were neither V nor VI. Results were not significantly different when broken down by association with I or II separately, nor when broken down by serogroup (Table 10). None of the combinations occurred in any of the five group A strains serotyped by Kasper, et al. [7], and the combination III-V-VI was absent from group Y and type I strains. Number of strains tested in these three categories were small, however, and the results probably reflect selection bias. Furthermore, there was no correlation between combinations of types III, V and VI and time or place of isolation for group C strains. Among group B and Y strains, however, type VI was infrequent at Fort Dix (2/11 B; 1/7 Y) and type V common (6/11 B; 7/7 Y), while the reverse was true of group Y strains from Fort Leonard Wood (4/6 type VI; 2/6 type V).

In order to confirm that the linked serotypes III-V-VI were independently associated with type I (or its associated pair, IV), but positively associated with type II, the frequency of association of types I, II or IV with any of the four combinations of III, V and VI was compared with their frequency among the total 102 strains (Tables 8 and 10). Types I and IV were no more frequent among III-V-VI strains than among all strains (11.7 vs. 9.8 for type I, 20.0 vs. 25.5 for type IV), but type II was significantly more common (88.3 vs. 68.6). This linkage of type II with some combination of III, V, and VI was seen in both epidemic and nonepidemic strains and was not changed by the addition of type V and VI strains which were negative for type III (vide infra).

Types III and IV were also positively associated independent of their respective associations with II and I. Seven of 16 type IV strains of serogroups B and C were also III, and an additional three group Y strains had the combination III, IV, V. Finally, three group B strains were type III alone, while another three strains (1A, 1B, 1C) were type IV alone.

Type V, although most commonly linked with III and VI or III and IV (75 percent), also occurred alone in six group A and Y strains (10.5 percent); type VI, however, never occurred alone.

#### Effect of growth and complement on serotype determination.

Two variables markedly effect the determination of certain serotypes by the bactericidal assay. Strains appear to express different antigens as loci of bactericidal activity at different stages during their growth, over time. This effect is shown in Table 11, where the optical density (O.D.) of the organisms at the time of harvest is used as a crude marker of stage of growth. Depending upon this final O.D., quite different results are obtained for serotypes III and V antisera, but not for serotype II antisera. Moreover, the point at which strains display sensitivity to each of the former two antisera varies between strains without any consistent pattern. During the experiments reported here every effort was made to grow strains so as to demonstrate type III positivity and, to a lesser extent, type V.

Weanling rabbit sera, purchased from a single source, were used as a complement source in all experiments. However, as can be seen in Table 12, not all lots of sera were equally efficient in killing strains in the presence of antisera against type III antisera. Again, this was not true in the presence of antisera against type II. The effect of complement lot on positivity with type V antisera was similar to that shown with type III antisera. Only complement lots which gave positive results with type III antisera were used for these experiments.

Table 11. Effect of growth-time on serotype expression as determined by bactericidal assay.

Organism	O.D.	Serotype:	(% maximum <sup>14</sup> C-release)		
			II	V	VI
C-6229 <sup>1</sup>	.550		97.9	23.2	100
	.636		87.0	68.1	100
	.694		95.1	87.9	100
	.707		100	85.2	63.0
	.783		83.2	76.5	100
	.960		90.0	76.8	100
			II	III	V
C-6271 <sup>2</sup>	.685		100	22.5	27.7
	.764		100	1	9.1
C-6247 <sup>2</sup>	.650		91.4	0	100
	.795		97.6	100	92.4

<sup>1</sup> Strain tested at six separate optical densities (O.D.) during one continuous growth.

<sup>2</sup> Strain tested at two separate O.D.'s on separate days.

Table 12. Effect of different complement lots on serotype as determined by bactericidal assay.

Organism	Complement Lot <sup>1</sup>	14C-release			% maximum release		
		Type	II	III	Type	II	III
B-6548	1		36.8	5.1		89.3	12.4
	2			6.9			16.7
	3			23.4			56.8
	4		41.2	24.0		100	58.3
	8			6.5			15.8
C-6259	3		41.2	31.5		100	76.5
	5		25.8	3.0		62.6	7.3

<sup>1</sup> All complement lots were weanling rabbit sera purchases from the same source at different times. No lot contained bactericidal antibodies against the indicated strain.

### Conclusions:

The data reported here provide further confirmation of the arbitrary but epidemiologically valid system utilized by Gold, et al. in their original paper [5]. Positivity for types III, V and VI was much greater in this series than in theirs or in the series reported by Kasper [7]. This can be accounted for by the use of 20 percent kill as the cut-off for positivity, as opposed to 50 percent; by the selection of complement lots and by the close attention to the stage of growth at which each strain was tested. The rationale for this approach was the belief that if any sub-population of a given strain expressed an antigen as a locus of complement-mediated bacteriolysis under any conditions, that strain possessed that antigenic potential and should be considered positive for it.

The wide cross-reactivity observed between strains of meningococci is analogous with that seen in other gram negative organisms, notably the Salmonellae [12]. In addition to their serogroup specific capsular polysaccharides, meningococci possess cell-wall proteins of multiple antigenic specificities [9,10], as well as endotoxic lipopolysaccharides (LPS) with multiple, separate specificities [13,14]. Lapine antibodies directed against the cell wall proteins have been shown to be bactericidal, and both humans and rabbits have been shown to respond immunologically to determinants of the LPS, although the bactericidal capacity of these antibodies is not known. To date, only the specificity of type II antisera (CII-B2), an outer cell membrane protein, is known with any precision.

Immunochemically, serotype determinants appear to be of two sorts. The first, typified by CII-B2, is a protein, expressed throughout the growth cycle of the organism. Lapine antibodies against it are able to invariably activate complement and its presence is associated with epidemic potential [5,10]. The second, typified by type CIII, is of unknown chemical type and expressed only during delimited stages of growth. Antibodies to it vary in their ability to activate complement, and its role in host resistance/susceptibility is unknown. Determinants of the two types may be linked with one another as in the II:III combination.

Experiments were designed to investigate either differences in complement lots or expression of antigenic determinants as a function of growth phase. Possible explanations for the first phenomenon include the presence of IgA blocking antibodies in the complement sera [11], synergy between antibodies of different classes or specificities in the complement and typing sera, or differences in the spatial configurations of antigens on the surface of the meningococcus which either facilitate or hinder complement activation [12]. The second phenomenon, the plasticity of antigenic expression, is well known for other gram negative and positive bacteria, including members of the Neisseriae [15], and its effect on the sensitivity of Escherichia coli to bactericidal antibody has been demonstrated [12]. The fact

that antigenic plasticity has been shown only for carbohydrate antigens [12,15], combined with the absence of such plasticity for the CII-B2 protein antigen, suggests that the plastic and complement variable antigens are contained within the lipopolysaccharide moiety of the outer membrane complex [8]. Such an hypothesis is in accord with those proposed for the Enterobacteriaceae [12].

It is furthermore clear that cross-absorptions to remove antibody components and render sera monovalent is no more likely to succeed with N. meningitidis, than it has with the Salmonellae [12], and that further understanding of the role of serotype antigens in meningococcal immunity must await more precise localization and chemical characterization.

The final system used for the study of epidemic strains from Fort Dix and Fort Leonard Wood (Table 9) differed little from that originally reported from this laboratory [5]. Seven strains positive for type I were considered to be type I whether occurring alone or linked with III-V-VI or IV. Sixty-seven strains positive for type II were considered type II, regardless of linked types. Three strains positive for III alone were considered to be III, while 13 strains were considered type IV, whether positive for it alone or in combination with type II (B and C strains), or both III and V (group Y strains). Type V strains consisted of those positive for this antisera alone. Finally, three strains (2 C, 1 Y) were found to be positive for five separate serotypes (I, II, III, IV, V or I, II, III, IV, VI). These were considered to represent a combination of the linkages I:IV and II:III-V-VI, and were thus considered to be of two serotypes, denominated I-II.

#### B. Epidemiology of serotypes.

Fort Dix: From 1 December 1969 through 31 October 1971, 137 strains of N. meningitidis were isolated from military recruit cases of meningococcal disease at Fort Dix, NJ. Of these, 119 were of serogroup C (86.9 percent), 11 (8 percent) of serogroup B and 7 (5.12 percent) of serogroup Y. There were no isolates of serogroup A. One hundred and five (76.6 percent) of these strains were isolated during one of the 17 months surveyed during this period (Table 13). All 18 strains of serogroups B and Y were serotyped, while 25 of the 87 (28.7 percent) group C strains were tested. Twenty-four (96 percent) of the group C strains, 7 (63.6 percent) group B, and 3 (42.9 percent) group Y strains were type II. One of the group C strains was also type I (I-II). The remaining group C and B strains were type IV, along with two group Y strains. One each of the remaining group Y strains were types I and V (Table 13)..

More importantly, from 1 December 1969 through 30 April 1971, all strains of both group C and group B (30/30) were type II (Table 13). Beginning in February 1971, all incoming recruit trainees were routinely vaccinated with the group C specific polysaccharide vaccine,

Table 13. Serotypes of 43 strains of pathogenic *N. meningitidis* at Ft. Dix, NJ: Dec. 1969-Oct. 1971.

Month	No. <sup>1</sup>	Serogroup C					Serogroup B					Serogroup Y						
		I	II	III	IV	V	No.	I	II	III	IV	V	No.	I	II	III	IV	V
Dec 69	3(5)	1	3	(1 strain:I-II)			2(2)						1					1
Jan 70	1(2)		1															
Feb 70	1(7)		1															
Apr 70	2(16)		2															
May 70	3(7)		3															
Jul 70	2(15)		2															
Aug 70	1(1)		1															
Sep 70	1(3)		1				3(3)	3										1
Oct 70	3(12)		3				3(3)	3										1
Nov 70	2(6)		2															
Feb 71	3(10)		3															1
<b>Vaccine 2</b>																		
Mar 71	1(1)		1															1(1)
Apr 71	(0)						1(1)	1										
May 71	1(1)		1				2(2)				2							1(1)
Jun 71	(0)					1	1(1)				1							
Jul 71	(0)						1(1)				1							
Oct 71	1(1)		1				1(1)				1							
<b>Total 25(87)</b>		24	1	11(11)	7	4	7(7)	1	3	2	1							

<sup>1</sup> Number of strains tested for each month of each serogroup. Number in parenthesis is number of confirmed cases of each serogroup for that month.

<sup>2</sup> Group C-specific polysaccharide vaccine administered to all in-coming recruits.

after which almost all group C disease ceased [16]. From May, 71, through July, 71, however, there were four cases of group B disease and one case of group C disease in an unvaccinated recruit. All five of these strains, of both serogroups, were of serotype IV, indicating a switch in the prevailing pathogenic serotype. One additional case of group C disease occurred in Oct, 71; this strain was again type II. No such temporal correlations of serotype were seen among disease-producing strains of group Y; the four separate serotypes were randomly distributed among these seven strains.

Fort Leonard Wood: Similar clusters of group C and B disease were not found at other recruit training centers during the same period; however, similarly concurrent group C and Y disease did occur at Fort Leonard Wood, and strains from this post were surveyed in a similar manner (Table 14).

During the period 1 Jan, 1970, through 31 Oct, 1971, there were 74 strains of N. meningitidis isolated from cases of meningococcal disease in recruits. Sixty-eight (91.9 percent) were of serogroup C and six (8.1 percent) serogroup Y. One additional isolate of group B was not available for testing. All 74 strains were isolated during one of the 15 survey months. All six strains of serogroup Y were serotyped and 17 (25 percent) of serogroup C.

It can be seen in Table 14 that, just as at Fort Dix, serotype II was the dominant serotype among group C strains, occurring in all 17, with one being a type I-II. The six group Y strains again were of several different serotypes. In contra-distinction to the findings at Fort Dix, however, serotype II was not only dominant (four of six), among group Y strains, but the four type II strains occurred sequentially between Nov, 1970, and May, 1971. There was no change in the prevailing serotypes of disease-producing group C strains after the advent of routine vaccination.

#### Relationship between serotype and susceptibility to sulfadiazine.

Ninety-three of the 102 strains were tested for sensitivity to sulfadiazine, using an MIC of one mcg/ml as the upper limit of sensitivity (Table 15). Forty-five of 50 (90 percent) group C strains were resistant by this criterion, while only six of 23 (26 percent) group B strains and one of five (20 percent) group A strains were resistant. All 15 group Y strains tested were sensitive. As can be seen in Table 15, there was no correlation between serotype and sulfonamide sensitivity except for group C strains which were type II. All 43 of such group C strains were resistant. Nineteen of 23 type II strains of serogroups A, B and Y, however, were sensitive.

Table 14. Serotypes of 23 strains of pathogenic N. meningitidis at Fort Leonard Wood, MO: Jan., 1970-Oct., 1971.

Month	No. <sup>1</sup>	Serogroup C					Serogroup Y						
		I	II	III	IV	V	No.	I	II	III	IV	V	
Jan 70	1(2)		1										
Feb 70	1(6)		1										
Mar 70	1(30)		1										
Apr 70	1(1)		1										
Jul 70	2(2)		2				1(1)						1
Aug 70	1(1)		1										
Oct 70	1(1)		1										
Nov 70	1(2)		1				1(1)			1			
Dec 70	1(1)		1				1(1)			1			
Feb 71	2(4)		2										
Mar 71	2(14)		2				1(1)			1			
Apr 71	1(2)	1	1		(1 strain: I-II)								
May 71	1(1)		1				1(1)			1			
Sep 71	0(0)						1(1)			1			
Oct 71	1(1)		1										
Total	17(68)	1	17				6(6)	1	4				1

<sup>1</sup> Number of strains tested for each month of each serogroup. Number in parentheses is number of confirmed cases of each serogroup for that month.

<sup>2</sup> Group C-specific polysaccharide vaccine administered to all in-coming recruits.

Table 15. Relationship between serotype and sulfadiazine sensitivity of 100 strains of N. meningitidis of four separate serogroups.

Serotype	Serogroup A		Serogroup B		Serogroup C		Serogroup Y	
	Sens <sup>1</sup>	Resis <sup>1</sup>	Sens	Resis	Sens	Resis	Sens	Resis
I	(7/7) <sup>2</sup>		2		3		2	
I-II	(3/3)					2	1	
II	(63/67)	1	10	4		41	7	
III	(1/3)			1				
IV	(11/13)	1	3	1	2	2	2	
V	(6/6)	2						3
NT <sup>3</sup>	(2/3)		2					
Total	(93/102)	4	17	6	5	45	15	

<sup>1</sup> Sensitive = MIC  $\leq$  1 mcg sulfadiazine per ml.  
Resistant = MIC  $>$  1 mcg sulfadiazine per ml.

<sup>2</sup> Numbers in parenthesis = number of strains of a given serotype tested/number of strains of that serotype.

<sup>3</sup> NT = nontypable.

### Conclusions.

These data confirm the epidemiological importance of determining the serotype of infecting strains of *N. meningitidis* regardless of serogroup, since meningococcal strains of different serotypes clearly vary in their ability to produce epidemic disease. It has previously been reported that group C strains of serotype CII-B2 were responsible for epidemics among military recruits at widely separated locations within the United States [5], and among civilians in North and South America [10]. The present data confirm this association for group C disease in military recruits and extend it to intercurrent group B disease. Up until the advent of routine immunization with the group C polysaccharide vaccine, all epidemic strains of both groups B and C at Fort Dix were of serotype CII-B2. It is intriguing that following routine vaccination four temporally related group B strains associated with one group C strain were of serotype CIV. Type CIV is antigenically similar to B11. Munford, et al. [10] found that 88 of 93 typable group C strains of wide geographic and temporal origin were either B2, B11 or both. Of the five strains which were neither, four were pharyngeal isolates. Gold, et al. [5] also found that CIV was the predominant serotype in military recruits at posts other than Fort Dix prior to the emergence of CII strains. Among military dependents and civilians, however, CIV was less common. These findings suggest that the epidemic potential of groups B and C meningococci of serotype CII-B2 exceeds that of serotype CIV-B11, but that both are responsible for most epidemic disease in closed populations.

It is unclear, however, whether the serotype represents a "virulence" factor or is a marker for host susceptibility. Kasper, et al [7] have demonstrated that protective bactericidal antibodies in human convalescent sera following group B disease are directed against serotype antigens rather than the capsular serogroup antigen, while isolated and purified group B capsular polysaccharide has been shown not to be immunogenic in humans [3]. It seems reasonable to assume that serotypes CII-B2 and CIV-B11 provide epidemic potential to group B meningococci by virtue of the relative absence of bactericidal antibodies against these determinants in a given population. The emergence of CII-B2 as the major epidemic strain for groups B and C might then be more an expression of the prior antigen experience of a population than of the inherent virulence of a given strain.

This hypothesis subsumes that the role of protective bactericidal antibodies against the capsular polysaccharide of group C meningococci is the same as that against group B capsular polysaccharide. The finding that these two serogroups track together, epidemiologically, by serotype, rather than serogroup, supports this assumption. Furthermore, though the group C polysaccharide is a potent immunogen in man after either infection or vaccination with the purified antigen, levels of antibody against it in recruit populations upon entrance into

military service are very low [17]. Functionally, therefore, the majority of incoming recruits may be assumed to have no bactericidal antibodies to either capsular polysaccharide. Protection for these individuals would then reside with bactericidal antibodies against subcapsular serotype determinants. The final epidemiologic event, meningococcal disease, would then reflect the relative proportion of serogroups B and C carrier strains of a given serotype against which bactericidal antibodies were absent.

These data further support the concept that the relevant serotype antigen is the protein determinant CII-B2, rather than the linked serotypes CIII, CV and CVI, since these serotypes, when linked with CI, do not increase its epidemic potential. However, it would be naive to assume that all the important serotype determinants are represented within this system. The CII-B2 determinant might be only a marker for other more important immunologic characteristics, such as an increased potential for pharyngeal carriage. Alternatively it is possible that the chemical link between CII-B2 and CIII itself represents the critical antigenic determinant.

Group Y meningococci have not, as yet, demonstrated significant epidemic potential in either civilian or military populations despite the fact that they are often the most prevalent serogroup in carriers [18,19]. Meningococcal disease produced by them, however, is indistinguishable from that produced by other serogroups [19], suggesting equal "virulence". When the ability of normal sera from incoming recruits to kill a single strain of groups A, B, C and Y was compared [18], over 90 percent of 253 sera were bactericidal for group Y vs. 67-78 percent for the other three serogroups. More impressively, when values for recruits destined to develop meningococcal disease were compared, 91 percent of 23 sera were bactericidal for group Y vs. 8.7-17.4 percent for the other three. These data strongly suggest that the low epidemic potential of group Y meningococci is a function of increased host resistance. The observed epidemiological independence of serogroup Y strains, as determined by serotyping, is therefore not surprising, and suggests chance selection of those few individuals nonimmune to group Y strains, independent of serotype.

The finding that the prevailing serotype of group B strains at Fort Dix switched from CII-B2 to CIV-B11 after eradication of group C disease by group-specific vaccination is highly intriguing, but unexplained.

Finally, it has been reported that serotype CII-B2 and sulfonamide resistance are significantly associated in group C strains. Although our data is in accord with this finding for group C strains, it is quite clear that no such association exists between serotypes and sulfonamide sensitivity for other serogroups. It would appear more likely that serotype antigens, chemical composition of capsular

polysaccharide and sulfonamide sensitivity are under separate genetic control. Only extensive serotyping of group C strains isolated before the emergence of sulfonamide resistance can answer this question.

### III. Studies of the immunochemistry and serology of *Neisseria meningitidis* lipopolysaccharides (LPS).

The immunochemical characterization of the lipopolysaccharide (LPS) moiety of the outer membrane complex (OMC) has been hindered by the complex nature of the antigen (a hydrophobic lipid moiety bound to a hydrophilic polysaccharide) and methodological problems in quantifying the immune response to it. Two approaches to this problem were developed during the past year. The first involved determination of the LPS serotype of meningococcal strains by inhibition of a standard hemagglutination assay (HAI). The second involved modification of a Farr-type primary radioactive antigen binding assay (RABA), which measures antibodies of all major classes, and which was used to obtain preliminary data on the antigenic diversity within the LPS molecular and variations of host response to them.

#### A. LPS serotyping by hemagglutination inhibition.

LPS for these studies was purified from intact organisms of their OMC by standard methods and were shown to be free of significant protein, capsular polysaccharide, unbound lipid or nucleic acid contaminants.

The standard hemagglutination assay (HA) employed high titered (1:128-1:2048), unabsorbed rabbit antisera raised to live meningococci and having a relatively low level of cross reactions with heterologous LPS's. Sheep red blood cells (SRBC) were optimally sensitized with alkaline treated LPS antigen [14].

For LPS serotyping by hemagglutination inhibition, 0.025 ml of each prototype antiserum, diluted to contain eight HA units of antibody, was mixed in the wells of a microtiter plate with 0.025 ml of either phosphate buffered saline (PBS) as a control or the inhibiting LPS antigen diluted in PBS to a standard concentration (125 µg/ml and 31 µg/ml). After incubation at 37°C for one hour, 0.05 ml. of 0.5 percent sensitized SRBC was added, the plate rotated to mix the reagents and then left at room temperature for two hours.

The agglutination pattern of wells containing inhibitor were compared to that of the negative control well containing no inhibitor and to the positive control wells containing homologous antigen as inhibitor. Agglutination was scored on a 0 to +4 scale where the negative control gave +4 and the positive control 0 or +1 at both antigen concentrations. A heterologous LPS was interpreted to have

the serotype(s) defined by a given HA reaction if the higher concentration gave 0 or +1 agglutination and the lower concentration 0 to +2. LPS antigens which gave 0 to +2 at the higher concentration but +3 to +4 at the lower concentrations were given that serotype in parentheses.

Eight rabbit antisera with distinctly different serologic specificities for meningococcal LPS's were chosen as prototype antisera. In Table 16 the results of a grid inhibition experiment are shown. The same results were obtained whether purified LPS or the OMC was used as the inhibiting antigen. Each of the homologous reactions was used to define a different LPS serotype determinant and if a heterologous LPS antigen inhibited one of these reactions it was designated as having that type. Some antigens inhibited only one reaction, while others inhibited two or more, suggesting the presence of multiple determinants on the LPS.

Using these eight prototype HA reactions we have determined the LPS serotype of 57 additional meningococcal strains. These results are given in Table 17. All strains were typable and all but 18 of the total of 65 strains inhibited more than one of the prototype reactions.

Table 18 gives the frequency with which the eight prototype reactions were inhibited by the OMC of the 65 strains tested. These results include both strong and weak inhibition. Types 3 and 7 were most common, often appearing together on the same antigen.

The eight LPS prototype strains were selected from among strains previously shown to have different standard serotypes. With one exception they are among the prototype strains used for preparation of serotyping antisera at Walter Reed Army Medical Center and elsewhere (4 Gold system, 3 Frasch system).

The relationship of the LPS and standard or "protein" serotype determinants is shown in Table 19 which lists both the LPS type and the "protein" type of the two sets of standard serotyping strains. One Gold strain, 32I (Type VI), is missing from the WRAMC set. The two systems have not yet been fully correlated and the typing of each set with the other set, based on a solid phase radioimmune inhibition procedure (vide infra) is preliminary. It may be seen from the results in this table that there is some degree of association between "protein" types and the LPS types. For example, in the strains tested, "protein" type III-8 and LPS type 8 were usually found together as were "protein" type IV-11 and LPS type 4. It is clear, however, from Tables 17 and 19 that no correlation exists between the serogroup and the LPS type.

The protein and LPS serotype antigens were clearly distinguished by inhibition of the LPS prototype HA reaction with several concentrations of the homologous purified outer membrane protein [14] and the

Table 16. Hemagglutination inhibition serotyping of *N. meningitidis* on the basis of the lipopolysaccharide antigens.

Serum No.	Vaccine Strain	Inhibiting Antigen - Outer Membrane Complex or Purified LPS								LPS Type
		126E(C)	35E(C)	6275(B)	89I(C)	M981(B)	M992(B)	6155(B)	M978(B)	
425	126E(C)	++*	--	--	--	--	--	--	--	1
414	35E(C)	--	++	--	--	--	--	--	--	2
813	6275(B)	--	--	++	--	--	--	++	++	3
264	89I(C)	--	--	--	++	--	--	--	+*	4
863	M981(B)	--	--	--	--	++	--	--	--	5
856	M992(B)	--	--	--	--	--	++	--	--	6
31	6155(B)	--	--	--	--	--	--	++	+	7
331	M978(B)	++	--	--	--	--	--	--	++	8

\* Inhibition of homologous HA reaction by antigen at 250 µg LPS/ml and at 31 µg LPS/ml.

\*\* Inhibition of homologous HA reaction by antigen at 250 µg LPS/ml but not at 31 µg LPS/ml.

Table 17. LPS serotyping of meningococcal strains.

Strain	Sero-group	LPS type	Strain	Sero-group	LPS type
138I	C	3	S-3032	B	3,7
60E	C	3,7,(4)	6586	B	3,4
126E	C	1,8	6611	B	3,4
118V	C	(2),4	NE	B	4
85E	C	2	99M	B	3,(8)
89I	C	4	190I	B	1,3,7,(4)
91III	C	3	135B	B	1,3,7,(4)
693840	A	6,7	7614	B	3,7
2E	A	4	7641	B	4
135M	Y	3,(4)	279	B	2,3
6236	Y	2,(6)	355	B	2,(7)
7062	Y	3	366	B	2
29E	29E	2,7	372	B	2,(3)
6308	135	3,7	53I	B	2
Z	Z	7,(3)	144I	B	2,3,(7)
X	X	6	298I	B	1,7
120	N.G.*	7	51I	B	3,(7)
6155	B	3,7	65V	B	3,7
6940	B	1,(8)	43II	B	3,7
6275	B	3	48I	B	2,3,5,7
6557	B	1,8	7510	B	7
M1080	B	3,7,8,(4)	7610	B	2,5
M981	B	5,(2)	7609	B	2,5
B16B6	B	2,(3)	7576	B	1,8(6)
M992	B	6	7583	B	3,7
M990	B	3,7,(4)	7523	B	2,6
M986	B	3,(4),(7)	7549	B	2,5
M978	B	3,8,(4),(7)	7599	B	2,5
M982	B	7	7566	B	2,5
M1011	B	3,(7)	7569	B	2,3,7,(1)
M136	B	4	7611	B	2,5
			7770	B	(2)
			B7	B	3,4,7
			6249	B	3,7

\* Nongroupable

Table 18. Frequency of occurrence of 8 meningococcal LPS serotypes among 65 strains.

Prototype reaction	Number of strains showing positive inhibition	Percentage
1	8	12
2	21	32
3	33	51
4	17	26
5	8	12
6	6	9
7	29	45
8	7	11

Table 19. Correlation of protein serotype and LPS serotype in the standard serotyping strains of Gold and Frasch.

Strain	Sero-group	Protein serotyping set	LPS type	Protein type <sup>1</sup>	
				Gold system	Frasch system
60E	C	Gold	3,7,(4) <sup>2</sup>	I	NT <sup>3</sup>
138I	C	"	3	II	2
126E	C	"	1,8	III	8
118V	C	"	4,(2)	IV	11
35E	C	"	2	V	NT
89I	C	"	4	VII,IV	11
6155	B	"	3,7	X,(I)	9
6940	B	"	1,(8)	XII,III	(8)
6557	B	"	1,8	XI,III	(8)
M1080	B	Frasch	3,7,8,(4)	NT	1
B16B6	B	"	2,(3)	II,(V)	2
M981	B	"	5,(2)	NT	4
M992	B	"	6	NT	5
M990	B	"	3,7,(4)	(X)	6
M986	B	"	3,(4),(7)	II	2,7
M978	B	"	3,8,(4),(7)	(V)	8,(3,1)
M982	B	"	7	X	9,(2)
M1011	B	"	3,(7)	II	2,10
M136	B	"	4	IV	11
S3032	B	"	3,7	NT	12

<sup>1</sup> As determined using indicated set of serotyping strains and antisera.

<sup>2</sup> Parentheses indicate minor types.

<sup>3</sup> NT = not typable.

homologous capsular polysaccharide. Although the homologous LPS inhibits strongly down to a concentration of 1-3  $\mu\text{g/ml}$  the proteins gave no inhibition below 100  $\mu\text{g/ml}$  and only a weak (+3 agglutination) inhibition between 100 and 1000  $\mu\text{g/ml}$ , which is consistent with a 1-2 percent cross contamination of the protein by LPS. The capsular polysaccharide gave no inhibition even at 1000  $\mu\text{g/ml}$ .

Determination of the LPS serotype of meningococcal strain permits serologic identification of strains on the basis of three distinct cell surface antigens: capsular polysaccharide, outer membrane proteins and LPS. Antibodies against all three have been demonstrated in immune rabbit serum and convalescent human serum following meningococcal infections. To avoid confusion when referring to the serotype of a strain it may be advisable to prefix the protein type by the letter "P", eg. P2,7 or PIII,V and the LPS type by the usual letter "O", eg. O3,7. This should not imply that meningococcal LPS is strictly analogous to the LPS antigens of the enterobacteriaceae or that the meningococcal LPS serotype determinants are known to reside on "O" side chains.

B. Radioactive antigen binding assay studies of meningococcal LPS.

The RABA developed, used a modification of the principles described by Farr. Meningococcal LPS was intrinsically labeled with  $^{14}\text{C}$  acetate during 16 hour growth, and purified. The LPS, which aggregates in aqueous solutions, was maintained in a monomeric form in three percent Sodium Desoxycholate (NaD) as determined by gel filtration over Sephadex G-100 (Fig. 1). Since NaD is insoluble in  $(\text{NH}_4)_2\text{SO}_4$ , 20 percent polyethylene glycol (PEG), which does not precipitate LPS, was used to precipitate immunoglobulins of all three major classes [20].

For the RABA, 100  $\mu\text{l}$  of an appropriate concentration of LPS, diluted in three percent deoxycholate in .1 M borate buffered saline of pH 8.3 is added to 50  $\mu\text{l}$  antiserum and 50  $\mu\text{l}$  deoxycholate buffer. After the mixture is allowed to react at  $4^\circ\text{C}$  for 15 min. an equal volume of 50 percent polyethylene glycol (PEG) of 6000 molecular weight, which precipitates immunoglobulins by molecular exclusion, is added and allowed to incubate at  $4^\circ\text{C}$  overnight. After incubation, an additional ml of 20 percent PEG is added to each tube which is then centrifuged at 1000 g for 30 min. The supernatant is discarded and the precipitate is dissolved in tissue solubilizer and washed into a counting vial with scintillation fluid.

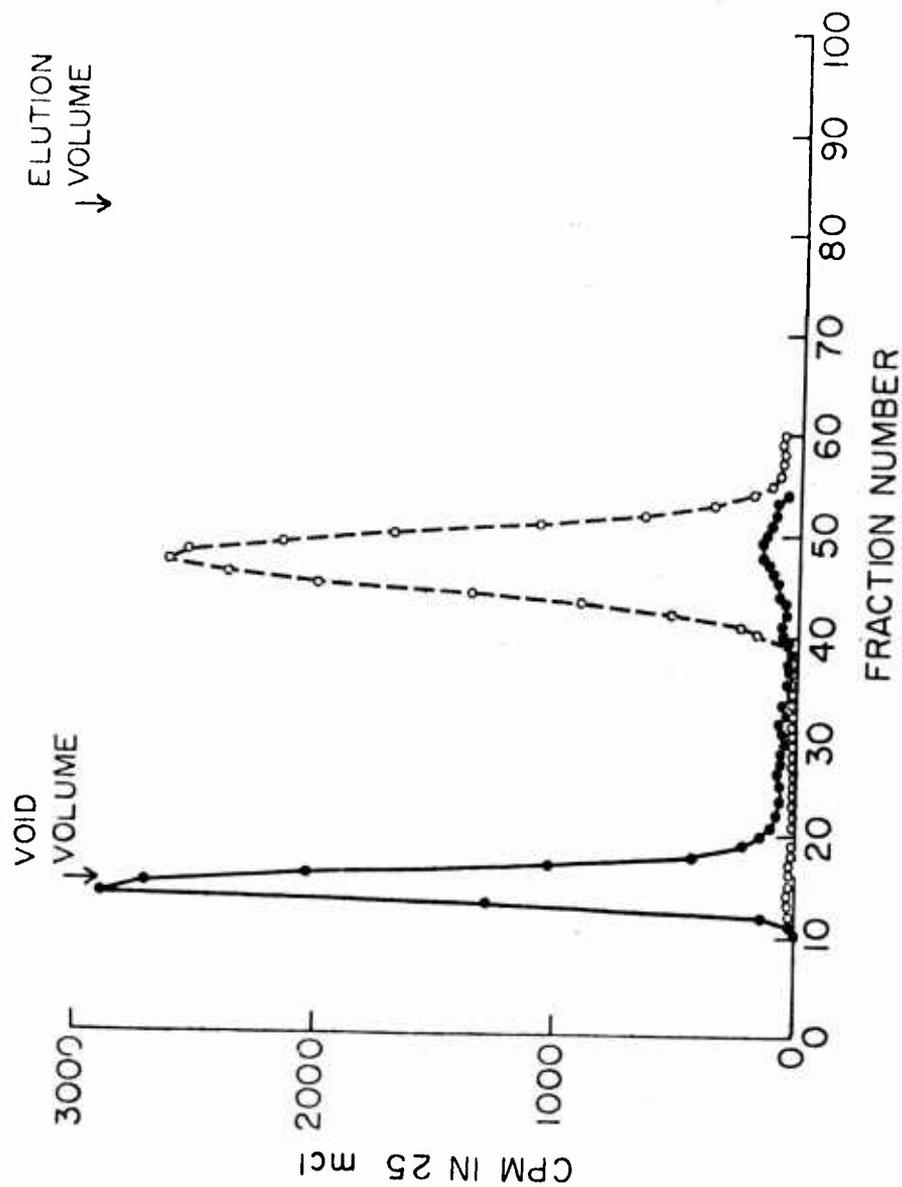


Fig. 1. Elution profiles of 126E lipopolysaccharide over Sephadex G-100 Fine equilibrated with .1M borate buffered saline pH 8.3 (BBS) (●) and with 1.5% sodium desoxycholate/BBS (○)

The percentage of LPS antigen bound to immunoglobulins precipitated from each test serum is then calculated.

$$\% \text{ Ag bound} = 100 \times \frac{\text{test serum cpm} - \text{background cpm}}{\text{antigen added cpm} - \text{background cpm}}$$

In the absence of serum, nonspecific precipitation of the LPS is less than one percent, and in the presence of fetal calf serum less than five percent. Reproducibility was determined from results compiled from one homologous hyperimmune rabbit serum tested on 14 separate occasions over a period of six months; the mean percentage antigen bound was  $78.9 \pm 5.9$ .

Rabbits immunized with N. meningitidis generally demonstrated a rise of antigen binding capability (Table 20); however, this response appears to be inversely related to the level of preexisting binding capacity, which is consistent with the concept of antibody mediated immune hyporesponsiveness.

Table 20. Rabbit response to meningococcal LPS as a function of preexisting antibody level.

Rabbit No.	Nanograms of 126 E LPS bound by 50 $\lambda$ serum 100% = 225 nanograms	
	Pre	Post
675	19.6	130.9
39	29.7	128.9
677	46.4	103.3
38	66.4	55.1

The ability of antisera to bind homologous and heterologous LPS's was compared using antisera to four different strains of group N. meningitidis raised in rabbits immunized by two injections of  $10^8$  viable organisms at six week intervals. Nine rabbit antisera to the four meningococcal strains were tested against three corresponding LPS's (Table 21). The four antisera to strain 126E bound 40-84

percent of its homologous LPS but only 10-19 percent of 35E and 118V LPS. Similarly, antisera to 35E bound 40 and 88 percent of its homologous LPS and only 25 percent of the heterologous 126E LPS. Moreover, antisera to 138I failed to bind more than 25 percent of any of the three heterologous LPS's, while a partial cross reaction between 118V and 35E LPS's was demonstrated by antisera to both organisms.

Table 21. Specificity of radioactive antigen binding assay: Hyperimmune rabbit sera and heterologous LPS strains.

Rabbit immunized with <i>N. meningitidis</i> serogroup-strain	Rabbit no.	% antigen bound		
		C-126E	C-35E	C-118V
C-126E	38	40	13	10
	39	84	15	18
	675	84	16	13
	677	60	19	19
C-35E	414	22	88	62
	415	25	40	35
C-118V	678	12	38	27
	738	17	80	70
C-138I	680	24	19	22

To determine if the failure to bind 100 percent of a homologous antigen was due to an inappropriately high antigen load the 126E LPS was titrated against its homologous sera (Fig. 2). Binding remained relatively constant over a 10-fold range of antigen load, suggesting an admixture of relevant and null antigenic determinants. Therefore, the 126E LPS was subjected to gel filtration by recycling chromatography over 9M of Sephadex G-50 Fine, equilibrated with the deoxycholate buffer. Selected fractions were assayed for reactivity in the RABA with high titered sera, #39, and those fractions demonstrating near 100 percent binding were pooled and concentrated.

Titration of this antigen pool against these same sera resulted in the expected decrease in antigen binding with increased antigen concentration for three of the four sera (Fig. 3), while the fourth serum, #677, though demonstrating decreasing antigen binding, never achieved an increase in binding over that seen with the unchromatographed LPS, suggesting that individual rabbits responded to different antigenic determinants.

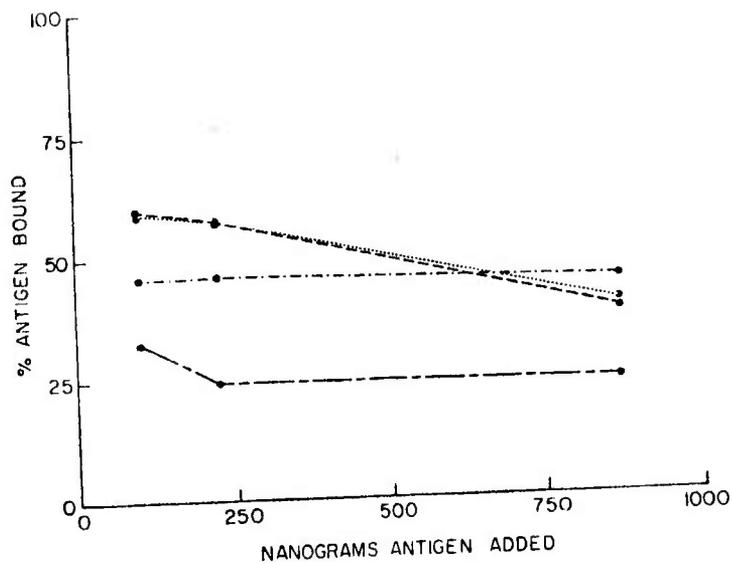


Fig. 2. Titration of 126E LPS in the RABA against four homologous sera: #38 (●- - - -●), #39 (●- - - - -●), #675 (●- - - - -●), #677 (●- - - - -●).

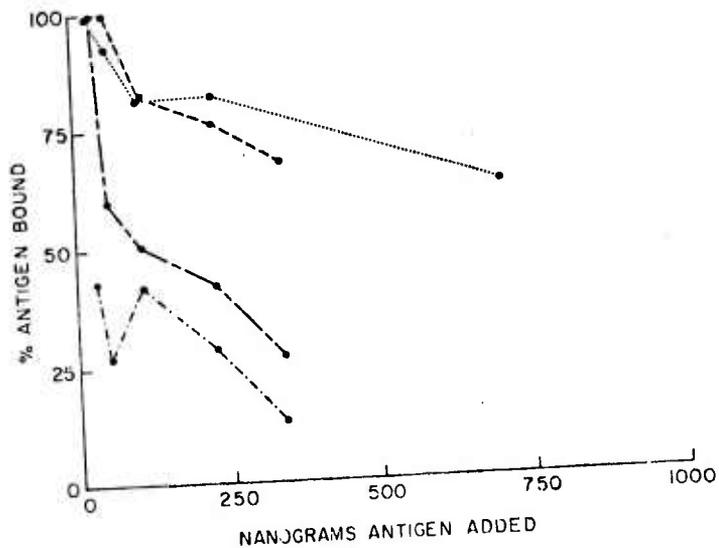


Fig. 3. Titration of chromatographed 126E LPS against four homologous sera: #38 (●- - - -●), #39 (●- - - - -●), #675 (●- - - - -●), #677 (●- - - - -●).

To investigate this possibility, 126E LPS was chromatographed over a total of 15 M Sephadex G-50 superfine; ribonuclease A, molecular weight of 13700 and Cytochrome C, molecular weight of 11700 were co-chromatographed and were separated by 125 tubes, or 60 mls, in this system. The labelled LPS was eluted with the Cytochrome C giving an average molecular weight of about 11500. Each one-half ml. sample over the mid-portion of the elution profile was assayed for reactivity in the RABA with the high titered serum, #675 (Fig. 4). Binding of the LPS by this serum was not constant throughout the elution profile. To determine if these variations were characteristic of all homologous antisera, selected fractions were also assayed against sera #38 and #677 (Fig. 5).

Serum #677 responded better, in general, to the smaller and #38 to the higher molecular weight determinants. In addition, a variation in response to individual fractions was seen. For each particular serum, the average of the antigen binding capacity across the elution profile of separated LPS was the same as the binding of unseparated LPS.

To determine if these variations to the individual fractions were influenced by preexisting binding capacity, pre-inoculation serum from rabbit #38 was assayed against selected fractions at either end of the elution profile and compared with the post-inoculation values. When binding values for the post-inoculation serum are ordered from highest to lowest with mid range points excluded, and plotted with their corresponding pre-inoculation binding values, an inverse correlation is shown (Fig. 6) A similar relationship, however, could not be demonstrated with the other homologous sera and it is clear that pre-existing antibody is not the only factor determining response.

The assay described here proved to be a reproducible and specific primary radioactive antigen binding assay for meningococcal LPS, the use of which has permitted preliminary observation of antigenic diversity within the LPS molecule, and of variations in individual host response to these determinants. The specificity determined for the four heterologous LPS's, as well as the low level of cross-reactivity is in agreement with that found using HA [14] and HAI.

The serogroup independence of LPS determinants was again demonstrated by the differential binding of homologous and heterologous LPS's within a single serogroup. Ultra-high resolution chromatography permitted the investigation of rabbit responses to meningococcal LPS's partially separated by molecular size and differences in binding across this elution profile were observed. Further understanding of such differences will only be possible after removal of the lipid moiety to facilitate chromatography of the polysaccharide moiety.

In addition, the previously reported role of endogenously produced antibody in the regulation of the primary response in mice to bacterial

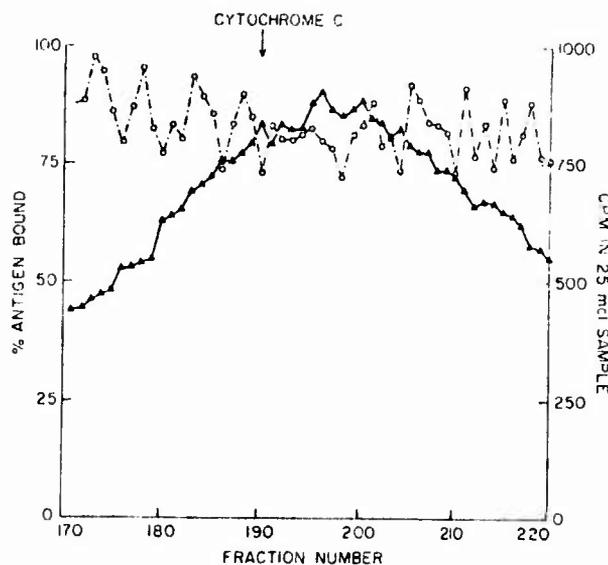


Fig. 4. Binding by the homologous serum #675 (○ — — — ○) of individual fractions across the elution profile of 126E LPS (▲ — — — ▲) chromatographed over 15M Sephadex G-50 superfine.

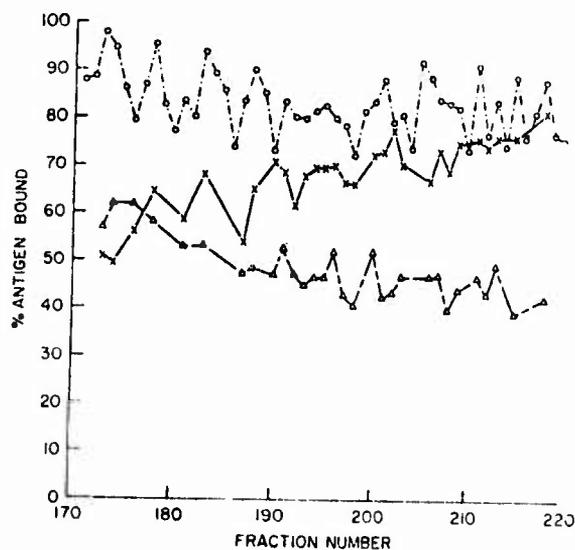


Fig. 5. Binding by homologous sera of selected fractions across the elution profile of 126E LPS chromatographed over 15 M Sephadex G-50 Superfine: #38 (Δ — — — Δ), #675 (○ — — — ○), #677 (x — — — x).

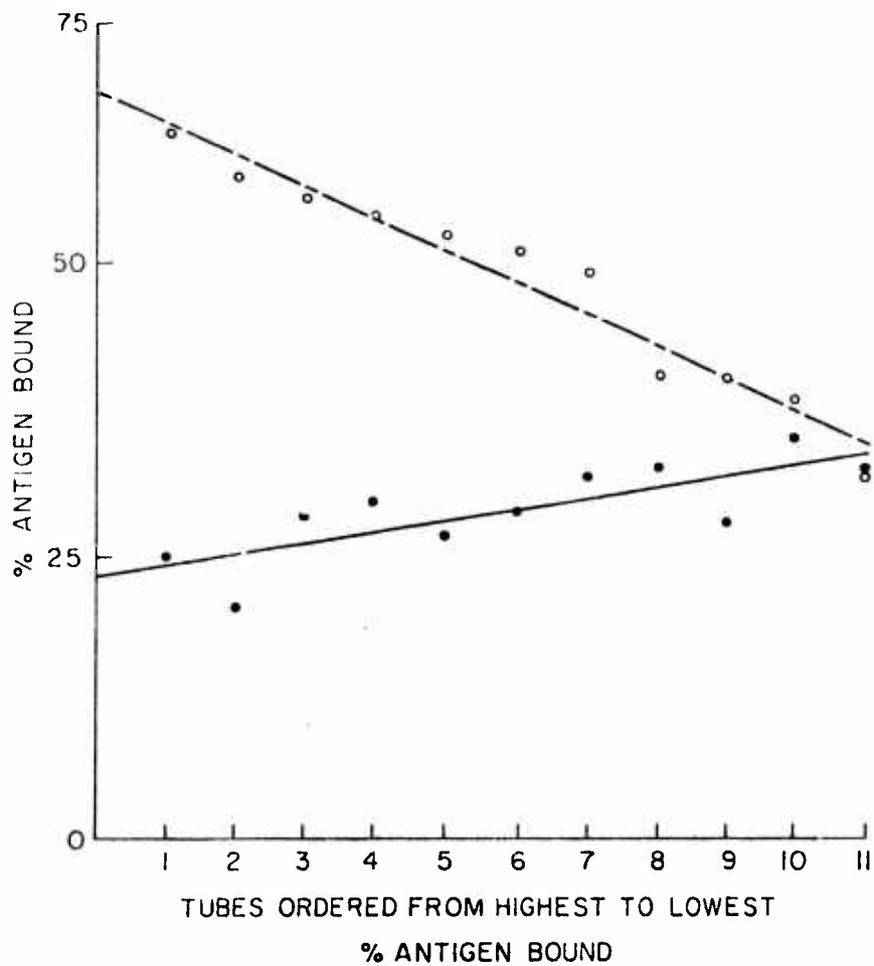


Fig. 6. Effect of preexisting binding capacity on response to individual fractions. Serum #38 vs. 126E LPS chromatographed over 15M Sephadex G-50 Superfine. Binding values for post-inoculation serum are ordered from highest to lowest with mid range points excluded (o— — —o) and plotted against corresponding pre-inoculation values (●————●).

LPS appears to hold for rabbits immunized with meningococci [21]. This was shown by the inverse relationship between pre-immunization binding capacity of sera and the post-immunization response to homologous unchromatographed LPS. Finally, it is clear that the RABA described lends itself to more definitive studies of antigenic diversity and host responses to meningococcal LPS.

#### IV. A solid phase radioimmunoassay for measurement of antibodies against meningococcal outer membrane antigens.

##### Introduction.

Studies on the human antibody response to bacterial membrane associated antigens such as membrane proteins and lipopolysaccharides have been hampered because of inadequate methods for accurate determination of antibodies to these antigens. A primary binding assay is most desirable for measurement of total antibody without regard to function or class since detection requires only that the antibody bind to its antigen.

In this report we describe a solid phase radioimmunoassay (SPRIA) which is based upon the assay described by Rosenthal et al. [22]. The basic parameters of the assay have been examined and conditions defined which permit standardization of the assay and possibly quantitation of serum antibody. Conditions have also been determined for performing antigen inhibition studies to investigate antigenic potency and antibody specificity.

##### Materials and Methods.

##### Iodination of immunoglobulins.

Immunoglobulins were iodinated by the lactoperoxidase method [23] using high concentration, carrier free  $\text{Na}^{125}\text{I}$  obtained from New England Nuclear, Boston, MA. Labelling efficiency was typically about 70 percent and the resultant specific activity was  $2-10 \times 10^6$  counts per minute per microgram of protein. After labeling, 0.25 ml of Phosphate Buffered Saline (PBS) containing 0.1 percent  $\text{NaN}_3$ , 10 percent fetal calf serum and 0.01M KI were added and the unbound iodide was removed by dialysis or by chromatography on Sephadex G-25. The labelled immunoglobulins were diluted in PBS containing 0.1 percent  $\text{NaN}_3$  and 10 percent fetal calf serum and stored as a 20-50X stock solution at 4°C.

##### Preparation of immunoglobulins for iodination.

Goat anti-rabbit gamma globulin (GARG) was obtained from Nutritional Biochemicals as the 7S fraction of the goat serum and was dialyzed vs. PBS to remove the preservative. Goat antisera against human IgG, IgA, IgM or IgG, A, M, K, L was obtained from Meloy Labs.

Purified antibodies were prepared from these antisera by absorbing onto glutaraldehyde insolubilized human gamma globulin, washing with PBS and eluting with 0.05M glycine-HCl pH 2.7. The eluted antibodies were neutralized, lyophilized, dissolved in a small volume of distilled water and dialyzed against PBS. Immunoglobulins prepared for labelling were stored at -60°C in 100-200 µg samples.

Purification of rabbit antibodies against meningococcal proteins was done by affinity chromatography using purified outer membrane protein [14] linked directly to CNBr-activated Sepharose 4B. After washing, antibodies were eluted with saline at pH 11.6, neutralized, lyophilized, dissolved in a small volume of distilled water and dialyzed against PBS pH 7.4.

#### Antigens.

Meningococcal outer membrane complex (OMC), purified outer membrane protein (OM protein), lipopolysaccharide (LPS), and capsular polysaccharides were prepared as previously described [14].

#### Other materials and reagents.

The assay was done in disposable polyvinyl "U" microtiter plates obtained from Cooke Engineering Co., Alexandria, Va. Human IgG for use as a standard for protein determinations was purified by DEAE cellulose chromatography and standardized to 1 mg/ml on the basis of its absorbance at 280 nm ( $e_{280}^{1\%} = 14.3$ ). Dulbeccos PBS buffer was obtained from Grand Island Biological Co., Grand Island, NY.

#### Standard Assay Procedure

After initial studies to determine optimum conditions the following procedure was adopted.

1. Antigen was diluted in PBS containing 0.01 percent phenol red to the optimal concentration (usually 50-100 µg/ml). Using a micro-pipette, 25 µl of antigen was placed in the bottom of each well of the microtiter plate and the plate was then placed in a humidity box at 37°C for one hour.
2. The antigen solution was carefully aspirated and 0.05 ml of filler (PBS pH 7.2 containing 10 percent fetal calf serum, 0.01 percent phenol red and 0.2 percent NaN<sub>3</sub>) was dropped in each well with a microtiter dropper. This solution was immediately aspirated and 0.1 ml of filler was dropped in each well. The plate was then placed at 37°C for 30-60 min.
3. The filler was flicked out and the plate washed twice with PBS pH 7.2 by filling the wells with a slow running stream of buffer from a reservoir and then flicking the solution out. After the last wash

the plate was held upside down and tapped on a clear absorbent towel to remove all liquid.

4. Serial dilutions of the sera to be tested were made in a separate plate or in tubes using filler as diluent. A tube containing diluent only is placed at the high dilution end of the series. Using a micropipette 25  $\mu$ l of each dilution was transferred to a well of the antigen coated plate. The plate was placed in a humidity box and the binding of antisera (primary antibodies) to antigen(s) allowed to continue at room temperature overnight. For screening of sera and other less exacting studies two-fold serial dilutions of the antisera were made directly in the antigen coated wells.

5. The liquid was aspirated from the wells and 0.05 ml of filler dropped in each well. This was aspirated out and the plate washed twice with PBS. Filler was then placed in the wells (0.1 ml) and the plate placed at 37°C for one hour. The solution was flicked out, the plate washed twice more with PBS and excess liquid removed by tapping on an absorbent towel as before.

6.  $^{125}$ I-anti-immunoglobulin (secondary antibody) of the desired specificity was diluted with filler to contain a standard concentration ( $\mu$ g antibody protein/ml) of specific antibody with sufficiently high specific activity to result in maximum binding of 3,000-20,000 cpm per well (25  $\mu$ l). Using a micropipette, 25  $\mu$ l of the diluted  $^{125}$ I secondary antibody was placed in the bottom of each well and the plate placed in a humidity box at room temperature for 6-18 hours.

7. The liquid was aspirated from the wells into a radioactive waste container. Then 0.05 ml of filler was dropped in the wells, aspirated out, and the plates washed five times with PBS and two times with tap water.

8. The plates were allowed to dry and the wells cut off with scissors into tubes for counting.

A high titered immune serum and a normal or baseline serum should be included in each test. A no serum control was included as mentioned in step 4. A no antigen control (filler only) was also found to be useful.

The procedure is shown schematically in Fig. 7. Typical binding curves obtained by this procedure using normal and immune human sera are seen in Fig. 8. In this case the antigen was  $^{138}$ I OMC, the antisera were serum from a healthy 14 month old child and three week convalescent serum from a patient with group B systemic meningococcal disease, and the secondary antibody was  $^{125}$ I-goat anti-human IgG, A, M, K, L. The curve obtained with the immune serum had a plateau in the region of primary antibody excess where essentially all available secondary antibody is bound. At higher dilutions of primary antibody

# SOLID PHASE RADIOIMMUNOASSAY

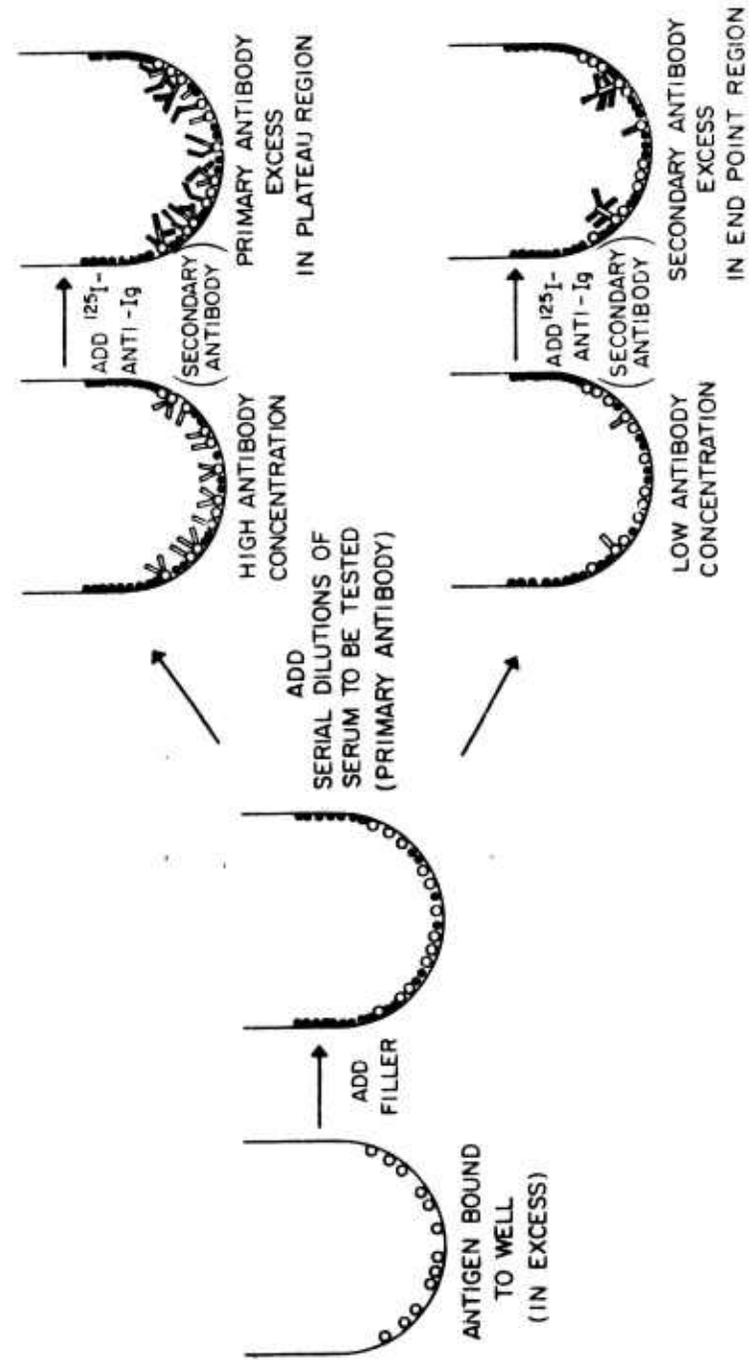


Fig. 7. Schematic diagram of the solid phase radioimmunoassay.

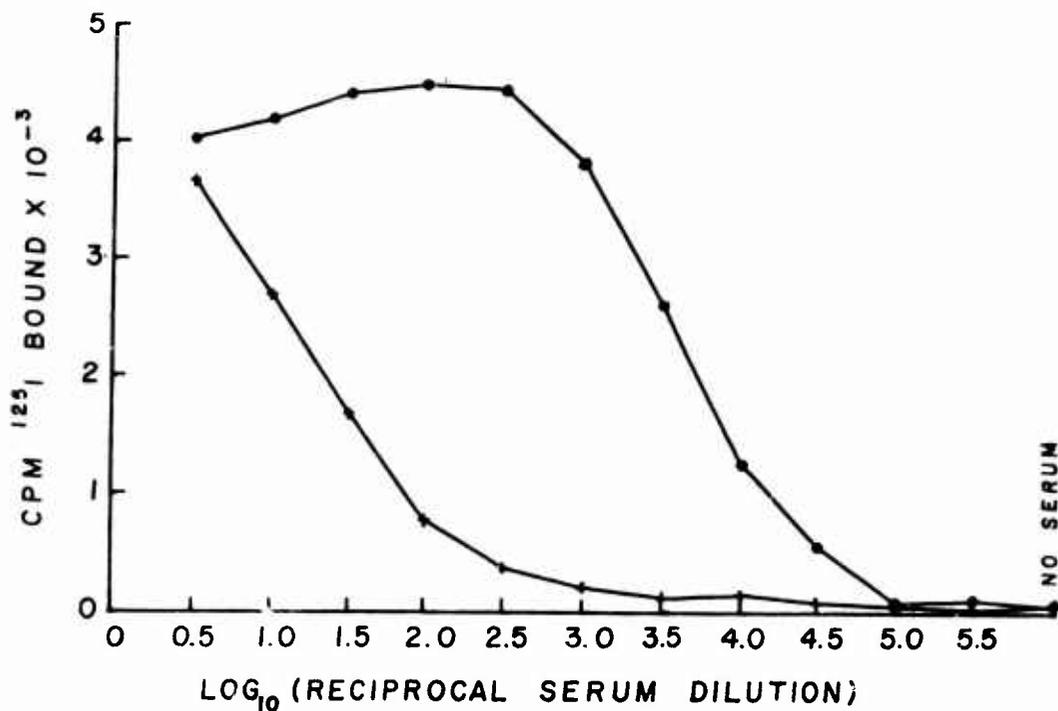


Fig. 8. Binding curves obtained with normal and immune human sera using the standard SPR1A procedure. Antigen was 138I(C) OMC, primary antibody was either normal serum from a health 14 mo. old child (+) or 21 convalescent serum from an army recruit with group B systemic meningococcal disease (●), and secondary antibody was <sup>125</sup>I-goat antihuman IgG,M,A,K,L (specific activity approximately  $2.5 \times 10^6$  cpm/ $\mu$ g).

a region of secondary antibody excess occurs ( $^{125}\text{I}$  cpm bound 30 per cent of the plateau level) where the primary antibody is limited and becomes essentially saturated with secondary antibody. These two situations are depicted in Fig. 7. The curve obtained with the normal serum is similar to the end point region of the immune serum curve and represents the background of nonspecific binding of serum immunoglobulin.

#### Results.

##### The binding of antigen to the plate.

The binding of antigen to the wells of the plate was investigated in order to determine conditions which would insure sufficient binding of antigen so that antigen would not be the limiting component in the assay. The amount of  $^{125}\text{I}$ -primary antibody bound per well as a function of the length of time the well was exposed to antigen (OM protein) and antigen concentration is shown in Fig. 9. A constant amount of  $^{125}\text{I}$ -primary antibody was added to all wells and became limiting at the higher antigen concentrations. Thus, the small increase in the amount of  $^{125}\text{I}$  bound between 100 and 500  $\mu\text{g}/\text{ml}$  of antigen reflects limiting  $^{125}\text{I}$ -antibody rather than saturation of the binding sites on the well with antigen. The results indicate that about half of the antigen bound after two hours was bound within the first five minutes.

The binding efficiency of each type of antigen was found to be different as judged by the concentration of antigen required for optimal coating of the wells. The OMC and LPS antigens were 10-15 times more efficient at binding than the purified OM protein; and the capsular polysaccharide was very inefficient. The effect of coating the wells with several different concentrations of capsular polysaccharide on the binding curve obtained with a hyperimmune rabbit serum is shown in Fig. 10. Even at 400  $\mu\text{g}/\text{ml}$  the amount of antigen bound was limiting as indicated by the fact that in the plateau region of the curve only about two-thirds of the specific secondary antibody added was bound. Under conditions of limiting antigen both the amount of secondary antibody bound in the plateau region and the apparent titer of the primary antibody are decreased. Thus, in order to obtain consistent, meaningful results the assay must be run under conditions of antigen excess.

##### Binding kinetics of primary and secondary antibody.

The binding kinetics of primary and secondary antibodies was examined to determine the length of time required to obtain maximum antibody binding (Fig. 11). The kinetics of primary antibody binding was determined under conditions of antigen excess using  $^{125}\text{I}$ -labeled anti-6275 OM protein antibodies (specific activity about 8000 cpm/ng) and 6275 OM protein as antigen. The binding kinetics of secondary antibody was investigated under conditions of primary antibody excess using  $^{138}\text{I}$  OMC as antigen, a 1:40 dilution of convalescent serum from

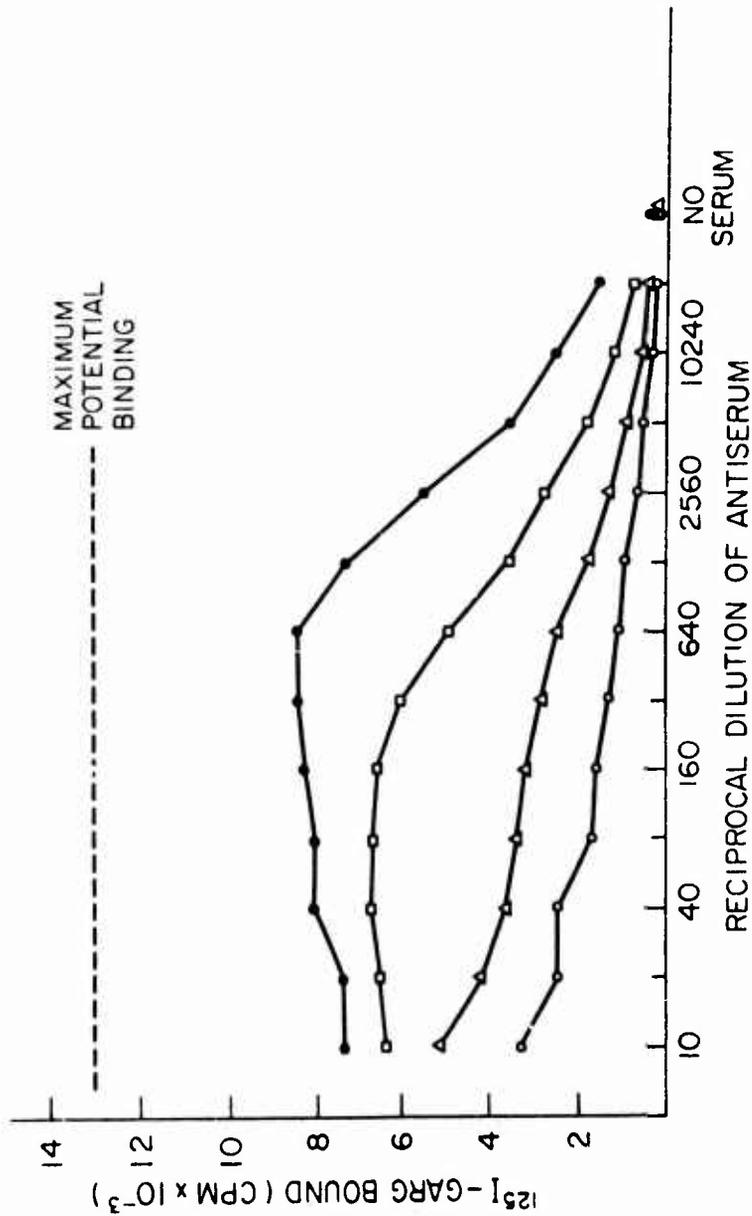


Fig. 9. Effect of limiting antigen on the resultant binding curve. Meningococcal group C capsular polysaccharide was used at concentrations of 6 (A), 25 (B), 100 (C), and 400 (D)  $\mu\text{g/ml}$  to coat a series of wells. The test was completed using the standard procedure and the same primary (rabbit anti-138I(C) antiserum) and secondary ( $^{125}\text{I}$ -GARG) antibodies at each antigen concentration. The maximum potential binding is the plateau binding level obtained under the same conditions but with excess antigen ( $^{138}\text{I}$ (C) OMC) bound to the wells.

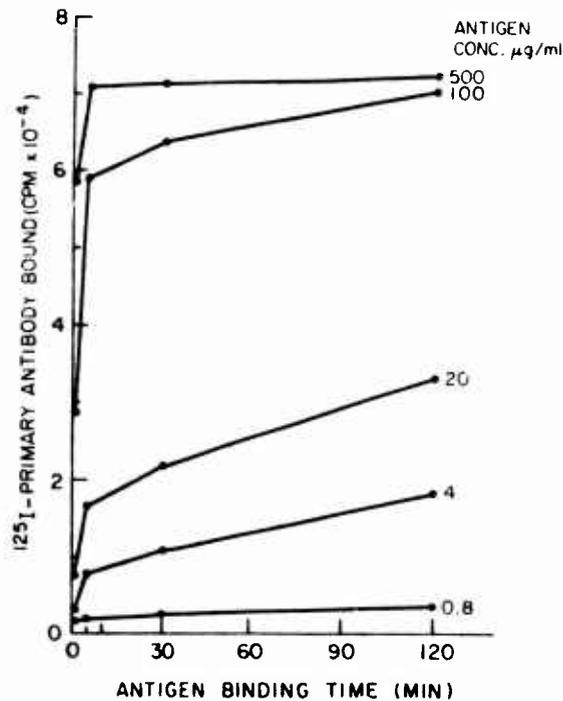


Fig. 10. Kinetics of antigen binding. Antigen (6275 OM protein) at concentrations of 0.8, 4, 20, 100 and 500  $\mu\text{g}$  protein/ml. was allowed to bind to the wells of a plate for 1 to 120 min. (0-30 min. at room temperature and 30-120 min. at  $37^\circ\text{C}$ ). The amount of antigen bound was monitored by measuring the binding capacity of the wells for labeled primary antibody ( $^{125}\text{I}$ -anti 6275 OM protein antibodies from rabbit serum). Binding of primary antibody was at  $37^\circ\text{C}$  for 4 hrs. The same amount of primary antibody was used at all antigen concentrations.

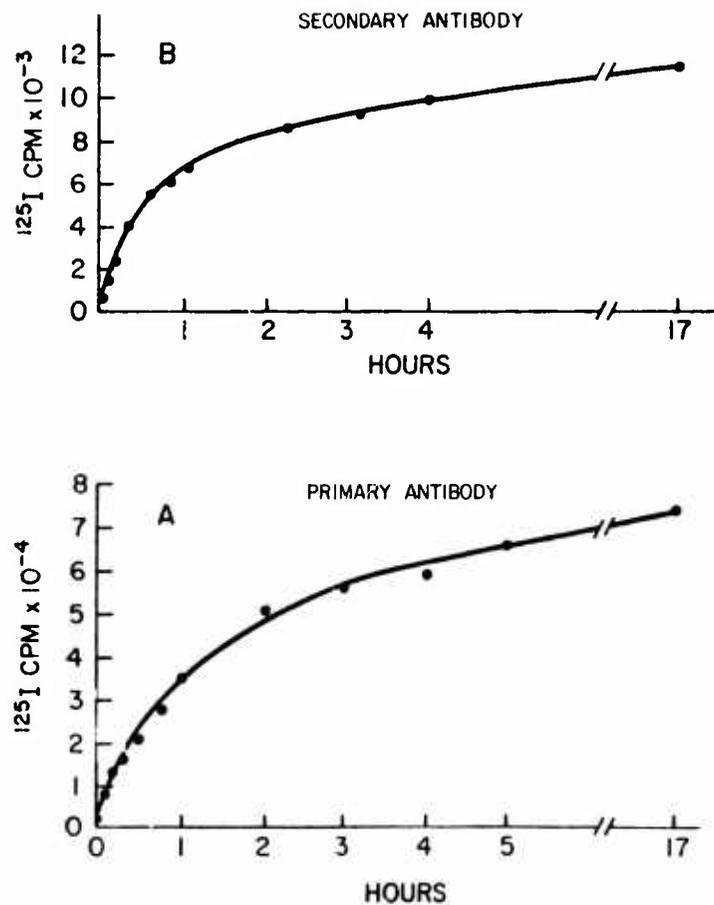


Fig. 11. (A) Binding kinetics of primary antibody. Rabbit  $^{125}\text{I}$ -anti 6275 OM protein antibodies (specific activity 5600 cpm/ng) were allowed to bind to excess antigen (6275 OM protein) on the wells of a plate for various lengths of time. Binding was at room temperature for 0-30 min., at  $37^\circ\text{C}$  for 30 min. to 5 hours, and at room temperature for 5 hours to 17 hours.

(B) Binding kinetics of secondary antibody.  $^{125}\text{I}$ -goat antihuman IgG (specific activity 630 cpm/ng) was allowed to bind at room temperature for various lengths of time to excess primary antibody (1:40 dilution of convalescent serum from a patient with systemic meningococcal disease) bound to antigen ( $^{138}\text{I}$ (C) OMC) on the wells of a plate.

a patient with group B systemic meningococcal disease as primary antibody, and  $^{125}\text{I}$  goat anti-human IgG as secondary antibody.

The binding kinetics of primary and secondary antibody are similar. About 50 percent of the total antibody bound after 18 hours is bound within the first hour and about 85-90 percent is bound within the first four-five hours.

#### Extent of primary antibody binding.

Another question we sought to answer was what proportion of the specific primary antibody added to the well became bound to the antigen after 18 hours. As an approach to this question, a two-fold dilution series of primary antibody was allowed to bind to antigen on the plate for 18 hours. The total liquid phase was then transferred to a second set of wells coated with the same antigen and any remaining antigen allowed to bind for an additional 18 hours. After washing  $^{125}\text{I}$ -secondary antibody was added and the extent of binding to the first and second set of wells determined. The results are shown in Fig. 12. The amount of primary antibody bound at a particular point on the curve is directly proportional to the amount of  $^{125}\text{I}$ -secondary antibody bound at that point only when  $^{125}\text{I}$ -secondary antibody is present in excess.  $^{125}\text{I}$ -secondary antibody excess occurs only in the endpoint region of the curve corresponding to dilutions  $\geq 1:64,000$  in Fig. 6. The dashed line represents the estimated binding of  $^{125}\text{I}$ -secondary antibody under the same conditions of secondary antibody excess as are present at a primary antibody dilution of 1:128,000. This curve was generated by back extrapolation from the end point region assuming two-fold increases in primary antibody bound.

The results indicate that in the end point region approximately 90 percent of the primary antibody was bound to antigen in the first well. This is evident both from the greater than eight-fold reduction in titer of the primary antibodies and the relative amounts of secondary antibody bound to the first and second wells in the end point region of the curves.

#### Effect of inadequate washing following binding of primary antibody.

Washing with tap water following binding of primary antibody as described by Rosenthal et al. [22] usually resulted in binding curves which had a very pronounced prozone. An investigation of the cause of the prozone revealed that it was due to neutralization of  $^{125}\text{I}$ -secondary antibody by nonspecifically bound serum immunoglobulins, principally IgG, which came loose from the plate during the reaction with secondary antibody and were washed away along with the  $^{125}\text{I}$ -secondary antibodies that had bound to them. Several observations which support this conclusions are: 1) Immune sera diluted beyond the prozone to the peak of the binding curve showed only very low binding of secondary

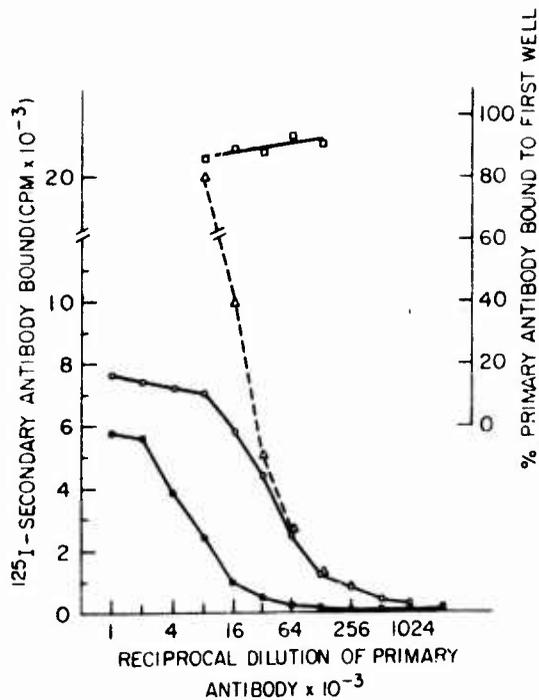


Fig. 12. Extent of primary antibody binding in the end point region of the binding curve. Antigen was 138I(C) OMC, primary antibody was rabbit anti 138I(C) antiserum, and secondary antibody was  $^{125}\text{I}$ -GARG. Serial dilutions of primary antibody were allowed to bind to antigen for 16 hours at room temperature after which the total liquid phase was transferred to a new set of wells coated with the same antigen and binding allowed to continue for an additional 16 hours. After washing, the amount of primary antibody bound to the first and second wells was determined by the capacity to bind a constant amount of  $^{125}\text{I}$ -GARG.  $^{125}\text{I}$ -GARG bound to the first set of wells (○), and to the second set of wells (●). Saturation binding of  $^{125}\text{I}$ -GARG to the first set of wells as estimated by back extrapolation from the primary antibody dilution of 1:128,000 ( $\Delta$ ). Calculated ratio of the amount of primary antibody bound to the first well to the total primary antibody bound to the first and second wells (see text) (□). A 20 percent loss of primary antibody due to transfer and incomplete binding was assumed.

antibody if first mixed 1:1 with normal serum at dilutions up to 1:50 i.e., normal serum acted like an inhibitor in the system. 2) This effect of normal serum was animal specific. Rabbit serum would not inhibit in the human system and vice versa. 3) DEAE-purified IgG from normal serum had the same effect as the whole serum while other serum fractions such as albumin had no effect. 4) Absorption of normal serum with antigen or immune complexes had no effect. 5) Addition of normal serum to the wells after completion of primary antibody binding and washing had the same effect as adding the mixture, but exposure of the antigen coated wells to normal serum before addition of primary antibody had no effect. 6) Using  $^{125}\text{I}$  primary antibody and unlabeled secondary antibody no difference in the amount of primary antibody bound at the completion of the assay could be detected as a result of adding normal serum as compared to diluent.

Investigation of the washing procedure suggested that the use of water rather than buffered saline or filler for washing the plates following binding of the primary antibody was responsible for the prozone. Extensive washing with water could not eliminate the prozone while thorough washing with buffered saline or filler essentially eliminated the prozone in most cases. Fig. 13 shows the difference in the binding curves that were obtained with an immune rabbit serum following washing with either PBS or water.

#### Effect of secondary antibody concentration.

The effect of secondary antibody concentration on the nature of the binding curve obtained is shown in Fig. 14. Using the same primary antibody dilution series and test conditions, binding curves were obtained with four different concentrations of secondary antibody. It is evident that the height of the plateau region is directly proportional to the concentration of secondary antibody and that the end point of the curves, i.e., the dilution at which the binding is 25-30 percent of the plateau level, is inversely related to secondary antibody concentration. In order to obtain reproducible results in terms of antibody titer one must, therefore, keep the concentration ( $\mu\text{g}$  antibody protein/ml) of active secondary antibody constant even though the amount of radioactivity added may vary due to radioactive decay or variations in specific activity of different preparations.

#### Quantitative aspects of the assay.

Expression of the results of serologic tests in quantitative terms, i.e., micrograms of specific antibody per milliliter of serum is desirable where possible since it is easily interpreted and allows data obtained in different systems and in different laboratories to be easily compared. We, therefore, undertook to determine the feasibility of quantitating this solid phase RIA.



Fig. 13. Effect of washing with tap water or buffer on the shape of the binding curve. For both curves antigen was <sup>138</sup>I(C) OMC, primary antibody was rabbit anti-<sup>138</sup>I(C) antiserum, and secondary antibody was <sup>125</sup>I-GARG. Following primary antibody binding the wells were washed with tap water 8-10 times (●) or with buffer as described in the standard procedure (O).

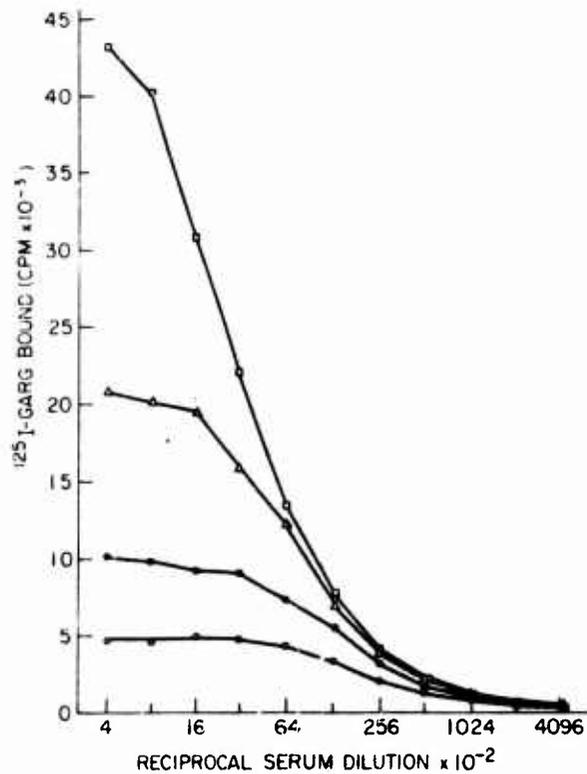


Fig. 14. Effect of secondary antibody concentration. Antigen was  $^{138}\text{I(C)}$  OMC, primary antibody was a single dilution series of rabbit anti- $^{138}\text{I(C)}$  antiserum, and secondary antibody was  $^{125}\text{I-GARG}$  (specific activity 2400 cpm/ng) at concentrations of 80 (O), 160 (●), 320 ( $\Delta$ ) and 640 ( $\square$ ) ng active antibody protein/ml.

Several relationships must be known in order to determine the amount of primary antibody present in the serum at a given dilution from the amount of  $^{125}\text{I}$  bound at that dilution: 1) the relationship between the counts per minute of  $^{125}\text{I}$  bound and the nanograms of secondary antibody bound, i.e., the specific activity of the secondary antibody; 2) the relationship between the amount of secondary antibody bound and the amount of primary antibody bound; 3) the relationship between the amount of primary antibody bound and the amount of specific primary antibody added to the well.

1) Determination of secondary antibody specific activity.

Although the amount of protein that is iodinated can be readily determined, accurate determination of the specific activity of the final preparation is not simple due to undetermined losses which occur during labeling and removal of unbound iodide. Direct determination of protein concentration following labeling is not feasible because the amount of protein labeled is very small and a protein carrier is routinely added prior to removal of the unbound iodide to minimize losses. Three methods were used for estimating the specific activity of secondary antibody preparations.

a) Estimation of protein losses during the labeling procedure.

If a known amount of protein is placed in the labeling mixture the final specific activity can be determined by measuring the total radioactivity and estimating the total protein recovered. This was done by measuring the radioactivity remaining on the reaction tube, micropipette tips, dialysis tubing and any other surfaces which had been in contact with the sample. It has been shown that nearly all of the radioactivity adhering to such surfaces which have been rinsed once is protein bound rather than free iodide [24]. When transfers were made all surfaces coming in contact with the sample were rinsed once and the rinse added to the sample. The sum of the radioactivity in the sample plus the radioactivity bound to the surfaces mentioned above was assumed to represent the total protein and the fraction of the total found in the sample was taken as the fraction of the original protein recovered. The mean recovery estimated in this way was 85 percent.

b) Inhibition of binding with unlabeled secondary antibody.

Under conditions of large excess of secondary antibody the binding of  $^{125}\text{I}$  secondary antibody to primary antibody (bound to antigen on a plate) can be competitively inhibited by varying amounts of identical but unlabeled secondary antibody of known concentration. Fifty percent inhibition should occur when equal amounts of labeled and unlabeled antibody are present. The validity of this approach depends upon the assumption that no loss in antibody activity occurs

as a result of iodination. This is because the competition is between active antibodies rather than total protein in the antiglobulin preparation.

The results of an experiment of this type are given in Fig. 15. The binding of a constant amount (excess) of  $^{125}\text{I}$ -GARG to a constant amount of rabbit antimeningococcal antibodies was inhibited by mixing with an equal volume of unlabeled GARG containing 3.6 to 36  $\mu\text{g}$  protein/ml. For the uninhibited control an equal volume of  $^{125}\text{I}$ -GARG rather than unlabeled GARG was added. A similar control containing  $^{125}\text{I}$ -GARG at double the concentration was also included to insure the condition of excess  $^{125}\text{I}$ -GARG was met. Background controls contained the same mixtures of labeled and unlabeled GARG but no primary antibody on the plate. The results plotted on log-probit paper as percentage inhibition (probit scale) versus concentration of unlabeled GARG added were linear in the range between 20 and 80 percent inhibition. The values plotted are the means of quadruplicate determinations. Fifty percent inhibition was obtained with 10.2  $\mu\text{g}/\text{ml}$  of unlabeled GARG. This result was used to calculate the total GARG protein in the labeled sample and yielded a result that corresponded to a recovery of 70 percent of the GARG protein labeled.

It is not clear whether the discrepancy between this value and the 85 percent recovery as determined by method a) is due to experimental error or inactivation of some antibody as a result of labeling.

c) Saturation binding to a constant amount of primary antibody.

Determination of relative specific activity for purposes of standardization of the sensitivity of the assay was done by binding serial dilutions of the two or more preparations of  $^{125}\text{I}$ -antiglobulin to be compared to a standard, constant amount of a suitable primary antibody bound to its specific antigen on the wells of a plate. A standard dilution of good immune serum near the end point (binding of secondary antibody equal to 50-75 percent of the plateau level) was used as the primary antibody. As the concentration of  $^{125}\text{I}$ -secondary antibody was increased relative to the constant amount of primary antibody the amount of  $^{125}\text{I}$  bound increased proportionally and then leveled off to a near plateau (a curve of this type is shown in Fig. 16. This was interpreted to mean that the binding sites on the primary antibodies had become essentially saturated with secondary antibodies. The nanograms of secondary antibody bound at this point of saturation should be constant for different labeled batches of the same lot of antiglobulin. The relative number of counts per minute bound at saturation was then taken as being directly proportional to the relative specific activity of the different batches.

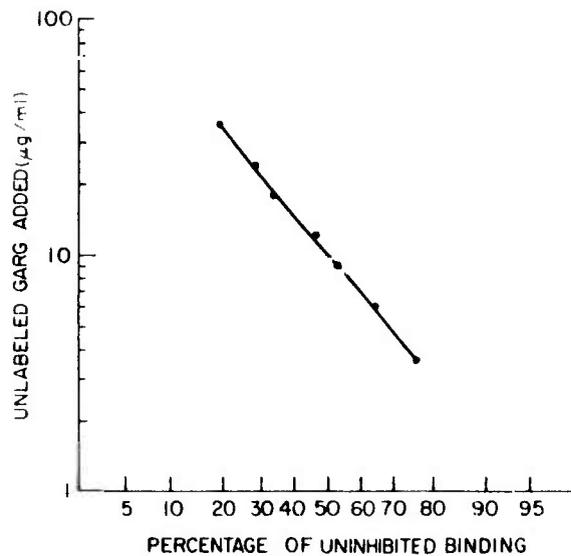


Fig. 15. Determination of  $^{125}\text{I}$ -GARG concentration by inhibition with unlabeled GARG of known concentration. The binding of a constant amount of  $^{125}\text{I}$ -GARG to a constant amount of primary antibody (1:10,000 dilution of rabbit anti-138I(C) antiserum) bound to antigen (138I(C) OMC) of the wells of a plate was inhibited with various concentrations of unlabeled GARG. Each point represents the mean of four determinations.

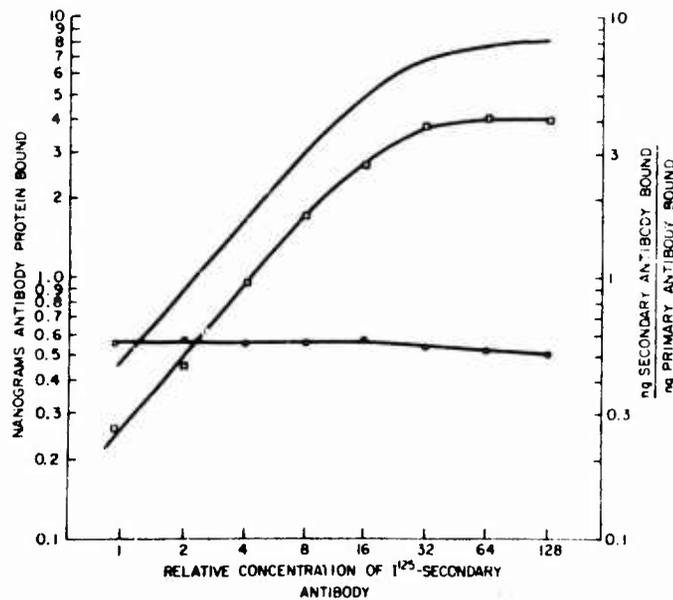


Fig. 16. Determination of the ratio of secondary antibody bound to primary antibody bound. A constant amount of  $^{125}\text{I}$ -primary antibodies (rabbit anti- $^{138}\text{I}$ (C) OM protein antibodies) was allowed to bind overnight at room temperature to antigen ( $^{138}\text{I}$ (C) OM protein). After washing, serial dilutions of the same concentration of either  $^{125}\text{I}$ -GARG or unlabeled GARG were added and allowed to bind overnight at room temperature. The amount of  $^{125}\text{I}$ -primary antibody bound ( $\bullet$ ) was determined from the wells to which unlabeled GARG was added. The amount of secondary antibody bound ( $\square$ ) was determined from the difference between the  $^{125}\text{I}$  bound to the wells to which  $^{125}\text{I}$ -GARG was added and the wells to which unlabeled GARG was added. Each point represents the mean of four determinations. Background binding to wells receiving no antigen (filler only) but the same amount of labeled primary antibody or no primary antibody but the same amounts of labeled secondary antibody has been subtracted.

2) Determination of the ratio of secondary antibody bound to primary antibody bound.

The relationship between the amount of secondary antibody bound and the amount of primary antibody bound was investigated using as primary antibodies  $^{125}\text{I}$ -rabbit antibodies against 138I OM protein and as secondary antibodies  $^{125}\text{I}$ -GARG. To a constant amount of  $^{125}\text{I}$  primary antibody bound to its antigen on the plate. Serial dilutions of either labeled or unlabeled GARG were added and after overnight binding the total amount of  $^{125}\text{I}$  bound was determined. The amount of  $^{125}\text{I}$ -secondary antibody bound was determined by subtracting the  $^{125}\text{I}$ -primary antibody bound as determined from the wells to which unlabeled GARG had been added from the total  $^{125}\text{I}$  bound in the corresponding wells containing  $^{125}\text{I}$  primary antibody and  $^{125}\text{I}$ -GARG. All samples were run in quadruplicate. The specific activity (as determined by method a) above) of the primary antibody was  $10 \times 10^6$  cpm/  $\mu\text{g}$  and of the secondary antibody was  $2.5 \times 10^6$  cpm/  $\mu\text{g}$ . The results of this experiment are presented in Fig. 16. The amount of  $^{125}\text{I}$ -GARG bound at first increases in proportion to the amount added and then levels off at a plateau of about four nanograms per well. The amount of  $^{125}\text{I}$ -primary antibody bound remains constant until saturation levels of GARG are reached where it begins to fall off slightly. This suggests that saturation binding of secondary antibody to primary antibody may cause a small percentage of the primary antibody to come loose from the plate. The ratio of nanograms secondary antibody bound to nanograms primary antibody bound increased with increasing concentrations of secondary antibody and then became nearly constant at approximately 7.5-8.0 as the primary antibody became saturated with secondary antibody.

The point on the binding curve of a serum at which the primary antibody becomes saturated with secondary antibody was determined by a similar experiment in which serial two-fold dilutions of  $^{125}\text{I}$  primary antibodies were allowed to bind to specific antigen in the wells of a plate and a constant concentration of  $^{125}\text{I}$ -GARG or unlabeled GARG at the same concentration was allowed to bind to the primary antibody using the standard assay procedure. The results are shown in Fig. 17. The binding of  $^{125}\text{I}$  primary antibody to antigen was directly proportional to the concentration added over the entire range of concentrations used. (This indicates that the condition of antigen excess was met over the entire range.) The binding of secondary antibody, however, gave a typical binding curve with a plateau in the region of primary antibody excess and then falling off as primary antibody became limiting until the binding paralleled the amount of primary antibody bound. The ratio of secondary antibody bound to primary antibody bound leveled off at a near plateau when the secondary antibody bound had fallen off to about 30 percent of the plateau level. Thus, the region of the binding curve where the binding of secondary antibody has fallen off to 30 percent or less of the plateau level was considered the end point region of the region of secondary antibody excess. In

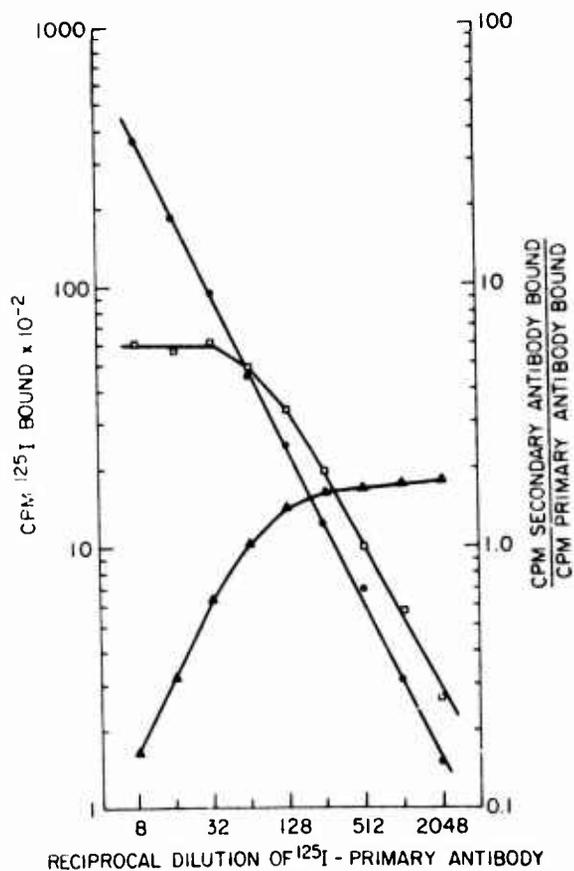


Fig. 17. Determination of the endpoint region of the binding curve. The test was performed using the standard procedure. Antigen was 138I(C) OM protein; rabbit  $^{125}\text{I}$ -anti 138I(C) OM protein antibodies were used as the primary antibody; and  $^{125}\text{I}$ -GARG or unlabeled GARG was used as the secondary antibody. The amount of primary antibody bound ( $\bullet$ ) was determined from the wells to which unlabeled GARG was added and the amount of secondary antibody bound ( $\square$ ) was taken as the difference between the total  $^{125}\text{I}$  bound in the wells to which  $^{125}\text{I}$ -GARG was added and the  $^{125}\text{I}$  bound to the corresponding wells to which unlabeled GARG was added. The ratio of secondary antibody bound to primary antibody bound ( $\blacktriangle$ ) was calculated from the other two curves. Each point represents the mean of triplicate determinations; and backgroundbinding to wells receiving no antigen (filler only) but the same dilutions of  $^{125}\text{I}$ -primary antibody, and no primary antibody but the same amount of  $^{125}\text{I}$ -GARG has been subtracted.

this region the ratio of secondary antibody to primary antibody is nearly constant at approximately eight.

3) Percentage of primary antibody bound in the end point region.

In the end point region of the curve antigen bound to the plate is in large excess over the amount of specific primary antibody added. As shown above (Fig. 5) approximately 90 percent of the specific primary antibody added was removed after 18 hours in an antigen coated well. It is possible that some of this antibody though probably bound to antigen does not stay bound to the plate during washing and subsequent binding of secondary antibody. Any antibodies bound to antigen that has come loose from the surface of the plate would not be detected. Studies designed to determine the extent of this problem are now in progress. Assuming such losses are not too large and can be reliably estimated one can then define the fraction bound  $F_B$  which equals the fraction of specific primary antibodies added which bind and remain bound to the well.

If the three relationships discussed above are known the amount of specific antibody in a serum may be calculated as follows. The counts per minute of  $^{125}\text{I}$  bound at a serum dilution in the end point region of the binding curve is related to the amount of specific primary antibody per milliliter of serum by the following expression:

$$\text{antibody protein } \left( \frac{\text{ng}}{\text{ml}} \right) = \frac{{}^{125}\text{I}_B \text{ (cpm)} \times \text{RD}_{\text{EP}} \times 40}{\text{SASAB (cpm/ng)} \times F_B \times R_{\text{SP}}}$$

Where  ${}^{125}\text{I}_B$  is the counts per minute of  $^{125}\text{I}$  bound at the end point dilution (a suitable dilution in the end point region of the curve),  $F_B$  is the fraction of primary antibody added that is bound to the well,  $\text{RD}_{\text{EP}}$  is the reciprocal dilution of primary antibody at the chosen end point.  $\text{SASAB}$  is the specific activity of the secondary antibody (cpm/ng protein),  $R_{\text{SP}}$  is the ratio of secondary antibody bound to primary antibody bound at the end point dilution, and the factor 40 adjusts the result from ng/25  $\mu\text{l}$  to ng/ml.

Providing the separate variables in the above expression can be determined with sufficient accuracy and reliability, quantitation of serum antibody by this procedure should be possible. Experiments are now in progress to compare quantitative results obtained by this procedure with results obtained by the standard quantitative precipitation test. An alternative approach to quantitation of antibody by SPRIA is to calibrate several sera by quantitative precipitin analysis and use these calibrated sera to empirically relate serum end point dilution to serum antibody content.

### Antigen inhibition of the SPRIA.

Preliminary experiments have demonstrated the feasibility of studying antibody specificity and antigen differences by antigen inhibition of the SPRIA. The conditions necessary for antigen-inhibition studies are a) excess antigen on the plate, b) a limiting concentration of primary antibody and c) excess secondary antibody.

An example of an inhibition experiment is given in Fig. 18. Various dilutions of antigen were mixed with an equal volume of diluent containing a constant amount of primary antibody and kept at 37°C for one hour before adding the mixtures to the antigen coated plate. The plate was further processed by the standard procedure. The homologous antigen inhibited up to 95 percent at 50 µg/ml whereas similar antigens from three heterologous strains inhibited a maximum of about 25 percent. Using this basic procedure we have demonstrated the feasibility of serotyping strains of meningococci on the basis of their protein serotype antigens. This method should also prove useful for determining the antigenicity of an antigen at various stages of purification.

### Discussion

Membrane associated antigens such as membrane proteins and LPS which are usually rather insoluble and difficult to handle in some serological assays appear to be particularly well suited as antigens for the SPRIA. The capsular polysaccharides, on the other hand, are soluble and easy to work with in most assays but are relatively poor antigens in this assay since they do not bind well to the plastic plate. In order for the assay to be reproducible and susceptible to quantitation it is essential that it be done under conditions of antigen excess and that the antigen be sufficiently tightly bound that no significant amount is released from the plate during the assay. For some antigens such as the meningococcal capsular polysaccharides these conditions maybe difficult to meet. Development of methods for modifying these antigens or the plate to enhance binding or for covalently coupling the antigens to the plate would enlarge the range of application of the assay.

The sensitivity of the assay is dependent on the concentration of active secondary antibody that is used. Standardization of the assay with respect to sensitivity requires, at a minimum, knowledge of the relative specific activity or relative concentration of active antibody of different preparations of antiglobulin that are labeled. Saturation binding of successive preparations of secondary antibody to a constant limiting quantity of primary antibody appears to be a relatively simple and effective method for obtaining this information. Alternatively, a value for the active secondary antibody concentration relative to that in the unlabeled antiglobulin may be obtained by determining the quantity of unlabeled secondary antibody of known

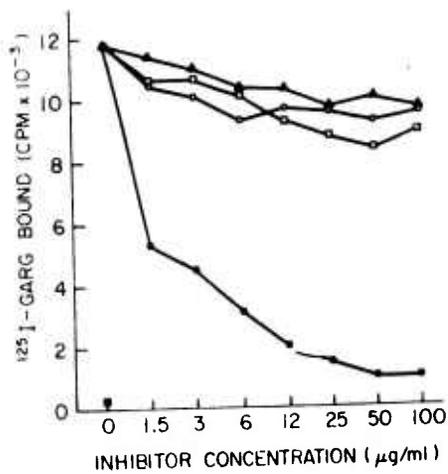


Fig. 18. Antigen inhibition of the SPRIA. Binding of rabbit anti-138I(C) antiserum (1:50,000 dilution) to 138I(C) OMC was inhibited by preincubation for one hour at 37°C with various concentrations of 138I(C) OMC (○), 35E(C) OMC (□), 126E(C) OMC (△) or 118V(C) OMC (●). The test was then completed using the standard procedure.

protein content required to give 50 percent inhibition of binding of  $^{125}\text{I}$ -secondary antibody. Depending on the extent of inactivation of antibody during labeling this method may also yield an acceptable value for the recovery of labeled protein.

The sensitivity of the assay is also limited by the level of non-specific binding of gammaglobulin at high serum concentrations. Normal sera devoid of specific antibody cause binding of significant secondary antibody at dilutions below 1:100 (see Fig. 8). Specific binding of antibodies at levels equal to or less than that of the nonspecific binding cannot be detected. Methods of reducing the nonspecific binding at high serum concentrations are presently under investigation.

The feasibility of quantitating specific antibody using the SPRIA depends in large measure upon one's ability to determine three parameters which are necessary to relate the amount of  $^{125}\text{I}$  bound to the amount of primary antibodies placed in the well. Of these three parameters, only one, the specific activity of secondary antibody, might be expected to vary over a wide range. This parameter, however, is most easily determined and must be known at least in a relative sense to even standardize the sensitivity of the assay. The specific activity values used in this study were determined by estimating protein losses during labeling and are probably subject to error of about  $\pm 10$  percent. Refinements in the procedure, however, should improve the accuracy.

The second parameter is  $R_{sp}$ , the ratio of the amount of secondary antibody bound to the amount of primary antibody bound. Since this value becomes nearly constant in the region of secondary antibody excess ( $\geq 30$  percent of the control plateau level of secondary antibody binding) one can minimize error by choosing a point in this region as the end point. Since counting statistics and background fluctuations become more of a problem at low levels of radioactivity we have used as the end point a dilution which binds 20-30 percent of the control plateau level of secondary antibody. Determination of  $R_{sp}$  requires use of labeled primary antibodies and knowledge of the specific activity of those antibodies. The accuracy of this ratio is clearly dependent on the accuracy of the specific activity determinations of the primary and secondary antibodies. The value we obtained (7.5-8.0) in the region of secondary antibody excess is consistent with the value of seven reported by Kabat [25] for the ratio of rabbit antihuman gammaglobulin to human gammaglobulin in immune complexes precipitated in the region of large antibody excess. The value of  $R_{sp}$  may be relatively constant for different gammaglobulin anti-gammaglobulin systems but in any event should only have to be determined once for each lot of anti-gammaglobulin. Experiments are in progress to determine the value of  $R_{sp}$  for a human gammaglobulin-goat anti-human gammaglobulin system.

The third parameter,  $F_B$ , is the most complex since it may be affected by a number of experimental variables. The length of time the binding of primary antibodies is allowed to continue, the release of antigen concomitant with primary antibody binding or of primary antibody concomitant with secondary antibody binding, and the nature and extent of the washing procedure all affect this parameter. The effects of several of these variables have been investigated but several more remain to be studied. Preliminary results suggest a value for  $F_B$  of about 0.8 when the test is performed using a good antigen (e.g. meningococcal OMC) and the standard assay procedure.

The ease and reliability with which the above three parameters can be determined for a given system will dictate the feasibility and practicality of using the SPRIA as a quantitative assay. Once three basic parameters are known for a given system, a large number of sera could be tested with a modest investment of time and effort.

V. Antibody dependent cellular cytotoxicity (ADCC) of bacteria: Lymphocyte-mediated killing of meningococci (mgc).

Antibody dependent cellular cytotoxicity (ADCC) is one of the mechanisms whereby effector lymphocytes can kill target cells independent of complement ( $c'$ ) [26,27]. It is thought to be an *in vitro* manifestation of *in vivo* lymphocyte-mediated immune cytolysis resulting in anti-viral and anti-parasitic host defenses, allograft rejection, tumor destruction and autoimmune disease. Although cellular aspects of antibacterial host defenses have been widely investigated *in vivo* [28], to date no *in vitro* lymphocyte-mediated bactericidal system has been described. Such a system, ADCC of meningococci (mgc) by purified lymphocyte populations, was investigated during the past year.

Materials and Methods

1. Cell separation: Human peripheral blood lymphocytes from normal adult volunteers were isolated by Ficol-Hypaque density centrifugation and termed undepleted lymphocytes (U-Lym). They were contaminated with 15-25 percent monocytes and one percent polymorphonuclear leukocytes (PMN) as determined by latex ingestion and peroxidase stain. These cells were then incubated for 18-24 hours, and the non-adherent (NA) cells gently decanted. They were found to contain only two-five percent monocytes. No PMN were noted. Adherent cell (Ad) enriched populations scraped from the flasks consisted of 35-55 percent monocytes and 45-65 percent lymphocytes. Further purification of the NA lymphocytes was obtained by passage over nylon wool columns, which resulted in 99.7 percent pure lymphocytes (NW) by stain and no monocyte contamination by latex ingestion.

2. Preparation of bacteria: Meningococcal strain 138-I (group C) was grown overnight, transferred to Mueller-Hinton broth and grown in a shaking water bath to a final O.D. of 0.62 over two hours.  $3 \times 10^3$  organisms diluted in cold test media (RPMI, 2.5 percent Hepes buffer and 1 percent L-glutamine, TM), were used as target cells in the ADCC test

3. ADCC bactericidal assay: 0.1 cc of cold diluted meningococci (mgc), approximately 3,000 organisms/tube, were added to 0.1 cc of heat inactivated human antiserum, known to contain antibodies against group C capsular polysaccharide, from volunteers who had been immunized with the group C polysaccharide vaccine. Final serum dilutions were 1/40-1/1280. Cold TM was added to bring the final volume to 0.5 cc. The bacteria were centrifuged and then incubated at 4°C for 30-90 min. to coat the target bacteria. Populations of U-Lym, NA, NW and Ad cells were added at mononuclear cell:bacteria (effector:target cell) ratios ranging from 5/1 to 550/1. The tubes were again centrifuged and then incubated at 37°C in five percent CO<sub>2</sub> for 20, 50, 60 or 120 min. The standard assay was performed at a 75/1 target:effector cell ratio, antisera dilution of 1:160 and an incubation time of one hour. All tests were performed in triplicate. Bacterial growth was determined by colony counting of 20 microliter samples after overnight growth on Mueller-Hinton agar.

In order to assess the cytotoxicity of the cells + Ab on the mgc three calculations were made. The first, percent total kill (% kill<sub>T</sub>), reflects the total percentage of bacteria killed at a given time period (usually one hour) in the experimental tubes (e.g. cells + Ab) compared to the number of bacteria present at the start of the 37°C incubation period (zero time) based on the formula -

$$\% \text{ kill}_T = 100 - \left( \frac{\text{no. colonies exptl.}}{\text{no. colonies zero time}} \right) \times 100$$

The second calculation, percent specific kill (% kill<sub>sp</sub>), was derived in order to control for the effects of Ab alone on mgc during the incubation period. % kill<sub>sp</sub> reflects the specific effect of the combination of cells + Ab compared to that which Ab would have accomplished alone at a given time period based on the formula -

$$\% \text{ kill}_{sp} = 100 - \left( \frac{\text{no. colonies cells + Ab}}{\text{no. colonies Ab}} \right) \times 100$$

When there were less colonies at the end of the incubation in the Ab tubes than there were at zero time, the % kill<sub>sp</sub> was correspondingly less than the % kill<sub>T</sub>.

The third calculation, percent relative kill (% kill<sub>R</sub>), was determined in order to account for the growth of bacteria during the test incubation period. % kill<sub>R</sub> reflects the ongoing inhibitory effect of cells + Ab compared to how the bacteria would have grown in the test media alone during the same incubation time period based on the formula -

$$\% \text{ kill}_R = 100 - \left( \frac{\text{No. colonies cells + Ab}}{\text{No. colonies media}} \right) \times 100$$

% kill<sub>R</sub> at one-two hours may also be indicative of the maximum percent specific kill at 20 min. as demonstrated in the Results Section. This is theoretically evident when it is understood that kinetically the smaller number of bacteria left viable in the experimental cells + Ab tubes at 20 min. grow to a proportionately smaller number at 60 min. than do the bacteria in the control tubes. Accordingly, % kill<sub>R</sub> may be more representative of the ongoing kinetic effects of cells + Ab than either % kill<sub>T</sub> or % kill<sub>sp</sub>. All three calculations are given so that the entire picture may be appreciated.

In addition to zero time and Ab, controls run in every experiment included test media, baby bunny complement (c'), Ab + c' and cells alone. Significance of cytotoxicity was determined by unpaired T-tests where P > 0.05 was not considered significant. Unless otherwise indicated, all cytotoxicity was significant above this level. P values were usually < 0.02.

### Results.

Table 22 documents the results of a representative experiment of one donor's Ficoll-Hypaque separated lymphocytes prior to and following removal of adherent cells (Ad) by overnight incubation of the cells in plastic flasks. Note that this method which depleted monocytic contamination from 10-25 percent to 2-5 percent also eliminated the one percent contamination by polymorphonuclear leukocytes (PMN). This PMN elimination was owing to their known propensity to undergo autolysis following 18 hours incubation in vitro. Undepleted (U-Lym) and non-adherent (NA) lymphocytes killed 46 percent and 44 percent of mgc respectively demonstrating that removal of Ad and PMN does not alter the ability of lymphocytes to kill mgc. Table 22 also shows that complement (C'), immunoglobulin-free serum (A<sub>γ</sub>FCS), C' and A<sub>γ</sub>FCS or viable cells alone caused marked stimulation of bacterial growth producing -43 percent, -123 percent, -94 percent and -39 percent kill, respectively. In other experiments, immunoglobulin free serum + cells as well as supernatant from cells antigenically stimulated with mgc antigens also repeatedly promoted growth of bacteria while nonviable lymphocytes (as determined by non-exclusion of trypan blue) always failed to produce either significant kill or stimulation of bacteria. The above is interpreted to mean that serum moieties or factors secreted by viable cells provide an enriched media for bacterial growth while only the

Table 22. ADCC of mgc by Ficoll-Hypaque separated lymphocytes A) prior to and B) following depletion of adherent cells.

Adherent cells	Variable	Mean no. colonies $\pm$ SEM	% kill <sub>t</sub> <sup>a</sup>	Kill <sub>sp</sub> <sup>b</sup>
A)	Zero time	158 $\pm$ 11	-	-
	Ab	167 $\pm$ 1	- 6 (NS) <sup>c</sup>	-
	C'	196 $\pm$ 23	-43	-
	Ab + C'	0	100	100
	A $\gamma$ FCS <sup>d</sup>	306 $\pm$ 3	-123	-
	A $\gamma$ FCS + C'	266 $\pm$ 9	-94	-
	Present U-Lym <sup>e</sup>	191 $\pm$ 24	-39	-
	" U-Lym <sup>e</sup> + Ab	86 $\pm$ 23	46	49 <sup>g</sup>
	" NVC <sup>f</sup> + Ab	151 $\pm$ 9	4 (NS) <sup>c</sup>	6 (NS) <sup>c</sup>
	B)	Zero time	253 $\pm$ 18	-
Ab		242 $\pm$ 15	4 (NS) <sup>c</sup>	-
Ab + C'		5 $\pm$ 3	98	98
Removed NA <sup>h</sup>		344 $\pm$ 6	-36	-
" NA <sup>h</sup> + Ab		141 $\pm$ 2	44 <sup>g</sup>	42 <sup>g</sup>

Footnotes to Table 22.

- a % kill relative to the zero time; negative values indicate stimulation. See text.
- b % kill relative to the effect of Ab; negative values indicate stimulation. See text.
- c NS = Not significant;  $p > 0.05$
- d A $\gamma$ FCS = A gamma fetal calf serum
- e U-Lym = Undepleted lymphocytes, i.e. prior to depletion of adherent cells. U-Lym/mgc ratio = 120/1.
- f NVC = Non-viable lymphocytes. See text.
- g  $p < 0.02$
- h NA = Non-adherent lymphocytes, i.e. after depletion of adherent cells. NA/mgc ratio = 63/1.

interaction of cells + Ab result in bacterial kill.

Table 23 demonstrates the variation of ADCC with Ab concentration. Note that cells + Ab concentrations both above and below serum dilutions of 1/80-1/160 resulted in decreased % kill. This optimal serum dilution was remarkably consistent for a given individual as can be seen in subsequent tables.

Table 24 demonstrates the variation of ADCC of mgc with effector/target cell ratio at two Ab concentrations. Once again, media, complement and cells alone promoted bacterial growth. Increasing NA;mgc effector:target cell ratios from 5:1 to 550:1 increase % specific kill from 43 percent to 94 percent at the optimal Ab concentration of 1/160, and from 11 percent to 71 percent at the more dilute Ab concentration of 1/640. Note that the highest concentration of NA lymphocytes + Ab resulted in % specific kills comparable to that produced by Ab + complement in both Ab concentrations.

Table 25 compares the ADCC effects of non-adherent (NA), nylon wool depleted (NW) and adherent (Ad) cells. In the three experiments shown, NA cells resulted in an average % total kill of 56 percent and an average % specific kill of 44 percent; NW cells resulted in an average % total kill of 42 percent and an average % specific kill of 26 percent, while Ad cells resulted in an average % total kill of 67 percent and an average % specific kill of 59 percent. Thus, nylon wool depletion of NA cells caused average decreases of 14 percent total kill and 16 percent specific kill from that caused by autologous non-nylon wool treated NA cells. These decreases ranged from one percent and two percent (expt. no. 1) to 25 percent and 30 percent (expt. no. 2) of % total and specific kills, respectively.

Table 26 describes the variation of ADCC of mgc by NA and NW cells with time. Of particular importance is that maximum % total and specific kills were found at 20 min. Furthermore, there was absolutely no difference between the cytotoxicity of NA + Ab and that of NW + Ab at this time period, both producing 89 percent kill<sub>T</sub> and 88 percent kill<sub>sp</sub>. As the incubation time continued through 40, 60 and 120 min., however, the bacteria that were not killed multiplied so that at 60 min. only 38 percent kill<sub>T</sub> and 33 percent kill<sub>sp</sub> was seen in the NA + Ab tubes and only 23 percent kill<sub>T</sub> and 17 percent kill<sub>sp</sub> was seen in the NW + Ab tubes. While even the latter values are, indeed, statistically significant ( $p < 0.05$ ), had we sampled only at 60 min., the true cytotoxic potential (i.e., the maximum % kill<sub>sp</sub>) would not have been evident by calculating only the total or specific kills at that time. Moreover, the apparent differences in cytotoxicity between the NW and NA cells + Ab (15 percent and 16 percent differences in kill<sub>T</sub> and kill<sub>sp</sub> respectively) were, therefore, merely increases in growth of mgc in the NW + Ab tubes during the extra 40 min. incubation.

Table 23. ADCC of mgc by non-adherent (NA) lymphocytes as a function of antibody concentration.

Variable	Mean no. colonies $\pm$ SEM	% Kill <sub>T</sub> <sup>a</sup>	% Kill <sub>SP</sub> <sup>b</sup>	% Kill <sub>R</sub> <sup>c</sup>
Zero time	229 $\pm$ 10	-	-	
Media	359 $\pm$ 19	-57	-	
NA <sup>d</sup>	317 $\pm$ 8	-38	-	
NA + Ab 1/20	101 $\pm$ 9	56	31	72
NA + Ab 1/40	74 $\pm$ 8	68	56	79
NA + Ab 1/80	52 $\pm$ 5	77	69	86
NA + Ab 1/160	53 $\pm$ 5	78	67	85
NA + Ab 1/320	75 $\pm$ 12	67	47	79
NA + Ab 1/640	105 $\pm$ 11	54	43	71
NA + Ab 1/1280	120 $\pm$ 7	47	35	67

a,b See footnotes of Table 22.

c % Kill<sub>R</sub> = % kill relative to the no. of mgc in the control (media tubes) following the same incubation.

d NA = Non-adherent lymphocytes used at lymphocyte:mgc ratio of 70:1.

Table 24. ADCC of mgc by non-adherent lymphocytes (NA) as a function of lymphocyte:mgc ratio.

Variable	Mean no. colonies $\pm$ SEM	% Kill <sub>T</sub> <sup>a</sup>	% Kill <sub>S</sub> <sup>b</sup>	% Kill <sub>R</sub> <sup>c</sup>
Zero time	288 $\pm$ 21	-	-	-
Media	390 $\pm$ 21	-35	-	-
C'	339 $\pm$ 44	-1.8	-	-
NA (5:1) <sup>d</sup>	425 $\pm$ 29	-48	-	9
NA (14:1)	441 $\pm$ 41	-53	-	-13
NA (55:1)	445 $\pm$ 24	-84	-	-14
NA (140:1)	448 $\pm$ 13	-56	-	-15
NA (550:1)	390 $\pm$ 9	-35	-	0
Ab 1/160	225 $\pm$ 15	22	-	-
Ab 1/160 + C'	7 $\pm$ 3	98	97	-
Ab 1/160 + NA (5:1) <sup>d</sup>	129 $\pm$ 8	55	43	67
Ab 1/160 + NA (14:1)	76 $\pm$ 4	74	66	81
Ab 1/160 + NA (55:1)	21 $\pm$ 6	93	91	95
Ab 1/160 + NA (140:1)	21 $\pm$ 4	93	91	95
Ab 1/160 + NA (550:1)	13 $\pm$ 3	96	94	97
Ab 1/640	338 $\pm$ 10	-17	-	-
Ab 1/640 + C'	103 $\pm$ 21	64	69	74
Ab 1/640 + NA (5:1) <sup>d</sup>	300 $\pm$ 7	-4 (NS) <sup>e</sup>	11 (NS) <sup>e</sup>	23
Ab 1/640 + NA (14:1)	260 $\pm$ 20	10 (NS) <sup>e</sup>	23	33
Ab 1/640 + NA (55:1)	215 $\pm$ 13	25	36	45
Ab 1/640 + NA (140:1)	118 $\pm$ 10	59	65	70
Ab 1/640 + NA (550:1)	98 $\pm$ 11	66	71	75

a,b,c See footnotes of Table 23.

d (X:y) = Lymphocyte:mgc ratio

e NS = Not significant: p >0.05

Table 25. Comparison of ADCC of mgc by non-adherent (NA), nylon wool depleted (NW) and adherent (Ad) cells.

Expt. no.	Variable	No. colonies ± SEM	% Kill <sub>T</sub> <sup>a</sup>	% Kill <sub>S</sub> <sup>b</sup>	% Kill <sub>R</sub> <sup>c</sup>
1	Zero time	122 ± 7	-	-	-
	Media	204 ± 8	-67	-	-
	Ab 1/80	90 ± 7	26	-	-
	NA (100:1) <sup>d</sup>	170 ± 9	-39	-	-
	NA + Ab	62 ± 4	49	31	67
	NW (100:1)	183 ± 7	-50	-	-
	NW + Ab	64 ± 4	48	29	66
	NA (200:1) <sup>d</sup>	151 ± 5	-24	-	-
	NA + Ab	55 ± 9	55	40	71
	NW (200:1)	168 ± 11	-38	-	-
NW + Ab	63 ± 13	48	30	66	
2	Zero time	131 ± 15	-	-	-
	Media	187 ± 5	-43	-	-
	Ab 1/160	109 ± 6	17	-	-
	NA (90:1) <sup>d</sup>	187 ± 10	-43	-	-
	NA + Ab	48 ± 5	63	56	74
	NW (90:1)	160 ± 6	-22	-	-
	NW + Ab	80 ± 8	39	27	57
	Ad (90:1)	160 ± 11	-22	-	-
	Ad + Ab	28 ± 4	79	74	85
3	Zero time	204 ± 1	-	-	-
	Media	282 ± 15	-38	-	-
	Ab 1/160	165 ± 3	19	-	-
	NA (60:1) <sup>d</sup>	279 ± 11	-36	-	-
	NA + Ab	84 ± 6	59	49	70
	NW (60:1)	259 ± 4	-27	-	-
	NW + Ab	134 ± 11	34	19	51
	Ad (60:1)	266 ± 4	-30	-	-
Ad + Ab	93 ± 5	54	44	67	

a,b,c,d See footnotes of Table 24.

Table 26. ADCC of mgc by non-adherent (NA) and nylon wool depleted (NW) lymphocytes as a function of time.

Variable	No. colonies			20 Min.			40 Min.			120 Min.		
	No. colonies ± SEM	% Kill <sup>a</sup> <sub>T</sub>	% Kill <sup>b</sup> <sub>SP</sub>	% Kill <sup>c</sup> <sub>T</sub>	% Kill <sup>b</sup> <sub>SP</sub>	% Kill <sup>c</sup> <sub>R</sub>	No. colonies ± SEM	% Kill <sup>a</sup> <sub>T</sub>	% Kill <sup>b</sup> <sub>SP</sub>	% Kill <sup>c</sup> <sub>T</sub>	% Kill <sup>b</sup> <sub>SP</sub>	% Kill <sup>c</sup> <sub>R</sub>
Zero time	71 ± 5	-	-	-	-	-	71 ± 5	-	-	-	-	-
Media	120 ± 11	-69	-	-	-	-	166 ± 5	-133	-	-	-	-
Ab 1/160	67 ± 1	6 (NS) <sup>e</sup>	-	-	-	-	66 ± 2	7 (NS) <sup>e</sup>	-	-	-	-
C'	66 ± 1	7	-	-	-	-	63 ± 1	11	-	-	-	-
Ab + C'	2 ± 1	97	97	-	-	-	0	100	100	-	-	-
NA (225:1) <sup>d</sup>	93 ± 7	-31	-	-	-	-	141 ± 4	-99	-	-	-	-
NA + Ab	8 ± 15	89	88	-	-	93	31 ± 2	56	53	-	-	81
NW (225:1) <sup>d</sup>	73 ± 1	-3	-	-	-	-	126 ± 6	-78	-	-	-	-
NW + Ab	8 ± 3	89	88	-	-	93	57 ± 6	20 (NS) <sup>e</sup>	14 (NS) <sup>e</sup>	-	-	66
Zero time	71 ± 5	-	-	-	-	-	71 ± 5	-	-	-	-	-
Media	214 ± 10	-120	-	-	-	-	800 ± 43	-1026	-	-	-	-
Ab 1/160	66 ± 3	7 (NS) <sup>e</sup>	-	-	-	-	97 ± 26	-37 (NS) <sup>e</sup>	-	-	-	-
C'	56 ± 0	21	-	-	-	-	180 ± 12	-7	-	-	-	-
Ab + C'	0	100	100	-	-	-	0	100	100	-	-	-
NA (225:1) <sup>d</sup>	190 ± 12	-168	-	-	-	-	560 ± 35	-520	-	-	-	-
NA + Ab	44 ± 2	38	33	-	-	70	50 ± 0	30	38	-	-	94
NW (225:1) <sup>d</sup>	190 ± 6	-168	-	-	-	-	690 ± 52	-857	-	-	-	-
NW = Ab	55 ± 4	23	17	-	-	74	60 ± 11	16 (NS) <sup>e</sup>	25 (NS) <sup>e</sup>	-	-	93

a,b,c,d,e See footnotes Table 24.

It is tempting to speculate by interpolating the 60 min. data shown in Tables 22-25 that at 20 min. the maximum percent specific kills of NA and NW cells varied between 85-98 percent. That this may be the case is suggested by calculating the percent relative kill (% kill<sub>R</sub>) which varies relatively little with time. As can be seen in Table 26, % kill<sub>R</sub> is quantitatively comparable at 20, 60 and 120 min. to the maximum percent specific kill seen at 20 min. % kill<sub>R</sub> of NA + Ab was equal to 93 percent, 79 percent and 94 percent at 20, 60 and 120 min., respectively while that of NW + Ab equaled 93 percent, 74 percent and 93 percent at these respective times. Since % kill<sub>R</sub> controls for the growth of bacteria during the incubation period, and since this calculation remains relatively constant and comparable to the maximum percent specific kill, % kill<sub>R</sub> may, therefore, be the most accurate expression of the total bactericidal effect of cells + Ab compared to the bacterial viability in the absence of cells + Ab. In light of the above, returning to Table 25, it may be seen that while Ad cells + Ab resulted in an average % kill<sub>R</sub> of 76 percent and NA + Ab gave 71 percent, even the 99.7 percent purified lymphocyte populations resulted in an average % kill<sub>R</sub> of 60 percent.

#### Discussion.

The contribution of the interaction of antibody with lymphocytes has never been explored in a complement free *in vitro* bactericidal system. The demonstration in this work that purified populations of peripheral blood lymphocytes (99.7 percent pure) can kill bacteria expands the cytotoxic capabilities of lymphocytes that have been demonstrated using lymphocytes, erythrocytes, parasites, and various normal and neoplastic cells as targets. Unlike nonbacterial target cell systems, however, we were unable to demonstrate any bacterial cytotoxicity in the absence of antibody, even when cells of individuals immunized with group C polysaccharide vaccine were used. In contrast, as has been shown in other ADCC systems, in cooperation with antibody, cells from nonimmunized individuals killed as well as those from immunized subjects. This lack of necessity for immunized cells in ADCC systems as opposed to their requirement in antibody-independent lymphocyte mediated cytotoxicity (LMC) is thought to reflect the mediation of killing by T-cells in LMC systems while only non-T cells reportedly mediate ADCC [26,27]. There is currently controversy in the literature as to whether or not B cells participate in ADCC. Whether B, T or Null(K) cells are the effector lymphocytes in bacterial cytotoxicity is currently under investigation. If, indeed, B cells are effective, the decrease in % kill seen in our experiments following nylon wool depletion (Table 25) (which depleted monocyte contamination from 2-5 percent to <0.3 percent) might be explained by the column's removal of B cells. That nylon wool columns deplete B cells has been amply demonstrated.

While it may be argued that the cytotoxicity obtained in our highly purified lymphocyte preparations was owing to the 0.05-0.3 percent monocyte contamination of these cells (as determined by Kaplow's peroxidase stain) no phagocytosis of bacteria or of latex particles comparable in size to mgc was ever seen in any nylon wool depleted cell population. Furthermore, (Table 22) undepleted lymphocytes (with 15-25 percent monocytes) yielded 46 percent total kill of mgc while autochthonous non-adherent cells (with 2-3 percent monocytes) resulted in 44 percent total kill (at similar lymphocyte:mgc ratios), and (Table 25, expts. 1 and 2), monocyte enriched populations of adherent cells (with 35-55 percent monocytes) averaged 67 percent total kill while autochthonous non-adherent cells (with 2-5 percent monocytes) averaged 61 percent total kill at the same mononuclear:mgc ratios. These differences in cytotoxicity were not statistically significant, thereby, supporting our contention that the bactericidal activity present in our assay is not a function of monocytic phagocytosis. Moreover, it has recently been shown that heat inactivation of human sera reduced the ability of human monocytes to phagocytose gram negative bacteria from 7.9 percent to <0.8 percent of available bacteria using a 10 percent concentration of serum. In contrast, only heat-inactivated serum was used in our experiments and the optimal serum concentration constituted only 0.6 percent of the test volume. That monocytes, when present in significant proportions, may contribute to the total cytotoxicity is entirely possible since they are capable of ADCC [28,29] as well as phagocytosis. This may explain the lack of significant decrease in cytotoxicity seen when Ad cells (with only 45-65 percent lymphocytes) were used instead of NA cells (with 95-98 percent lymphocytes).

The kinetics of our studies indicate that in any given experiment, ADCC varies with Ab concentration, effector/target cell ratio and time. Thus, any given serum sample was most effective at a specific dilution, e.g. 1/160. This optimal Ab concentration corresponded within one dilution to that concentration of Ab which was bactericidal when used with complement. It was posited that more dilute Ab concentrations did not provide enough effective Ab to coat the target bacteria. Higher concentrations of antibody on the other hand were less effective, possibly due to the interaction of cells with excess free Ab (i.e. Ab not attached to bacteria), thereby, competitively inhibiting the cells from reacting with Ab-coated bacteria. Alternatively, blocking factors, such as IgA [11] present in the higher concentrations of antisera may have been diluted out at the optimal concentration, thereby, allowing interaction of cells with bactericidal Ab and bacteria.

It should be noted that while increasing the effector:target cell ratio invariably increased the resultant cytotoxicity within any specific experiment, significant day to day (person to person) variation of bactericidal activity was evident. Thus, 5:1 and 55:1 NA:mgc ratios resulted in 43 percent and 91 percent specific kills

respectively in one experiment (Table 24), while when using the same Ab concentration with another donor (on another day), only 56 percent specific kill at an NA:mgc ratio of 90:1 was found. This variation may reflect temporal differences in the expression of either relevant antigens on the growing bacteria or specific receptors on the effector lymphocytes.

Variation of bacterial ADCC was also manifested in experiments examining the rate of cytotoxicity within a given experiment. As shown in Table 25, the maximum percent specific kill can occur following only 20 min. of incubation. Over the subsequent one-two hours the bacteria remaining alive may multiply so that the 20 min. 89 percent kill<sub>T</sub> may be reduced to only 23 percent kill<sub>T</sub> at 60 min. compared to the number of bacteria at time zero. In other experiments, however, the rate of growth was not as dramatic so that maximum specific and total kills were occasionally found at 40, 60, or even 120 min. of test incubation. These dynamic differences in the growth potential of the bacteria were often reflected in the calculation of % kill<sub>R</sub> which compared the number of viable bacteria in the experimental tubes (cells + Ab) to those in tubes with media alone at any given time period. Since the calculation % kill<sub>R</sub> controlled for bacterial growth, the cytotoxicity evidenced varied little at 20, 60 and 120 min. (Table 26). Moreover, in those experiments in which the bacteria grew more slowly allowing for maximum percent total kills at later time periods, the % kill<sub>R</sub> corresponded to the % kill<sub>T</sub>.

In light of the above, it is significant that % kill<sub>R</sub> of Ad, NA and NW cells + Ab were not substantially different averaging 76 percent, 72 percent and 63 percent respectively at 60 min. (Tables 25 and 26). Furthermore, there was no difference at all in % kill<sub>R</sub> between NA and NW cells, both equaling 93.5 percent  $\pm$  0.5 percent at 20 min. and at 120 min.

In summary, we have demonstrated the ability of purified populations of lymphocytes to kill mgc *in vitro* in cooperation with specific antisera. This ADCC varied with Ab concentration, effector/target cell ratio, and growth rate of the target bacteria. We have given evidence to support the recent report [29,30] that the critical lytic membrane lesion in ADCC occurs within the first 15 min. Moreover, the cytotoxic potential of lymphocytes, well known for their ability to kill a variety of normal and neoplastic animal and human cells has thus been expanded to include bacteria. While the protective effect of the humoral bactericidal reaction of Ab and complement is indisputably important in resistance to meningococcal disease [18], the clinical role of the lymphocytic ADCC has yet to be determined. In this regard, the switch of predominantly polymorphonuclear leukocytes to the lymphocytosis seen in spinal fluid during recovery from meningitis may be relevant. Furthermore, resistance to those diseases involving bacteria that are not sensitive to Ab + complement (e.g. serum-resistant strains of

pseudomonas) might very well require the ADCC potential of effector lymphocytes. This possibility, as well as the relevant antigens and antibodies involved in lymphocytes-dependent bacterial ADCC, is currently being explored.

#### Acknowledgement.

This work was done in collaboration with Dr. Richard P. MacDermott, Department of Gastroenterology, WRAIR.

#### Summary and Conclusions.

Cell wall protein antigens from meningococci of serogroups B and C have been prepared as vaccines and shown to be safe and immunogenic in animals. Two of these protein vaccines have been tested in five human volunteers and shown to be safe. Greater than four-fold rises in bactericidal antibody activity against groups B and C organisms occurred in the volunteers. Rises in bactericidal activity was detected against all group B strains tested indicating group specificity in contrast to rabbit responses which demonstrated type specificity. Analysis of 102 organisms typed by the radiobactericidal system indicated that a protein determinant was a marker for epidemic potential in terms of absence of bactericidal antibody directed against it. The immunoepidemiology of groups B and C is similar, while that of group Y is different. A solid phase radioimmunoassay has been developed to investigate protein antigens and quantitate antibody responses to them. A hemagglutination inhibition system has identified eight different lipopolysaccharide (LPS) types of the meningococcus. An LPS Farr test has also demonstrated serologic differences. Lymphocytes participate in antibody dependent cellular cytotoxicity against the meningococcus.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 120 Antigenic components of the cell wall of Neisseria meningitidis

Literature Cited.

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2. Griffiss, J. M. Bactericidal activity of meningococcal antisera: Blocking by IgA of lytic antibody in human convalescent sera. *J Immunology* 114:1779-1784, 1975.
3. Griffiss, J. M., Bannatyne, R. M., Artenstein, M. S. Recurrent meningococcal infection with an antigenically identical strain. *JAMA* 229:68-70, 1974.
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5. Zollinger, W. D., Pennington, C. L., Artenstein, M. S. Human antibody response to three meningococcal membrane antigens: Comparison by specific hemagglutination assays. *Infect Immunity* 10:975-984, 1974.
6. Artenstein, M. S. Development and use of meningococcal vaccines. *Arch Roum Path Exp Microbiol* 33:127-132, 1974.
7. Brandt, B. L., Artenstein, M. S. Duration of antibody responses after vaccination with group C Neisseria meningitidis polysaccharides. *J Infect Dis* 131:S69-S72, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA OB 6517	75 07 01	DD-DR&F(AK)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8A. DR&F INSTR <sup>f</sup>	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM A WORK UNIT
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10. NO./CODES <sup>g</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	61101A	3A161101A91C	00	185			
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Speciation of Biomolecules by Mass Spectrometry							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
002300 Biochemistry 008300 Inorganic Chemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER: <sup>j</sup>				FISCAL YEAR		C. CURRENT	
C. TYPE:				75		4	
D. KIND OF AWARD:				76		3	
E. AMOUNT:						142	
F. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Bass, B.G., MS			
				NAME: Kelley, CPT., J.A. DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Mass Spectrometry; (U) Drug Metabolism; (U) Gas Chromatography; (U) Analytical Chem							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The technical objective of this work unit is to develop and establish methodology and analytical techniques for the detection, identification and characterization of important biochemical compounds and their principal metabolites using coupled gas chromatography-mass spectrometry (GC-MS) for use in military medicine.</p> <p>24. (U) Computer-controlled GC-MS systems will be used to definitively characterize and quantitate compounds of biochemical importance for both developmental and research uses. Efforts will be focused on rapid, accurate identification of a variety of compounds and on feasibility of the implementation of these systems for research and practical general laboratory use. Efforts will be concentrated on metabolites of compounds of interest in drug abuse programs and in the study of metabolism of biomolecules both natural and foreign to mammalian biosystems. A library of mass spectra of drugs of abuse of military significance will be developed.</p> <p>25. (U) 74 07 - 76 06 Updated GC-MS with chemical ionization source installed and debugged. Adaptation of system to capillary columns has been completed. Successful in detecting small concentration of 11-hydroxy-delta-9-tetrahydrocannabinol in urine of a casual marijuana smoker. Deuterated (D4) THC synthesized for use as standard. Mass spectra of 6 and 8 spin-labeled codeine and 3-methylated 6 and 8 spin-labeled morphine found to be identical. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.</p>							

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 185 Speciation of biomolecules by mass spectrometry

Investigators.

Principal: LTC (P) Douglas J. Beach, MSC;  
Associate: Billy G. Bass, M.S.; LTC Gale E. Demaree, MSC;  
Laurence R. Hilpert, B.S.; Leo Kazyak, B.S.;  
CPT James A. Kelley, MSC; Robert C. Permisohn,  
M.S.; SP4 Steven J. Weise, B.S.

The objective of this work unit is to develop and establish methods and analytical techniques for the detection, identification, characterization and quantification of important biochemical compounds and their principal metabolites using coupled gas chromatography-mass spectrometry systems. The major effort during the past year was in the following areas:

1. Capillary column development in gas chromatography.
  2. Chemical ionization source installation.
  3. GC-MS applications.
1. Capillary column development in gas chromatography.

Advances have been made in the development and use of capillary columns for gas chromatography. Glass capillary columns 50 meters in length can now be readily prepared that have separation efficiencies of 1100-1500 theoretical plates per meter. An efficiency of over 80,000 theoretical plates has been achieved with a somewhat longer column. (By comparison, the maximum efficiency of an average packed column for gas chromatography is about 3000 theoretical plates). Moreover, up to one microliter of sample can be injected into the column without adversely affecting the column or its performance through the use of a modified gas chromatograph inlet. This modification has improved analytical precision by eliminating unpredictable sample splitting normally employed in capillary column operations. The columns are support coated open tubular (SCOT) columns with an internal diameter of approximately 0.6 mm. The column substrate is a methyl silicone polymer, SE 30, supported on silonox to improve and increase surface area.

## 2. Chemical ionization source installation.

The Model 5930A Hewlett-Packard quadrupole gas chromatograph-mass spectrometer has been modified and augmented. A new Model 5982A dual source (chemical ionization/electron impact) GC-MS system has been installed and checked out. The new instrument, in addition to having the chemical ionization source, includes an extended mass range to 1000 amu, improved electronics, jet orifice molecular separator, and a more sensitive detector. Over 180 samples have been analyzed with the new system. A marked improvement in reliability and ease of operation over the old system has been observed.

## 3. GC-MS Applications.

Applications of the GC-MS system to biochemical problems have steadily increased with equipment reliability improvements and more experienced personnel support. The following items are the most notable applications.

a. Characterization of methaqualone metabolites and confirmation of gas chromatographic analysis of methaqualone in human urine.

b. Characterization of cannabinoids in human urines. The combination of capillary column separation capability, isotope dilution techniques and selective ion monitoring with the GC-MS has improved remarkably the detection and identification of marijuana metabolites in human urine, even in very low concentrations.

c. The characterization of materials used in spin labeling experiments has been accomplished with mass spectrometry. Since the stable free radicals do not give satisfactory proton magnetic resonance spectra, mass spectrometry has become the technique of choice for characterizing these molecules. Mass spectra of synthetic 6 - and 8 - spin labeled codeine and 3-methylated 6 - and 8 - spin labeled morphine have been found to be identical. Further investigation has shown that the morphine and codeine molecules have the piperidine nitroxide free radical attached to the 6 - position in all cases. Mass spectral methods were employed to unambiguously determine the position of attachment of the free radical in a 3 - spin labeled morphine molecule.

d. Combined GC-MS of m-chloroperbenzoic acid generated nitroxide free radicals indicated an unusual alcohol oxidation reaction. This knowledge was exploited to improve the yield of nitroxide free radicals and to develop a general method for transforming secondary alcohols to ketones. GC-MS analysis of alcohol oxidation mixtures was employed to characterize products, improve yields and delineate a reaction mixture.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA 08 05 70	15 07 01	DD FORM 1498 (AR) 6 68	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8. ORGN INSTR <sup>6</sup>	9a. SPECIFIC DATA - CONTRACTOR ACCESS	9b. LEVEL OF SUM
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A. PRIMARY	61101A	3A161101A91C	00	191			
B. CONTRIBUTING							
C. CONTRIBUTING							
11. TITLE <sup>7</sup> (Precede with Security Classification Code)							
(U) Immunosuppressive Activity of Medicinal Compounds							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>8</sup>							
002600 Biology							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD			
72 07	CONT	DA		C. In-house			
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)
A. DATE/EFFECTIVE NA				PRECEDING			
B. NUMBER <sup>10</sup>				FISCAL YEAR	75	.3	25
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D. KIND OF AWARD				E. AMOUNT		F. CUM. AMT.	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. and include institution)			
NAME: Buescher, COL E. L.				NAME: Kinnamon, K. E., LTC			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2292			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				[REDACTED]			
22. KEYWORDS (Precede each with Security Classification Code)				23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)			
(U) Immunosuppression; (U) CFUs; (U) Spleen Colony Assay; (U) Simonsen Assay; (U) Bone Marrow; (U) Allogeneic				23. (U) To conduct testing to evaluate the immunosuppressive activity of various medicinal compounds. Knowledge re immunosuppressive activities of new drugs is essential to the Army Drug Development Program.			
				24. (U) Certain chemical agents which are being tested primarily for certain properties, e.g., antimalarial properties, may also have immunosuppressive properties. These immunosuppressive properties in some cases may serve to guide the development of potential drugs.			
				25. (U) 74 07 - 75 06 Certain chemical agents which may be used for their other properties may also have profound effects upon the immune system. For example certain chemical agents with radiation protective properties have been found to be immunosuppressively active. This system has been used to evaluate WR 122,455, WR 14,997, WR 30,090, WR 33,063, WR 1544, WR 158,122, WR 2721, WR 347, WR 3689, WR 108,503, WR 151,331, WR 2529, WR 348, WR 638 and WR 2823. The degree of immunosuppression was assessed by (1) injecting the candidate compound into the spleen cell donor beginning four days before death and (2) by incubating normal spleen cells with the compounds before injecting them into the infant mice. The data are being evaluated and will be the subject of a later report. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.			

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 191 Immunosuppressive activity of medicinal compounds

Investigators.

Principal: LTC Kenneth E. Kinnamon, VC

Associate: CPT Lyle L. Ketterling, MSC

Certain chemical agents which may be used for their other properties may also have profound effects upon the immune system. For example certain chemical agents with radiation protective properties have been found to be immunosuppressively active. The infant mouse assay system (1-3) has been used to evaluate the immunosuppressive activity of certain compounds in U.S. Army Drug Development programs. The assay for immunosuppressive activity consists of recording the spleen and liver enlargement which results in the recipients after transplantation of adult spleen cells to recipients that are less than 10 days old. From the spleen and liver enlargements, organ indices are computed. Recipient mice are first generation hybrids between the donor strain and the other strain against which the donor is expected to react, i.e. (C57BL/6 x CBA) F<sub>1</sub>. The response depends upon the absence of host antigens in the donor. Contrariwise, the presence of donor antigens which are absent in the host does not lead to an immune reaction under the condition of the assay system. In the system the recipient host is young enough at the time of transplantation that it is not yet immunologically competent and is therefore tolerant of the foreign cells.

The system has been used to evaluate WR 122,455, WR 14,997, WF. 30,090, WR 33,063, WR 1544, WR 158,122, WR 2721, WR 347, WR 3689, WR 108,503, WR 151,331, WR 2529, WR 348, WR 638 and WR 2823.

The degree of immunosuppression was assessed by (1) injecting the candidate compound into the spleen cell donor beginning four days before death and (2) by incubating normal spleen cells with the compounds before injecting them into the infant mice. The data are being evaluated and will be the subject of a later report.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 191 Immunosuppressive activity of medicinal compounds

Literature Cited.

References:

1. Cock, A. G., & M. Simonsen: Immunological attack of newborn chickens by injected adult cells. *Immunology* 1: 103-110, 1958.

2. Simonsen, M., & E. Jensen: The graft versus host assay in transplantation chimaeras. In: *A Symposium on Biological Problems of Grafting*, edited by F. Albert and P. B. Medawar. Oxford: Blackwell, 1959, p. 214-238.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL DD-DR&T(4)1636	
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B. CONTRIBUTING							
C. CONTRIBUTING							
11. TITLE (Precede with security Classification Code) <sup>9</sup>							
(U) Antiarrhythmic Effects of Aliphatic Amines							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>10</sup>							
012600 Pharmacology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER <sup>11</sup>				FISCAL YEAR		CURRENCY	
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E. AMOUNT:				1.0		44	
F. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Div of Medicinal Chemistry			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academy affiliation)			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				22. ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Korte, CPT D.			
				NAME: Einheber, Dr. A. DA			
23. KEYWORDS (Precede each with security Classification Code)							
(U) Pharmacology; (U) Drugs; (U) Medicinals; (U) Hypotension; (U) Arrhythmia							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security Classification Code.)							
<p>23. (U) Research is directed toward investigating the pharmacology of promising medicinal aliphatic amines, drug interactions, developing and refining animal models for the study of arrhythmias and associated cardiovascular effects produced by drugs or injury. Candidate drugs will be tested in these model systems and compared with standard drugs. The goal of this research is to develop a highly effective, non-toxic drug which would be useful in the treatment or prevention of arrhythmias occurring spontaneously or as a complication of therapy or injuries involving military personnel.</p> <p>24. (U) Drugs are tested in animal models for efficacy in preventing or treating experimental arrhythmias and associated problems. These may be induced by chemical agents or by injury.</p> <p>25. (U) 74 07 - 75 06 The mechanism of initial hypotension and bradycardia induced by intravenously injected WR 2823 was investigated in anesthetized cats; results of drug pretreatment and of spinal transection indicate that the effect may be mediated by an action on central vasomotor centers. The negative isomer is a more potent anti-arrhythmic than racemic primaquine which is more potent than the positive isomer. Lidocaine, procainamide and quinetholate were equally effective in reversing digitalis-induced arrhythmias in dogs. Propranolol and quinidine produced different effects on certain aspects of canine cardiac automaticity. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.</p>							

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DD FORM 1498  
1 MAR 68

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 192 Antiarrhythmic effects of aliphatic amines

Investigators.

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1. Introduction.

Simple aliphatic amines have a number of potential medical uses. Some of the series studied have important cardiac and autonomic nervous system effects whereas closely structured analogues may not have these actions. In addition to affording some protection against lethality of shock and of trauma, some of these compounds offer some protection against ionizing radiation. Some of these compounds also exhibit important antiarrhythmic activity. Studies have been carried out to extend our knowledge of these various pharmacological activities. These studies have centered around two major areas. The first is the utilization of screening procedures to determine antiarrhythmic potential of various compounds. The second is the continued pharmacological investigation of the important new drug, the aliphatic amine designated WR 2823.

2. Protection against chloroform-induced ventricular fibrillation in the mouse.

a. Background:

Lawson (1968) utilized the sensitivity of the mouse ventricle to chloroform-induced fibrillation as the basis for rapid screening of a number of potentially antiarrhythmic compounds. Silver and Aviado (1969) reported that quinidine and WR 81,844 protected against chloroform-induced ventricular fibrillation in the mouse. Bass et al., (1972) reported that primaquine and other 8-aminoquinolines also protected against chloroform-induced ventricular fibrillation in mice. Modifications of the various procedures have been incorporated into the development of the antiarrhythmic model for screening. This screening procedure produces ED50, LD50 and therapeutic index values for the test agents, and thus the test agents may be compared with one another and also with established antiarrhythmic agents.

b. Methods:

The ED50 and LD50 calculations were based on procedures established by Litchfield and Wilcoxon (1949) which require three doses of varying effectiveness excluding doses which produce a 0% or 100% effect. Previous studies or range-finding techniques were used to establish the appropriate doses of the test substances. Albino female mice, Charles River CD-1, were utilized in this study. Ten was the minimum number of mice employed at a particular dose level of the agent. Drugs were dissolved in 0.9% saline in a concentration such that each animal received the appropriate dosage in a volume equal to 1% of the total body weight. Drugs were administered intraperitoneally 10 min prior to chloroform exposure. Each mouse was placed individually in a covered 600 cc beaker saturated with chloroform vapors. Following respiratory arrest, the mouse was removed from the beaker and the heart exposed by excision of the sternum. The heart was visually inspected for presence or absence of fibrillation and electrocardiographic confirmation of this observation was obtained. The criterion for protection by the agent was establishment of a ventricular rhythm of less than 200 bpm. LD50 doses were calculated from the number of mice that had expired within 24 hr of the intraperitoneal administration of the test drug in a volume equal to 1% of the total body weight.

c. Results:

Studies were begun to determine the ED50 of primaquine (WR 2975) its negative isomer (WR 211,537) and its positive isomer (WR 211,536). Results of these studies are given in Table 1. The ED50 was computed for the negative isomer to be 27.0 mg/kg, for primaquine to be 36.2 mg/kg, and for the positive isomer to be 49.5 mg/kg. The slope functions for the negative isomer, primaquine, and the positive isomer were 1.36, 1.27, and 1.30, respectively. These data indicate that the negative isomer is more potent than primaquine, which is itself more potent than the positive isomer; however, they apparently protect the heart in a similar manner since the slope functions, an index of parallelism, is essentially equal for the three compounds.

3. Reversal of digitalis toxicity.

a. Background:

Reversal of arrhythmias produced by digitalis overdose is a primary criterion for potential antiarrhythmic agents. Smith and Nash (1973) developed a method which determines the potential

of an agent to reverse digitalis toxicity in the dog. This procedure yields information about the efficacy of an agent in reversing digitalis-induced arrhythmias, demonstrates the relative potency of the agent, and provides a preliminary dosage for electrophysiological and automaticity studies.

b. Methods:

Open-chest, mongrel dogs anesthetized with sodium pentobarbital were bilaterally vagotomized. Positive pressure artificial respiration was provided by a Harvard respiratory pump; blood pressure was measured from the left common carotid artery; and Lead II ECG was obtained via needle electrodes. The minimum amount of ouabain required to induce 80% ventricular tachycardia was administered dissolved in a 0.9% saline solution. The initial dose of ouabain was 40  $\mu\text{g}/\text{kg}$ , and additional ouabain was administered in 2 increments of 10  $\mu\text{g}/\text{kg}$  followed by increments of 5  $\mu\text{g}/\text{kg}$  every 15 min as needed. Lidocaine, procainamide, and quinetholate were infused at a rate of 0.5  $\mu\text{g}/\text{kg}/\text{min}$ .

c. Results:

Lidocaine, procainamide, and quinetholate were equally efficacious in reversing the digitalis-induced arrhythmias. The relative potency for procainamide was 1.8 and quinetholate was 10 with the antiarrhythmic dose of lidocaine assigned as unity. This procedure was shown to provide the basic information required of the antiarrhythmic screening procedure for digitalis toxicity.

4. Electrophysiology of antiarrhythmic agents.

a. Background:

Antiarrhythmic agents produce alterations in the electrophysiology of the heart. Many antiarrhythmic agents have membrane depressant actions and are loosely grouped under a heading of "quinidine-like" agents. Characteristic alterations in cardiac electrophysiology produced by quinidine include an increase in diastolic threshold, depression of excitability and conduction velocity, and prolongation of refractoriness of the canine ventricle (Duncan and Nash, 1970; Wallace *et al.*, 1966; and Brooks *et al.*, 1955). A procedure has been developed to measure the electrophysiological alterations produced by chemical agents in order to further define their antiarrhythmic potential.

b. Methods:

Mongrel dogs were anesthetized with sodium pentobarbital, the chest was opened, and the heart was denervated by bilateral vagotomy and stellate ganglionectomy. Heart rate, mean arterial pressure, pH, and rectal temperature were monitored. Three pairs of electrodes were sewn onto the surface of the right ventricle. One pair of electrodes was connected to the drive and test stimulators, and the output was synchronized so that one test impulse would arrive after every tenth drive impulse. This circuitry permitted measurement of the following: diastolic threshold, conduction velocity, excitability, and refractoriness. Diastolic threshold was defined as the minimum current strength necessary to overcome the spontaneous heart beat and drive the heart at 150 bpm. Conduction velocity was calculated by driving the heart and determining the time interval during which an impulse passed between the 2 pairs of recording electrodes placed 25 mm apart on the right ventricle. Excitability was determined by strength-duration studies which were carried out by placing a test impulse at a fixed interval following every tenth drive impulse and varying the duration while recording the minimum current strength necessary to depolarize the ventricle. Strength-interval studies were employed as a measurement of refractoriness by placing a test impulse of fixed duration at various intervals following the tenth drive stimulus and determining the stimulus strength necessary to elicit a response.

The animals were divided into 3 groups of 5 animals each. These groups were: the control group which was given only the vehicle, isotonic saline; the group administered 0.4 mg/kg propranolol; and the group administered 10.0 mg/kg quinidine. Propranolol was dissolved in 6 cc of isotonic saline and injected slowly over a 3 min period. Quinidine was dissolved in 20 cc of isotonic saline and injected slowly over a 10 min period. An equilibration interval of 15 min for quinidine and 10 min for propranolol was observed before recording experimental results. The results were statistically compared with control observations obtained prior to drug administration using the Student t test ( $P=0.05$ ).

c. Results:

Table 2 contains the results obtained from the diastolic threshold, conduction velocity, excitability, and refractoriness studies. Results from control animals indicate that the preparation is stable and is not affected by artificial stimulation of the heart. Quinidine, 10 mg/kg, produced a significant increase in

diastolic threshold and refractory period, and significantly depressed excitability and conduction. These findings are in agreement with published reports. Comparison of the propranolol response with that of quinidine indicates that propranolol produced a "quinidine-like" depression of conduction and excitability and prolongation of the refractory period. Although propranolol appeared to increase the diastolic threshold by a greater magnitude than quinidine, the increase produced by propranolol was not significant due to greater variation in the response. These types of measurements should provide a reliable estimate of the electrophysiological alterations produced by the test agents and as such may be used to define further the antiarrhythmic potential of these agents.

5. Automaticity studies:

a. Background:

All antiarrhythmic agents have a direct action in depressing phase 4 depolarization or automaticity in pacemaker cells. This depression of automaticity produces a decreased rate of firing of the pacemaker cells and is manifested as a slowing of heart rate. Automaticity is studied basically by two methods. The first is simply to monitor the heart rate both before and after administration of a compound. The second is to measure the asystole interval following cessation of a period of overdrive. A procedure was devised to permit recording of both heart rate and asystole interval simultaneously for the atrium and ventricle.

b. Methods:

Mongrel dogs were anesthetized with sodium pentobarbital, the chest was opened, and complete heart block was produced by injection of formalin into the AV node according to the method of Steiner and Kovalik (1968). Two pairs of electrodes were sewn onto both the right atrium and right ventricle. One pair of electrodes from each chamber was connected to separate stimulators so that the atrium and ventricle could be driven simultaneously at 200 and 150 bpm, respectively. The other pair of electrodes from each chamber was used to record atrial and ventricular electrograms. This allowed the determination of both the intrinsic rate and the asystole interval for both the atrium and ventricle. The intrinsic rate was equal to the spontaneous rate obtained from the electrograms. The asystole interval was defined as the time interval following cessation of a 2 min drive period before the heart produced its first depolarization. The same protocol for dividing the animals into groups and administering the drugs

as used in the electrophysiological studies was used in this study except that the dose of quinidine given was 4.0 mg/kg and the dose of propranolol given was 0.16 mg/kg. These changes were made to protect the animal from ventricular standstill.

c. Results:

The results of this study are presented in Table 3. Propranolol produced a significant decrease in both atrial and ventricular heart rate and produced a significant prolongation of both atrial and ventricular asystole interval. These results indicate that propranolol produced the depression of automaticity typical of antiarrhythmic agents. However, quinidine produced no significant alteration of intrinsic rate or asystole interval in the atrium or ventricle. It actually shortened, although not significantly, the ventricular asystole interval--an action similar to that produced by agents which enhance automaticity. This is consistent with the report of Roberts *et al.* (1962) that quinidine increased automaticity, possibly through indirect autonomic mechanisms. Cavoto *et al.* (1972) reported that quinidine depressed atrial and ventricular automaticity in isolated rat hearts, a preparation that eliminated the indirect autonomic effects of quinidine. Thus, the nonsignificant alterations in automaticity probably reflect the interactions of the direct effects of quinidine in depressing automaticity with its indirect action in enhancing automaticity.

6. Pharmacology of WR 2823 in cats and isolated preparations.

a. Background:

The pharmacological effects of WR 2823 were first described by Heiffer *et al.*, 1969. This aliphatic sulfur-containing compound was found to exert both immediate and prolonged actions on the cardiovascular system. The intravenous administration of WR 2823 produced an immediate hypotension and bradycardia in several animal species (Heiffer *et al.*, 1969, Herman *et al.*, 1971). These initial responses are followed by a long lasting alpha adrenergic blockade (Heiffer *et al.*, 1969). Perhaps because of this latter property, WR 2823 has been shown to have efficacy in the prevention of mortality from shock due to hemorrhage or endotoxin in animals (Vick *et al.*, 1969, Vick and Heiffer, 1970, Vick *et al.*, 1973). The clinical usefulness of WR 2823 in human shock conditions remains to be determined but could be limited because of the initial cardio-depressor responses. The present study was initiated to determine in detail the nature and possible attenuation of the initial hypotension and bradycardia induced by WR 2823.

b. Methods:

60 cats (2.5 to 3.5 kg) were anesthetized with 40 mg/kg pentobarbital sodium. The right femoral vein was cannulated for drug injection. Cannulae were also inserted into the right femoral artery and trachea for monitoring blood pressure and respiration. Needle-tipped electrodes were inserted into the appropriate limbs to record lead II of the electrocardiogram. Heart rate was determined from the interval of the R wave by means of a Cardiotach preamplifier. In some experiments carotid artery blood flow was determined by means of a Biotronex flow probe placed around a carotid artery and connected to an electromagnetic flow meter. All recordings were made on a Hewlett-Packard polygraph.

In 5 experiments the spinal cord was sectioned between the first and second cervical vertebrae. Immediately thereafter, the cats were placed on artificial respiration by means of a Harvard positive pressure respirator. An additional 3 cats were decerebrated at the intercollicular level according to the method of Sherrington (1898). Both types of animals preparations were allowed to stabilize for 30 min prior to the administration of WR 2823.

The drugs utilized in the present experiments, the dosage (calculated as the free base) and the equilibrium period before WR 2823 administration were as follows: control (5 ml isotonic saline, 10 min); atropine (1.0 mg/kg, 10 min); mecamlamine (2.5 mg/kg, 15 min); hexamethonium (10.0 mg/kg, 15 min); phenoxybenzamine (5 mg/kg, 60 min); dl-propranolol (0.5 mg/kg, 10 min); reserpine (0.1 mg/kg, 2 injections, 48 and 24 hr); reserpine (0.1 mg/kg, 2 injections 48 and 24 hr) plus mecamlamine (2.5 mg/kg, 15 min); lyergic acid diethylamide (50 µg/kg, 10 min); and diphenhydramine (10 mg/kg, 10 min). These agents were either dissolved or diluted in normal saline so that the amount given was contained in a volume of 5 ml or less. Each compound except for phenoxybenzamine and reserpine was injected over a 3 to 5 min period; phenoxybenzamine was injected intraperitoneally while reserpine was given intraperitoneally 48 and 24 hr before the experiments.

Dithiothreitol, a sulfhydryl group binding agent, was administered to 2 cats. In one, doses of 6.25, 12.5, 25.0 and 50.0 mg/kg were administered at 15 minute intervals. The second cat received a single injection of 50 mg/kg.

Following the equilibrium period, WR 2823 (50 mg/kg), dissolved in 5 ml of isotonic saline, was injected over a 4 min period. The

various physiological parameters were monitored for up to 60 min after WR 2823 injection and the experiment then terminated.

Control observations of the various physiological parameters were made just before either surgical or pharmacological pretreatment and again before administration of WR 2823. Changes in these parameters were also determined at 1, 5, 15, 30 and 60 min after injection. The mean and standard errors of the mean were calculated when three or more values were obtained for each parameter.

Three adult mongrel dogs (10-12 kg) were anesthetized with phenobarbital sodium (30 mg/kg) and the heart removed and perfused with autologous blood (Vick & Herman, 1971). The force of contraction was measured with a Walton-Brodie strain gauge sutured to the left ventricle. Coronary perfusion pressure was obtained by means of a needle-tipped catheter inserted into the perfusion circuit and attached to a pressure transducer. The electro-cardiogram and heart rate were determined by means of needle-tipped electrodes inserted into the left and right ventricles. Each was allowed to stabilize for approximately 15 min after which control responses to 1.0 µg epinephrine were obtained. WR 2823, dissolved in 5 ml saline, was injected into the perfusion circuit over a 2 min period at doses of 50 mg (2 hearts) or 100 mg (1 heart). Each heart was rechallenged with epinephrine at 15, 30 and 60 min after WR 2823 injection.

#### c. Results:

WR 2823, 50 mg/kg intravenously, induced immediate hypotension and bradycardia in all 7 intact control animals. These results, together with the effect of WR 2823 in the surgical pretreatment cats are summarized in Table 4. Changes were noted to begin during the infusion and reached maximum decreases of 32% to 48% in mean arterial pressure and 30% to 35% in heart rate by 1 min post injection. Although recovery began within 5 min, blood pressure ultimately stabilized below control levels. Heart rate returned to control levels by 60 min. Carotid artery blood flow, recorded in 2 experiments, increased 175% and 200% by 1 min post injection but returned to control levels within 15 to 30 min. In 3 of 7 experiments, WR 2823 also induced brief minor increases in respiratory rate and in the amplitude of the lead II R wave.

The 3 intercollicular decerebrated cats responded in nearly the same way to WR 2823 as did the saline controls except that heart rate did not recover as completely (Table 4). Complete

interruption of the spinal cord caused a prolonged decrease in both mean arterial pressure and heart rate. The administration of WR 2823 after 30 min produced a brief hypotensive response during the injection but by 1 min post injection both blood pressure and heart rate were slightly elevated. Within 5 min, and during the remainder of the experiment, blood pressure stabilized at 10% to 20% below control levels, while during the same period heart rate was at or near control levels.

Both of the ganglionic blocking agents (mecamylamine and hexamethonium) produced an almost immediate decrease in heart rate (10% to 41%) and blood pressure (10% to 35%). During the administration of WR 2823 brief episodes of hypotension and bradycardia occurred in 2 experiments while increases in both parameters occurred in 4 other animals. However, in all experiments, by 1 min post injection blood pressure was at or slightly above control levels while heart rate increased to preganglionic-blockage levels or above. Pulse pressure and carotid artery blood flow were also elevated during this period. All parameters declined slightly over the remainder of the 60 min experimental period. The results of these and all drug pretreatment experiments are summarized in Tables 5 and 6.

In 3 experiments phenoxybenzamine produced a 10 to 24 mmHg decrease in mean arterial blood pressure. Heart rate increased slightly in 2 of the 3 cats. Alpha-adrenergic blockade was confirmed before the infusion of WR 2823 by injection of epinephrine. WR 2823 caused a decrease in mean arterial pressure of between 36 to 60 mmHg. The changes in heart rate were much less pronounced than those seen in control animals, and at the end of 60 min were at or above pre-phenoxybenzamine levels in all 3 experiments.

Within 5 min of the injection of 0.5 mg/kg of dl-propranolol heart rate was depressed 5% to 16% and the typical cardiovascular responses to isoproterenol were inhibited. The injection of WR 2823 produced a 28% to 48% decrease in mean arterial pressure. The magnitude of the depression was similar to that seen in control animals. Heart rate, carotid artery blood flow and pulse pressure remained relatively unchanged during and following WR 2823 administration.

The effect of reserpine pretreatment was determined in 3 experiments. Blood pressure and to a certain extent heart rate were found to be lower in these animals than in the untreated controls. WR 2823 caused a moderate but brief (< 5 min) fall in blood

pressure (23%). Heart rate fell but to a lesser extent than in control animals. Carotid artery blood flow was increased the first 5 to 15 min after WR 2823 administration.

Mecamylamine had little effect on heart rate and depressed blood pressure in only 1 of 3 reserpinized animals. The infusion of WR 2823 caused a 37% decrease in mean arterial blood pressure within 1 min but by 5 min complete recovery was noted. At the end of 60 min a slight but significant increase above control levels had occurred. WR 2823 had relatively little effect on heart rate in these animals.

In 11 experiments pretreatment with atropine (4), lysergic acid diethylamide (4) or diphenhydramine (3) had little effect on resting blood pressure or heart rate. These animals responded in nearly the same way to WR 2823 as did the saline controls except that heart rate fell to a lesser extent in the atropinized cats.

The administration of 6.25, 12.5 and 25.0 mg/kg dithiotherotol to one cat caused brief hypotensive responses. The 50 mg/kg dose caused irreversible cardiovascular and respiratory depression. Similar effects were seen in a second cat given a single 50 mg/kg dose. Additional experiments with lower doses of this agent will be performed to determine the effect on WR 2823 activity.

The injection of either 50 mg or 100 mg WR 2823 into the isolated dog heart caused a brief 5% to 10% decrease in force of contraction with little or no change in heart rate. Coronary perfusion pressure fell slightly following the injection. Both the force of contraction and coronary perfusion pressure had returned to control levels 1 to 2 min after WR 2823. The positive inotropic and chronotropic responses to epinephrine (1  $\mu$ g) were essentially unchanged after either 50 mg or 100 mg of WR 2823.

d. Discussion:

The intravenous administration of WR 2823 consistently induced hypotension and bradycardia in the anesthetized cat. In the present experiments a dose of 50 mg/kg of WR 2823 was infused over a 4 min period. The cardiodepressant effects appeared before the injection was completed. Doses as low as 12.5 mg/kg have been shown to lower blood pressure and heart rate in the rat (Herman *et al.*, 1971). The magnitude of the fall was similar to that seen after 25 mg/kg. In the dog WR 149,024, a dimer of dephosphorylated WR 2823, produced hypotension and bradycardia over a dosage range

of 6.25 to 25.0 mg/kg (Caldwell et al., 1972). It was noted that the 6.25 mg/kg dose depressed blood pressure and heart rate to the same extent as the 25 mg/kg dose. Thus the initial cardiovascular effects are not dose dependent.

A critical dosage might be necessary if WR 2823 exerted direct non-specific depression on the myocardium or vascular smooth muscle or both. However, in the present experiments WR 2823 had little effect on the force of contraction and heart rate in the isolated dog heart. Likewise the tone of isolated rabbit aortic strips was not altered when WR 2823 was added to the bathing fluid (Demaree et al., 1971). In spite of the lack of direct WR 2823 effect on heart and vascular smooth muscle, the fact that both carotid artery blood flow and pulse pressure increased suggests that the initial hypotension is due to a decrease in total peripheral vascular resistance.

The decrease in peripheral resistance was not the result of parasympathetic nervous system stimulation since atropine failed to prevent the fall in blood pressure. This fact would also tend to rule out stimulation of the Bezold-Jarisch reflex mechanism which can be blocked by atropine. Histamine can also produce a hypotensive response in the intact animal. Although plasma histamine levels were not determined in the present experiments, the fact that pretreatment with the antihistaminic diphenhydramine failed to influence the magnitude of the responses tends to rule out histamine release as a prime factor in the hypotension. Likewise lysergic acid diethylamide pretreatment failed to alter the initial effects of WR 2823, suggesting that the release of 5-hydroxytryptamine is also not involved in the responses. These results tend to suggest that the locus of the hypotensive response resides with the sympathetic nervous system.

WR 2823 has been shown to possess alpha-adrenergic blocking properties (Heiffer et al., 1969). However, this action required 30 to 60 min to develop and thus could not be responsible for the immediate alterations in blood pressure and heart rate. When the peripheral portions of the sympathetic nervous system were blocked with either phenoxybenzamine or propranolol, WR 2823 administration still induced hypotension and bradycardia. However, some components of the response were modified. For example, propranolol prevented the increase in carotid artery blood flow and pulse pressure. Also, the decline in heart rate was less in animals pretreated with either of these agents than in the untreated controls. A more significant attenuation of the initial WR 2823 responses was noted in the reserpinized cats. In these animals both the hypotension and

bradycardia were less than in the untreated controls. Reserpine, by biogenic amine depletion, alters sympathetic nervous system activity both peripherally and centrally.

That higher autonomic centers may be involved has been suggested by the demonstration of both blood pressure and heart rate increase after WR 2823 in spinal transected or ganglionically blocked animals. The positive inotropic and chronotropic actions may reflect a peripheral action of WR 2823 which is masked by the intact nervous system. It is not known if reserpinization will prevent this. These responses are opposite to those occurring in decerebrate or control cats suggesting that the brain stem may be a critical area for WR 2823 activity. The importance of these areas has been emphasized by the demonstration that  $\alpha$ -methyl-dopa (Henning, 1969), L-dopa (Henning and Rubenson, 1970) and clonidine (Kobringer and Walland, 1967) have centrally mediated hypotensive effects. Also, intracisternal injection of phentolamine, an alpha-adrenergic antagonist, caused hypotension and bradycardia in vagotomized rats (Ito and Scharberg, 1974). These responses were found to be elicited from the medullary brain areas. The comparison between cardiovascular responses of these 2 agents is not absolute since alpha-adrenergic blockage appears to be involved in phentolamine action but is not necessarily the case for WR 2823. The areas of the brain stem affected, however, may be similar. In any event, the results of the present experiments tend to indicate that the immediate cardiovascular effects of WR 2823 can be attenuated by agents which alter sympathetic nervous system activity at central levels.

Table 1

Protection from Chloroform-induced Arrhythmias in Mice by Primaquine and its Purified Optical Isomers

Compound	ED50 <sup>a</sup> (mg/kg)	Slope <sup>a</sup>
WR 211,537 (- primaquine)	27.0 (23.7-30.8)	1.36 (1.12-1.66)
WR 2978 (± primaquine)	36.2 (32.1-40.8)	1.27 (0.94-1.71)
WR 211,536 (+ primaquine)	49.5 (43.4-56.4)	1.30 (1.08-1.56)

<sup>a</sup> Numbers in parenthesis represent the 95% confidence interval.

Table 2

Electrophysiological Alterations in the Canine Ventricle Produced by Quinidine and Propranolol<sup>a</sup>

Measurements	Control Vehicle		Propranolol 0.4 mg/kg		Quinidine 10 mg/kg	
	Con. Exp. Diff.					
Threshold (mA)	Con. <sup>b</sup>	0.120 ± 0.010	0.092 ± 0.010	0.096 ± 0.010		
	Exp.	0.120 ± 0.010	0.114 ± 0.010	0.114 ± 0.010		
	Diff.	- - - -	+0.022 ± 0.008	+0.018 ± 0.006*		
Conduction (m/sec)	Con.	0.527 ± 0.028	0.593 ± 0.041	0.550 ± 0.030		
	Exp.	0.513 ± 0.017	0.538 ± 0.028	0.484 ± 0.017		
	Diff.	- - - -	+0.055 ± 0.015*	+0.066 ± 0.015*		
Excitability <sup>c</sup> (mA)	Con.	1.36 ± 0.11	1.12 ± 0.06	1.22 ± 0.09		
	Exp.	1.40 ± 0.08	1.36 ± 0.10	1.48 ± 0.12		
	Diff.	- - - -	+0.24 ± 0.06*	+0.26 ± 0.04*		
Refractoriness <sup>d</sup> (msec)	Con.	166.0 ± 4.8	171.0 ± 3.3	161.0 ± 6.4		
	Exp.	165.0 ± 4.2	184.0 ± 1.0	177.0 ± 6.2		
	Diff.	- - - -	+13.0 ± 3.4*	+16.0 ± 1.9*		

<sup>a</sup> Mean ± standard error for five animals for each group.

<sup>b</sup> The abbreviations refer to control value, experimental value and the change (Diff.) produced by the drug.

<sup>c</sup> Data obtained from strength-duration studies for a stimulus duration of 0.1 msec.

<sup>d</sup> Data obtained from strength-interval studies for a stimulus strength of 7 mA.

<sup>e</sup> \* = Significant change (p = 0.05).

Table 3  
Alterations in Automaticity Produced by Quinidine and Propranolol<sup>a</sup>

Measurements	Control Vehicle	Propranolol 0.16 mg/kg	Quinidine 4 mg/kg
<u>Atrium</u>			
Intrinsic Rate			
Control	158.8 ± 5.5	155.8 ± 8.6	144.4 ± 9.1
Experimental	158.8 ± 5.8	132.6 ± 7.8	136.4 ± 6.4
Difference	---	±23.2 ± 5.5*	± 8.0 ± 6.5
Asystole Interval			
Control	0.52 ± 0.01	0.51 ± 0.03	0.54 ± 0.04
Experimental	0.52 ± 0.01	0.60 ± 0.03	0.56 ± 0.03
Difference	---	±0.09 ± 0.02*	±0.02 ± 0.04
<u>Ventricle</u>			
Intrinsic			
Control	51.0 ± 5.2	49.8 ± 7.0	50.0 ± 6.3
Experimental	50.4 ± 5.4	39.4 ± 6.7	49.2 ± 4.4
Difference	---	±10.4 ± 2.7*	±0.8 ± 2.3
Asystole interval			
Control	4.8 ± 1.6	6.7 ± 1.8	8.5 ± 3.1
Experimental	4.9 ± 1.5	11.3 ± 3.5	5.3 ± 1.4
Difference	---	±4.6 ± 1.6*	±3.2 ± 2.3

a Mean ± standard error for five animals for each group.

b \* = Significant change (p = 0.05).

Table 4  
 Effect of Surgical Pretreatment on Initial Cardiovascular Responses Following  
 a Four Minute Intravenous Infusion of WR 2823 (50 mg/kg) in the Anesthetized Cat

Time Postdose (min)	% of Predosing (Control) Values					
	Mean Arterial Pressure <sup>a</sup>			Mean Heart Rate <sup>a</sup>		
	No Operation <sup>b</sup>	Decerebrate <sup>b</sup>	Spinal <sup>b</sup>	No Operation <sup>b</sup>	Decerebrate <sup>b</sup>	Spinal <sup>b</sup>
1	67±6	52±6	123±17	87±10	71±8	137±11
5	66±4	54±2	86±9	81±7	68±8	126±10
15	59±5	57±4	75±5	77±7	64±7	108±7
30	68±5	67±1	75±5	88±7	71±11	101±5
60	72±4	73±3	78±7	105±7	83±10	98±3
	128±12 mmHg	104±23 mmHg	70±11 mmHg	176±9 bpm	204±12 bpm	135±15 bpm

<sup>a</sup> Values are mean ± S.E.M. and represent at least three cats per group.  
<sup>b</sup> Number of experiments are seven for "no operation", three for "decerebrate" and five for "spinal."

Table 5  
 Effect of Pharmacological Pretreatment on Initial Mean Arterial Pressure Responses Following  
 a Four Minute Intravenous Infusion of WR 2823 (50 mg/kg) in the Anesthetized Cat

		% of Predosing (Control) Values <sup>a</sup>							
		Mecamyl- amine 2.5mg/kg	Hexameth- onium 10.0mg/kg	Phenoxy- benzamine 5.0mg/kg	d1-Propran- olol 0.5mg/kg	Reserpine 0.1mg/kg x2	Atropine 1.0mg/kg	LSD .05 mg/kg	Diphenhy- dramine 10.0mg/kg
After Pretreat- ment		64+1	86+4	83+5	98+3	-	96+3	99+3	109+5
After WR 2823 (min)									
1	67+6	120+2	96+4	46+3	62+6	77+10	55+5	68+5	54+10
5	66+4	106+13	79+5	53+9	68+9	96+3	59+6	63+5	67+7
15	59+5	104+13	79+5	57+8	60+7	93+3	62+6	70+10	72+4
30	68+5	107+16	79+5	66+11	66+7	96+5	66+6	78+12	87+1
60	72+4	104+9	91+8	78+8	68+10	101+6	77+5	86+12	89+3
Predosing Values (mmHg)	128+12	112+9	88+6	118+16	111+16	78+5	111+8	102+6	119+19
Number of Experiments	7	3	3	3	3	3	4	4	3

<sup>a</sup>Values are mean ± S.E.M. and represent at least three cats per group.

Table 6

Effect of Pharmacological Pretreatment on Initial Mean Heart Rate Responses Following a Four Minute Intravenous Infusion of WR 2823 (50 mg/kg) in the Anesthetized Cat

		% of Predosing (Control) Values <sup>a</sup>								
		No Pretreatment	Mecamylamine 2.5mg/kg	Hexamethonium 10.0mg/kg	Phenoxymethonium 5.0mg/kg	dl-Propranolol 0.5mg/kg	Reserpine 0.1mg/kg	Atropine 1.0mg/kg	LSD 0.05mg/kg	Diphenhydramine 10.0mg/kg
After Pretreatment		67+7	82+6	106+3	90+3	-	102+1	99+1	104+5	
After WR 2823 (min)										
1	87+10	151+6	144+9	103+5	90+3	94+1	95+4	82+9	77+2	
5	81+7	117+8	125+7	96+9	87+4	90+3	93+3	79+6	73+1	
15	77+7	109+12	98+10	89+9	89+5	91+7	85+8	76+6	73+1	
30	88+7	104+6	95+8	88+8	91+6	97+12	89+6	86+13	83+6	
60	105+1	100+1	99+11	112+6	104+9	92+9	96+1	104+6	115+11	
Predosing Values (bpm)		176+9	152+19	173+15	163+13	141+13	179+20	184+11	180+4	
Number of Experiments		7	3	3	3	3	4	4	3	

<sup>a</sup>Values are mean ± S.E.M. and represent at least three cats per group.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 192 Antiarrhythmic effects of aliphatic amines

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OB 6523	75 07 01	DD-DR&E(AR)636	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8. DOD'S INSTR. <sup>d</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>e</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
6. PRIMARY		61101A		3A161101A91C		00	
7. CONTRIBUTING						194	
8. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code)							
(U) Development of an Organ Culture Method from Intestinal Biopsies							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>f</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
73 01		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER: NA				FISCAL YEAR		75	
C. TYPE:				CURRENCY		1.5	
D. KIND OF AWARD:				76		1.5	
E. CUM. AMT.						72	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Division of Pathology			
				Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Publish ISSN if U.S. Academic Institution)			
NAME: Buescher, COL E.L.				NAME: Takeuchi, A.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2024			
				SOCIAL SECURITY ACCOUNT NUMBER:			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Richardson, MAJ W			
				NAME:			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Organ culture; (U) Intestine; (U)Phase Microscopy; (U)Electron Microscopy							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRAM (Publish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23(U) To develop a reliable and reproducible method in which organ cultures of small and large intestines of experimental animals may be cultured and maintained without producing alterations of function and metabolism. The cultured gut will be employed in studies on interactions between the mucosa and various bacteria, viruses and microbe-derived toxins. These results will provide new information which should clarify the pathogenesis of acute diarrheal diseases in military personnel.</p> <p>24 (U) Conventional morphologic techniques including phase contrast, light and electron microscopy, and histochemistry and isotope tracer methods are being used. Methods such as interference microscopy and cinematography will also be added as the work progresses.</p> <p>25 (U) 74 07 - 75 06 Utilizing a modified Rose tissue culture chamber and falcon tissue culture bottles, the viability and growth of the gut mucosa of guinea pigs and mice have been studied. Fetal intestinal epithelial cells including principal columnar cells, Paneth cells, mucous-secreting goblet cells, could be grown and remain viable up to 2 weeks while the adult counterparts do not survive more than 2 days. The fetal guts of guinea pigs were found to survive best in both types of tissue culture compartments, which have been confirmed by protein synthesis with radio-tracer methods, histochemistry, histology and electron microscopy. A method for electron microscope preparation of cultured guts has been successfully established. Current efforts have been directed toward the establishment of immunoglobulin synthesis and absorption of macromolecular substances in the growing gut.</p> <p>For technical reports, see Walter Reed Army Institute of Research Annual Reports, 1 Jul 74 - 30 Jun 75.</p>							

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method from Intestinal Biopsies

Investigators.

Principal: Akio Takeuchi, M. D.

Associate: MAJ Walter Richardson, MC

Description

To develop a reliable and reproducible method for the organ culture of the small and large intestine of experimental animals and man. The cultured gut will be employed in: (1) Studies by various parameters of responses of the gut mucosa and submucosa to various enteric microbes and microbe-derived toxins; special attention will be paid to cinematographic recordings of as well as conventional static observations on various cellular interaction of the gut epithelium mucosa with various microbes and toxins. (2) Studies of replication sites of certain enteroviruses in the cultured gut.

These studies should provide valuable new information which will clarify as yet unsolved problems in pathogenesis of acute infectious diarrheal diseases common in military personnel at home and overseas.

Background

Organ culture techniques have proven to be of considerable value in the study of respiratory tract infections in man and animals. Recently, interest has developed in the organ culture of gut tissues for the cultivation of enteric viruses. Rubenstein and Tyrrell (1971), Dolin and Stenhouse (1970), and Derbyshire and Collins (1971) provided evidence of the multiplication of viruses in organ cultures of the small intestine of the human embryo. Dolin et al. (1972) reported that viral antigens from viruses which belong to different viral groups were successfully detected in human fetal intestinal organ cultures by immunofluorescent techniques. Kagnoff et al. (1972), in their organ culture study of adult rabbits, have reported that the metabolic function and synthesis of macromolecular substances including secretory IgA were still active after 24 hours culture in a Petri dish. Finally, Eastwood and Trier were able to culture the human small intestine of normal subjects and patients with ulcerative colitis up to 24 hours (Eastwood and Trier 1973).

Most of the chambers used by the above investigators appeared adequate for the growth of certain viruses and for evaluation of limited metabolic activities in the cultured bowel. These chambers do not allow for an immediate and accurate and also sequential method of morphologic evaluation of intestines growing in cultures. This shortcoming prompted the development of a new chamber allowing a convenient observation during actual organ culture. The chamber we have developed by modifying Rose's chamber provides easier handling of tissues and better visualization of growing cells and tissues under the phase contrast microscope than conventional culture chambers. The viewing unit consists of a phase microscope, which can be attached to a time-lapse cinematographic instrument, enclosed in a plastic housing connected with a thermo-control device which maintains the temperature of the unit at 37° C (Takeuchi et al. 1974).

#### Progress

Utilizing our new culture chamber together with the conventional Falcon chamber, we have cultured fetal small and large intestine. Contrary to the general belief that a constant supply of oxygen is imperative for maintaining any organ culture, the fetal gut can grow well without it for up to 48 hours and shows no structural alteration. For the last six months, we have obtained a better growth and survival of intestinal epithelial cells, the most sensitive cell population of the gut, in the culture medium 199 with 20-10% adult horse serum containing either gentamicin or the combination of streptomycin and penicillin in 100% CO<sub>2</sub> at 37° C.

The following techniques have been used to monitor the growth and survival of the gut in our organ cultures:

1. Phase Contrast Microscopy: The direct visualization of the gut in the culture chamber by this technique has provided continuous morphologic observation and record of epithelial renewal by still microphotography.
2. Time-Lapse Cinematography: This technique has been perfected to record rhythmic movement of the smooth muscle of the cultured gut. (See 1973-74 Annual Report of Dept. of Experimental Pathology, WRAIR.) However, the continuous recording of the movements of mesenchymal cells such as macrophages and lymphocytes in the mucosa and of intestinal villi of cultured guts had to be discontinued because of frequent breakdowns of the obsolete time-lapse cinematographic equipment.

3. Electromicroscopy: A successful technique for electron microscopy of cultured intestine has been developed. Sequential ultrastructural observations have been initiated on the growth of intestinal epithelial cells of cultured guts.

4. Cytochemical Reaction of Absorbed Horseradish Peroxidase (HRP): Investigation of absorption of macromolecular substances by absorptive cells of cultured small intestine has been initiated. In order to determine functional cellular activity of cultured guts, horseradish peroxidase (HRP) has been used as a marker for macromolecular transport through intestinal absorptive cells, since it may be detected cytochemically by light and electron microscopy even when present in very small concentrations. HRP was added to the culture medium up to 48 hours after the start of the culture in both our organ chamber and Falcon bottles. The cultured small intestines were removed 1 hr. or 3 hrs. afterward, then briefly fixed in chilled glutaraldehyde with cacodylate buffer, followed by washing in the buffer. They were then frozen and cut at 10-15 microns. Frozen sections were incubated with diaminobenzine solution containing  $H_2O_2$ , then washed and processed for electron microscopy.

In tissue harvested 1 hr. after adding HRP into medium, HRP activity was strongly positive at the brush border and was also detected at the intercellular space between epithelial cells of intestinal villi (Fig. 1). By electron microscopy, unstained sections clearly demonstrated HRP activity over and along the microvilli of the intestinal absorptive cells. Occasional activity of HRP was also noted in membrane-bound vesicles in the epithelial cytoplasm. Some of them represented peroxidase activity in pinocytotic vesicles, while others appeared to be of endogeneous origin (Fig. 2). At 3 hrs., HRP was totally absent in the lumen and at the surface of the microvilli. Moderate HRP activity was still present between epithelial cells (Fig. 3). On the other hand, the intercellular spaces of the lamina propria revealed strong HRP activity (Fig. 4).

5. Protein, DNA Synthesis and Localization of Immunoglobulins in Cultured Guts: Using radio-tracer methods, determinations of both protein and DNA synthesis in the cultured gut mucosa have been initiated. Likewise monitoring of the immunofluorescent localization of immunoglobulin as another parameter of the functional activity of the cultured gut has also been started.



Fig. 1 Portion of mid-villus of jejunal mucosa showing absorbed horseradish peroxidase (HRP) after 1 hr. incubation. Reaction produce appears as a dark material along the brush border, between epithelial cells, and in the lamina propria. Unstained Epon embedded sections, phase contrast microscopy. X 1,700.



Fig. 2 Intestinal absorptive cells, mid-villus, jejunal mucosa cultured for 3 days after 1 hr. incubation with HRP. This low mag. EM photo illustrates HRP reaction produce (arrow), covering the microvilli in pinocytotic vesicles (PV), and intercellular space below the tight junction (TJ). Unstained, EM photo. X 7,800.

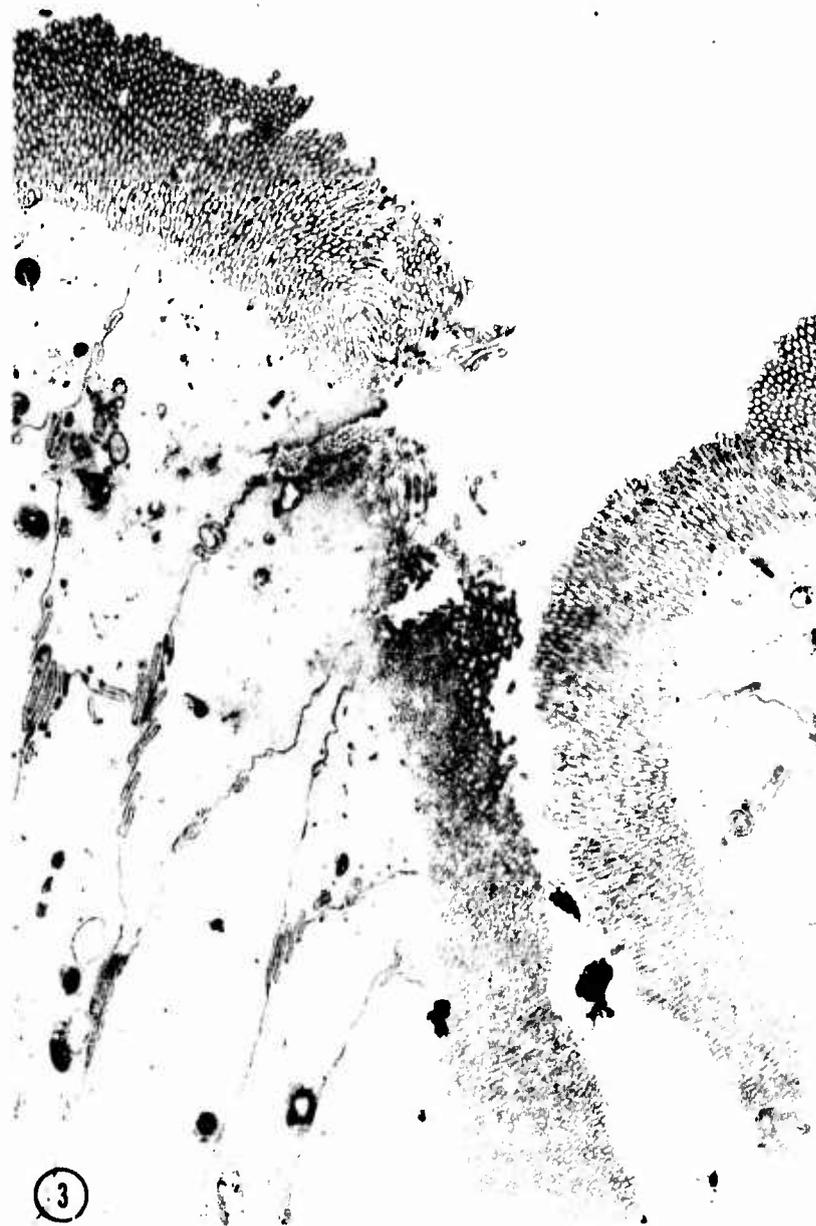


Fig. 3 Intestinal absorptive cells, jejunal villus cultured for 3 days, after 3 hr. incubation with HRP. HRP activity is absent in the gut lumen but is identifiable in some pinocytotic vesicles and between absorptive cells. Unstained, EM photo. X 7,500.

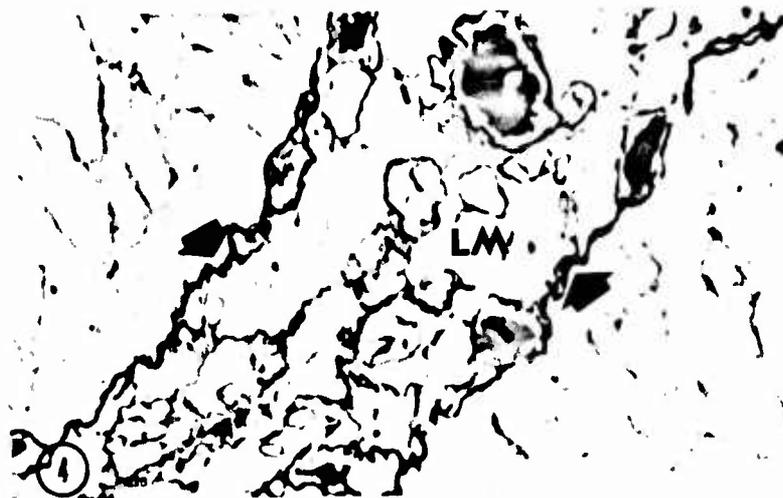


Fig. 4 Lamina propria (LM), jejunal mucosa cultured for 3 days after 3 hr. incubation with HRP. Most of absorbed HRP has been transported into the extracellular space in the lamina propria. Basement membrane of epithelium (arrows). Unstained, phase contrast microscopy. X 1,700.

### Conclusions and Recommendations

With improved organ culture techniques, fetal guts can be cultured up to 2 weeks without structural and functional alterations. Sequential observations can be successfully made on growing guts in our organ culture chamber by direct visualization by phase contrast microscopy and can be recorded sequentially by time-lapse cinematography. Electron microscope procedures specifically for cultured guts have been established and used to determine absorption and transport of macromolecular substance in the small intestinal epithelial cells in vitro. Protein synthesis and localization of immunoglobulins in both small and large bowels in the culture chamber and bottle have been initiated. All these techniques are to be utilized in determining the viability of growth of cultured bowel and will form the basis for the study of various experimental infections and injuries of the intestine in vitro.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method from Intestinal Biopsies

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
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3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8A. DES'N INSTR <sup>d</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS	
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10. NO./CODES <sup>e</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
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b. CONTRIBUTING							
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>f</sup>							
(U) Biochemical Characterization of Arbovirus Antigens							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>g</sup>							
003300 Biochemistry 002600 Virology 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 07		CONT		DA		C. In-House	
17. CONTRACT/GRAANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDENCE		b. FUNDS (in thousands)	
b. NUMBER:				FISCAL YEAR		1.0	
c. TYPE:				CURRENT		90	
d. KIND OF AWARD:				76		1.0	
e. AMOUNT:				76		120	
f. CUM. AMT.							
20. RESPONSIBLE S&D ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Division of CN&I			
				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Punish S&D if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: TOP, COL Franklin H., Jr.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3757			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Dalrymple, Dr. Joel M.			
				NAME:			
22. KEYWORDS (Precede with Security Classification Code)							
(U) Arbovirus; (U) Antigen; (U) Immunology; (U) Immunopathology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23 (U) To define the antigenicity and immunogenicity of the structural and non-structural proteins of arboviruses of military importance and to describe the role of such antigens in immune response and immunopathology of arbovirus infections, such as dengue hemorrhagic fever.</p> <p>24 (U) Antigens from virions or infected cells are separated and purified by ultracentrifugation, column chromatography, and isoelectric focusing. Sensitive assays for detection of viral antigens and antibody are developed using purified antigens and specific antisera to them.</p> <p>25 (U) 74-07-75-06. Antigens of Sindbis virus (an alphavirus) were used to develop the basic methodology for a solid phase radioimmunoassay test for the dengue viruses. The Sindbis virus model allowed more rapid development of the test because of the higher antigen and antibody titers that could be obtained. The selection of a useful solid phase carrier, the requirements for antigen purity and concentration, and the kinetics of binding were determined with this system. An extension of these studies has been defining the preparation of Sindbis virus suspensions containing defective particles. Investigation of the replication and antigenic characteristics of such preparations was necessary for comparative methodology with the dengue viruses where large quantities of defective particles or non-infectious antigens appeared to be involved in the immune response and the disease process. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 July 1974 - 30 June 1975.</p>							

<sup>a</sup> Available to contractors upon originator's approval.

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 196, Biochemical characterization of arbovirus antigens

Investigators.

Principal: COL F. H. Top, Jr., MC; J. M. Dalrymple, Ph.D.

Associate: COL P. K. Russell, MC; W. E. Brandt, Ph.D.; K. A. Kos;  
K. M. Langenbach; G. P. Onley; R. J. Jackson;  
PFC K. J. Belleville

Description

To define the antigenicity of arbovirus structural and non-structural components as potential immunogens of prophylactic value. To devise and evaluate virus components as antigens for evaluation of immune status and description of the role of such antigens in the immune response and immunopathology of arbovirus infections. Studies have emphasized the biochemical and biophysical characterization of arbovirus antigens from appropriate model systems.

Progress

Studies on the biochemical and biophysical characterization of arbovirus antigens have been initially structured toward the use of prototype alphaviruses as models to be used for subsequent investigations with some of the flaviviruses (e.g., dengue viruses) which are of more direct relevance to military health problems. The use of an alphavirus (Sindbis) offers many advantages over flaviviruses in preliminary studies concerning methodology because of its faster replication to high titers, greater efficiency of radiolabeling, and more complete characterization. Many of the projects and experiments carried out with dengue viruses have been based on preliminary pilot experiments with Sindbis virus to establish methodologies and experimental conditions.

I. Development of a solid phase radioimmune assay (RIA)

Sindbis virus antigen preparations were used to conduct the preliminary development of an RIA for subsequent application to dengue

virus infections. These studies resulted in the standard procedure for the dengue RIA described elsewhere in the current Annual Report (WU-166) and are not detailed here. However, some general experimental results will be given.

The search for a solid phase matrix for the RIA necessitated examination of an assortment of plastic tubes, round beads and microtiter plates. Antigen adsorption could be demonstrated with all of these, but the flexible microtiter plates made from combinations of polyvinyl chlorides appeared to bind Sindbis antigens as well as any, and its flexible plastic offered advantages in handling the many samples to be tested. Microtiter plates commercially treated for use with cell culture appeared to bind more antigen, but this advantage was negated by variation in binding efficiency observed between plates and lots from different companies. Binding of antigen appeared constant using untreated plates from the same company, with little or no difference observed between the "u" or "v" well configuration.

Many of the parameters of antigen adsorption to the plastic plates have been measured using Sindbis virus antigens in preliminary experiments, followed by confirmatory experiments using purified dengue virion antigens. General statements concerning the results of these experiments are as follows:

A. Antigen adsorption proceeds rapidly at 37°C and is essentially 95% complete within 30 min.

B. The diluent used for the antigen must be isotonic for good protein solubility and the presence of divalent cations ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ) appears to have a slight enhancing effect.

C. Antigen adsorption occurs readily above pH 6 and is increased only slightly by raising the pH to 8 - 8.5. Alkaline conditions above pH 10 appear detrimental to binding.

D. Antigen binding is concentration dependent to the point of saturation of available sites; however, at limiting concentrations the unadsorbed antigen in a well can be transferred to at least 4 successive wells with subsequent antigen adsorption at 70-90% maximum.

E. Antigen purity is an obligate prerequisite for binding to the solid phase surface, since the addition of small amounts of extraneous protein (0.1% BSA) to purified antigens completely inhibits antigen attachment.

F. Non-specific binding of normal serum globulins occurs at low serum dilutions; however, it can be reduced by the utilization

of a 10% fetal bovine serum containing diluent to saturate unbound sites on the plastic surface prior to addition of the test sera. Lower concentrations of fetal bovine serum, bovine serum albumin, or gelatin, were less efficient in reducing the nonspecific binding of serum globulins.

G. Elimination of non-specific globulin binding of certain sera has been attempted using a variety of extraction procedures. Acetone and kaolin extraction failed to remove these inhibitors and silicic acid and DEAE sephadex chromatography were only moderately successful.

H. Direct water washing of plates containing serum resulted in a marked prozone - i.e., reduced binding at lower serum dilutions. This effect was greatly reduced if rinsing with isotonic salt solutions preceded or replaced the water washes.

I. Antigen-antibody reaction times have not been studied in extensive detail; however, they appear to follow the pattern established for other serological reactions. Two hrs at 37°C appeared adequate.

Modifications of this standard procedure (see Work Unit 166) are currently being pursued to extend its application to antigen detection on electrofocus gradients, differentiation between homologous and heterologous reactions and increased sensitivity for both antigen and antibody detection.

## II. Preparation of Sindbis virus suspensions containing increased concentrations of defective particles.

Sindbis virus preparations are known to contain a certain number of noninfectious defective particles. Such particles contribute to the antigenic mass of a virus antigen preparation, yet cannot be detected by infectivity assays. The presence of a high concentration of these defective particles in an infective inoculum can greatly reduce the infectious virus yield of infected cultures. Many of the replication characteristics of the dengue viruses (slow replication, low titer, etc.) suggest that dengue virus suspensions may contain large numbers of defective or noninfectious particles. Again, Sindbis virus was chosen to establish the experimental procedures necessary for the understanding of the influence of defective particles on the antigenic and replication characteristics of various virus preparations.

Sindbis virus was passed in susceptible cells to yield defective particle rich preparations. Routine propagation of infective

Sindbis virus suspensions included the infection of primary chick embryo cell cultures with infected suckling mouse brain suspensions diluted to yield an moi of approximately 10. In these experiments, Sindbis virus was passed sequentially 10 times through chick embryo cell cultures at an moi of 10 for each infection. To check for variation in the ability of the different cell preparations to support virus replication, a reference mouse brain virus suspension was used as a control infective inoculum at each passage. The infectivity of these preparations is shown in Table 1.

Table 1. Passage of Sindbis virus through primary chick embryo cell cultures at a constant multiplicity of infection

Passage	Sequential (Infectivity)		Reference (Infectivity)
	A line <sup>a</sup>	B line	
1	$2.6 \times 10^{8b}$	$1.2 \times 10^8$	-
2	$1.7 \times 10^8$	$1.2 \times 10^8$	$7.5 \times 10^7$
3	$1.1 \times 10^8$	$9.5 \times 10^7$	$5.8 \times 10^7$
4	$6.5 \times 10^7$	$4.0 \times 10^7$	$3.3 \times 10^7$
5	$5.8 \times 10^7$	$1.8 \times 10^7$	$5.5 \times 10^7$
6	$1.7 \times 10^8$	$1.0 \times 10^8$	$4.8 \times 10^8$
7	$1.9 \times 10^8$	$1.1 \times 10^8$	$2.8 \times 10^8$
8	$1.7 \times 10^8$	$9.2 \times 10^7$	$9.5 \times 10^7$
9	$2.0 \times 10^8$	$1.6 \times 10^8$	$2.7 \times 10^8$
10	$5.0 \times 10^7$	$1.4 \times 10^7$	$2.5 \times 10^7$

<sup>a</sup>Duplicate experiments (A line and B line) were passed separately

<sup>b</sup>PFU/ml

Considerable differences in infectious virus yield were noted between the various chick cell cultures, as evidenced by the variability in titers from cultures infected with the reference mouse brain seed virus. Sequential infections exhibited a reduction over the first five passes with a subsequent increase and later variation as the chick cell preparations varied with reference mouse brain seed virus infection. From these studies it was concluded that the variation in the ability of the different batches of chick embryo cells to produce virus was too great to produce defective virus preparations using this moi.

The continuous BHK-21 cell line was investigated as a more consistent cell line for the production of defective Sindbis virus suspensions. Procedures differed in that the moi was no longer held constant, but rather, 3 mls of the preceding virus yield was added directly to 32 oz bottle cultures of the BHK cells. Cells were initially infected at passage number one, using mouse brain reference virus seed. Results are shown in Table 2.

Table 2. Sindbis virus passage in BHK-21 cells

Passage No.	A line			B line		
	Input MOI	Virus yield		Input MOI	Virus yield	
		PFU/ml	PFU/cell		PFU/ml	PFU/cell
1	0.34	$6.0 \times 10^7$	180	0.34	$5.5 \times 10^7$	170
2	8.6	$7.8 \times 10^7$	150	7.9	$1.4 \times 10^8$	270
3	11.0	$1.9 \times 10^8$	360	20.0	$3.6 \times 10^8$	680
4	40.7	$3.0 \times 10^8$	860	77.0	$7.3 \times 10^7$	210
5	300.0	$5.5 \times 10^6$	73	72.5	$3.1 \times 10^6$	41
6	4.7	$9.3 \times 10^7$	1050	2.6	$3.5 \times 10^7$	400
7	15.8	$5.5 \times 10^7$	160	6.0	$1.6 \times 10^7$	46
8	22.0	$5.8 \times 10^7$	310	6.4	$2.5 \times 10^7$	130
9	17.2	$1.7 \times 10^7$	68	7.5	$9.8 \times 10^6$	39
10	1.6	$1.6 \times 10^7$	20	0.9	$6.5 \times 10^7$	83
11	3.1	$4.0 \times 10^6$	11	13.0	$9.3 \times 10^5$	2.5

Infectivity titers appeared to increase through the first three to four passages; however, subsequent passages decreased in titer. Cell counts were performed on each set of cell cultures and the PFU/cell ratio calculated as a more exact indication of virus production. These data do not exhibit a pronounced linear reduction in virus production until 11 passages; subsequent experiments using material beyond the 11th passage indicate a continued low yield of virus. Material from the 11th passage in BHK-21 cells was considered a candidate defective-particle-virus-suspension for further studies.

Single step growth curves were performed on BHK-21 cells in an attempt to detect replication differences. A reference mouse brain seed virus suspension was compared with both low passage No. 2 (BHK cell adapted) and high passage No. 11 (defective particle rich) virus suspensions over an moi range of from 10 to 0.0001 PFU per cell (Fig. 1). No real significant differences were apparent between any of the seed virus suspensions when examined at moi's of 10 or .01. A pronounced delay in detectable virus replication was noted, with both mouse brain reference virus and high passage defective virus at the .0001 moi level. We cannot attribute this delay in detectable virus release to the presence of defectives because a) defective particles should manifest their inhibitory effect on virus replication at their highest concentrations, 2) near maximum levels of virus production were obtained in these cultures even though delayed, and 3) we did not initially suspect that the infectious mouse brain seed virus preparations would contain high concentrations of defective particles.

This effect of delayed replication at low moi with both mouse brain seed virus and high passage BHK cell virus has been reproduced and similarly demonstrated with other virus seeds from continued passage experiments. Although this effect cannot yet be ascribed to the presence or absence of defective particles, further experiments are planned because such a pattern of replication bears a strong resemblance to the growth curves of many of the flaviviruses and is poorly understood.

In all studies of defective particles and reduced virus replication, the possible contribution of interferon must be considered. As a simple check for interferon (species specific) effects we assayed low and high passage virus seeds from BHK-21 and CEC experiments on both BHK-21 and CEC monolayers. These data are compiled in Table 3.

In this experiment the CEC monolayers appeared less sensitive than the BHK cells for virus detection, with the exception of the high passage CEC propagated virus. The BHK propagated virus was lower titered on CEC monolayers, suggesting that interferon did not contribute significantly to the reduction in titer observed upon passage. The low passage CEC propagated virus was higher titered when assayed on BHK cells; however, the high passage CEC virus in

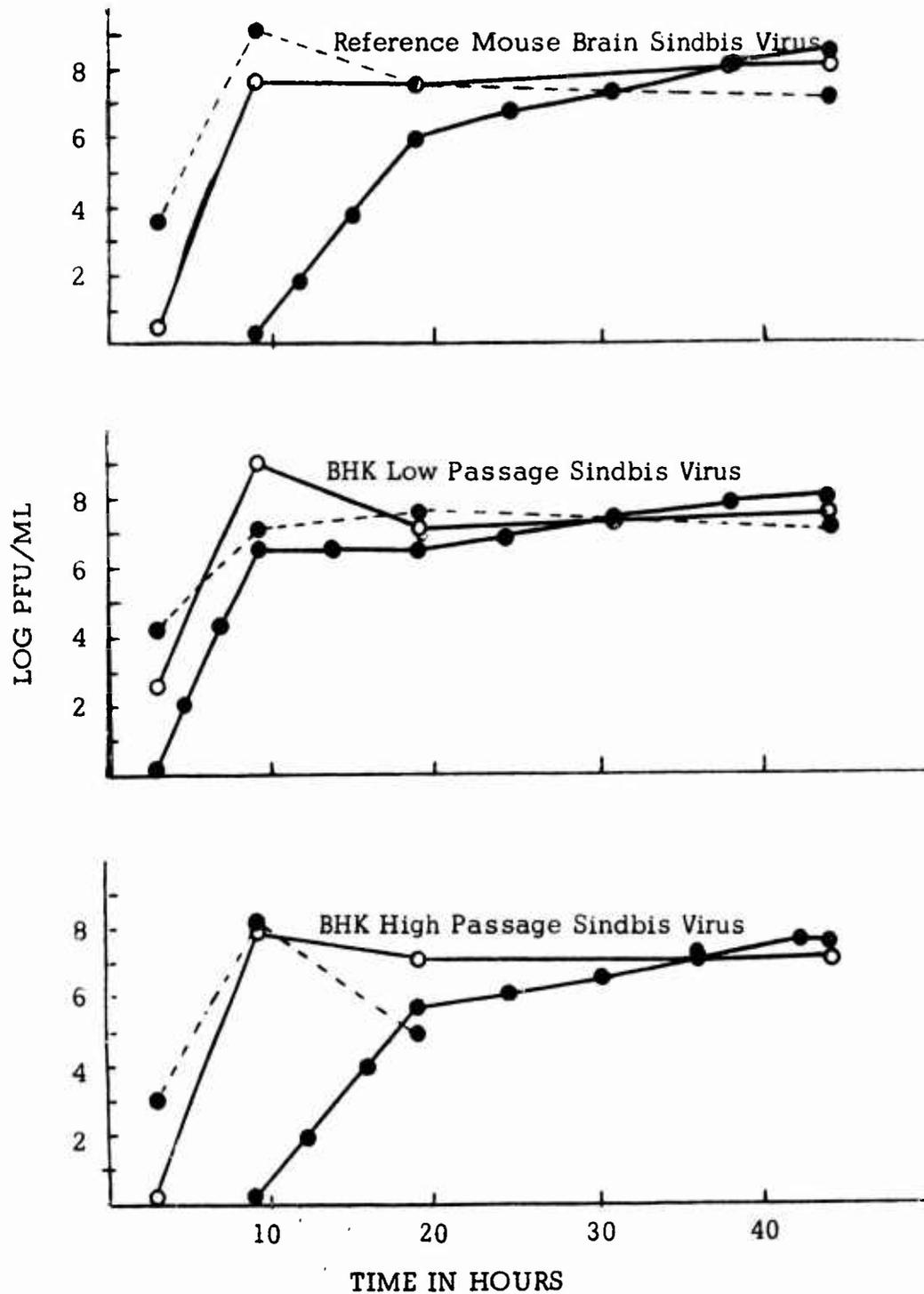


Figure 1. Sindbis virus growth curves at indicated multiplicity of infection: ●---● 1.0; O—O 0.01; ●—● 0.0001.

which interferon would be expected to be most abundant was even lower titered on BHK cell monolayers. Although not conclusive, these experiments do not immediately suggest a major contribution of interferon.

Table 3. Plaque assay of low and high passage virus suspensions on BHK-21 and CEC monolayers

Host cell for propagation	Passage level	Titer (PFU/ml) on indicator cell	
		BHK-21	1 <sup>0</sup> CEC
BHK-21	2nd pass	$1.5 \times 10^9$	$4.0 \times 10^7$
BHK-21	11th pass	$5.6 \times 10^7$	$5.8 \times 10^6$
CEC	2nd pass	$1.4 \times 10^9$	$8.0 \times 10^7$
CEC	11th pass	$2.5 \times 10^6$	$7.5 \times 10^6$

As a further test for the presence of defective particles or "interfering substances" in the high passage BHK propagated virus preparations, flasks of BHK cells were infected with either low passage virus, high passage virus, or a mixture of the two, and the resultant virus yields compared. The results of these experiments are presented in Table 4.

The low passage virus inoculum resulted in high titered virus yield, while yields of high passage material were greatly reduced as anticipated. Interestingly, the yields of the mixture infection resembled infection with the high passage inoculum, indicating the presence of interfering material such as defective particles.

High passage and low passage virus suspensions were used for a comparison of the efficiency of radiolabeling, using radioactive amino acids and uridine. Primary chick embryo cells were infected at an moi of one with each of the high and low passage virus preparations and radioactive amino acids and uridine added to separate cultures of each. Supernatant virus yields were clarified by slow speed centrifugation, concentrated by ammonium sulfate precipitation, and purified on tartrate-glycerol density gradients. The distribution of radioactivity on each of these gradients is illustrated in Fig. 2.

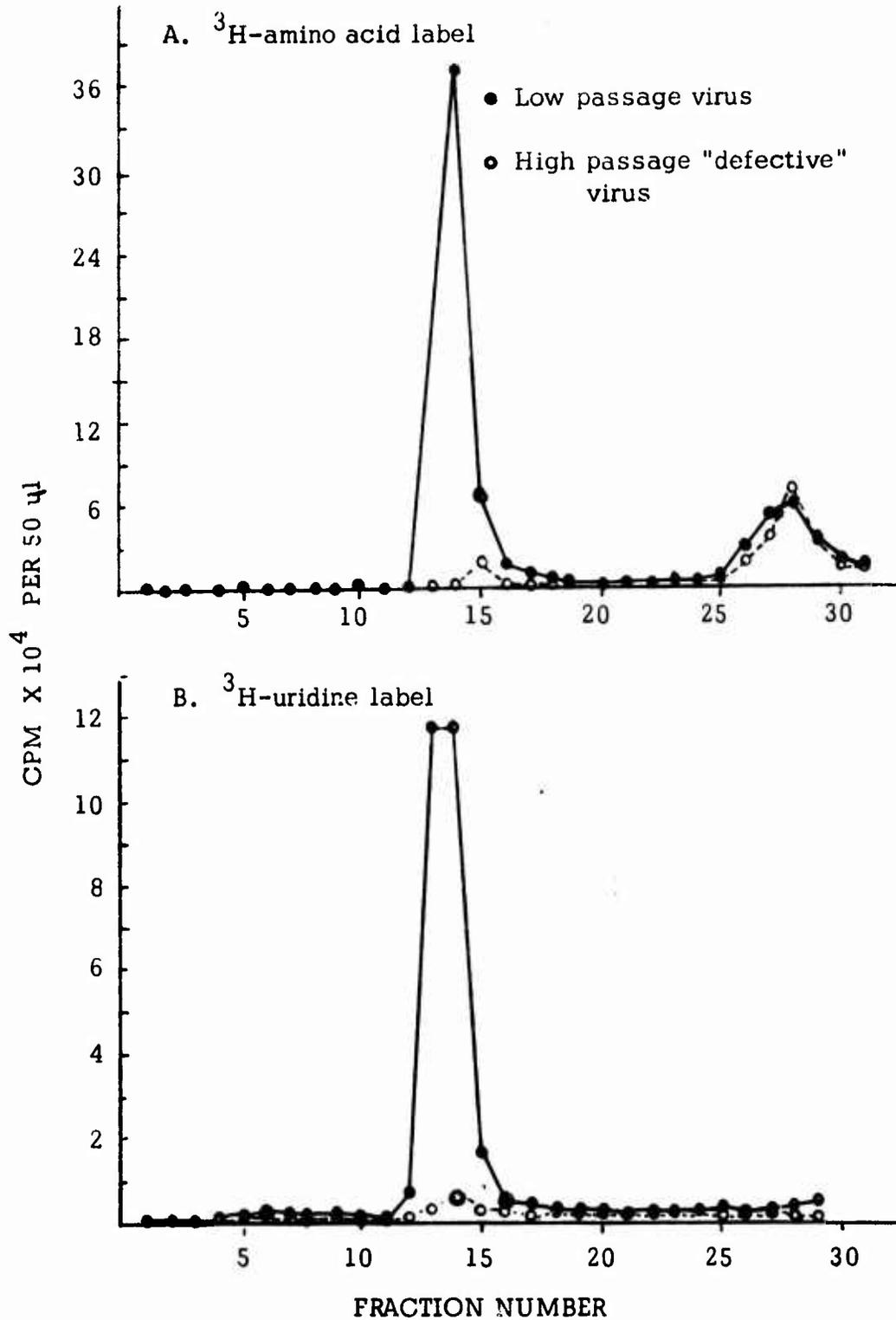


Figure 2 . Tartrate-glycerol density gradient of Sindbis virus prepared and labeled as indicated.

Table 4. Single step replication of low and high passage Sindbis virus individually and combined

Passage	Infecting virus inoculum		Virus yield
	Titer (PFU/ml)	Volume	
2nd passage (low)	$3.0 \times 10^8$	0.5 ml of $10^{-2}$ dil'n	$5.0 \times 10^9$
11th passage (high)	$8.0 \times 10^6$	0.5 ml undiluted	$8.5 \times 10^7$
low and high passage mixture	$2.0 \times 10^6$	0.5 ml of mix of above	$5.0 \times 10^7$

The entire yield of a 32-oz bottle culture was added to each of the gradients, allowing quantitative comparisons of radioactive uptake. In each instance the low passage virus inoculum resulted in much greater incorporation of radioactivity. Only a single peak was observed with each virus, suggesting that this represented the virion peak and that defective particles, if radiolabeled, could not be separated from infectious virus.

Infectivity titrations were performed on the peak fractions of each of the four gradients and calculations of specific activity are presented in Table 5. It is quite obvious that the virus yield from low passage virus inocula contained much more infectious virus than the virus yields from high passage virus inocula containing defective particles. These differences in infectious virus concentration would be expected to account for the majority of the huge discrepancies in radioactivity obtained in the peak. It was important to determine if the labeling efficiency was the same in preparations expected to contain defective virus particles compared to controls to more completely characterize such preparations.

Nucleic acid labeling with radioactive uridine showed that the efficiency of labeling was reduced when high passage or defective virus containing inocula were employed. This reduced labeling - plus the greatly reduced virus yield makes nucleic acid labeling of defective particles containing virus suspensions extremely difficult, if not impossible. In contrast, radioactive amino acids labeled

Table 5. Specific activity of low and high passage Sindbis viruses radiolabeled with uridine or amino acid mixtures

Infecting virus	Radioactive precursor	Virus yield		Specific activity (cmp/PFU)
		Infectivity/ml	CPM/ml	
Low passage	<sup>3</sup> H-uridine	5.75 x 10 <sup>8</sup>	1.15 x 10 <sup>7</sup>	0.020
High passage	<sup>3</sup> H-uridine	3.5 x 10 <sup>7</sup>	4.62 x 10 <sup>5</sup>	0.013
Low passage	<sup>3</sup> H-amino acids	2.53 x 10 <sup>9</sup>	3.70 x 10 <sup>7</sup>	0.015
High passage	<sup>3</sup> H-amino acids	1.89 x 10 <sup>7</sup>	2.10 x 10 <sup>7</sup>	1.111

proteins much more efficiently in preparations containing defective virus - i.e., produced following infection with high passage virus inocula. These data suggest that although infectious virus production is suppressed following infection with high passage virus seeds, the resultant virus yield probably contains a proportion of particles containing protein components, but little or no nucleic acid.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 196, Biochemical Characterization of Arbovirus Antigens

Literature Cited.

Publications:

1. Mooney, J. J., Dalrymple, J. M., Alving, C. R., and Russell, P. K. Interaction of Sindbis virus with liposomal model membranes. J. of Virol. 15: 225, 1974
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION NO. DA OC 6430	2. DATE OF SUMMARY 75 07 01	REPORT CONTROL SYMBOL DD DR&E(AR) 10 10
3. DATE PREV. SUMMARY 74 07 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY. U	6. WORK SECURITY U	7. REGRADING NA	8A. OTHER INSTN. NL	8B. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO. CODES: <sup>a</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		61101A	3A161101A91C	00	197		
b. CONTRIBUTING							
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>b</sup> (U) Rickettsial Genetics							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>b</sup> 010100 Microbiology							
13. START DATE 74 07		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House	
17. CONTRACT/GRAANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE NA				EXPIRATION		PREVIOUS	
b. NUMBER <sup>c</sup>				c. TYPE		d. AMOUNT	
e. KIND OF AWARD				f. CUM. AMT.		e. FUNDS (in thousands)	
18. RESPONSIBLE DOD ORGANIZATION				19. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				Oaks, Stanley, Jr., CPT, MSC			
				Simon, Christine, GS7			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>b</sup> (U) Genetics; (U) Biochemistry; (U) Structure-Function Relationship; (U) Structure-Antigenicity Relationship; (U) Vaccines; (U) Antibiotics							
23. TECHNICAL OBJECTIVE, <sup>b</sup> 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) 1. Prevention of rickettsial diseases. 2. Genetic manipulation of rickettsiae to provide strains with optimum immunogenicity. 3. Isolation and purification of sub-cellular components responsible for eliciting immunological protection. 4. Determination of the basis for intracellular parasitism. Studies required to improve rickettsial vaccines to prevent disease in troops operating in the field.							
24. (U) 1. Mutagenization and selection of appropriate rickettsial strains by tissue culture techniques. 2. Growth and purification of rickettsiae followed by polyacrylamide gel electrophoresis and immunoelectrophoresis of subcellular fractions. 3. Use of selective antibiotics and inhibitors to determine basis of intracellular parasitism.							
25. (U) 74 07-75 06: 1. Growth of rickettsiae in cultured cells has been studied to determine those most suitable for either vaccine development or the study of rickettsiae-host cell interaction. Representative continuous and diploid cell types were selected for detailed studies on temperature and pH providing optimum growth conditions. 2. Conditions for selection of temperature sensitive mutants of rickettsiae have been established. A technique for plaque assay and plaque transfer has been developed with gamma-irradiated L-929 cells and appropriate conditions for rickettsial mutagenesis with nitrous acid have been determined. 3. Biochemical studies of rickettsial macromolecules have focused on polyacrylamide gel electrophoresis of whole rickettsiae. This technique provides a sensitive method for comparison of various rickettsial species and has allowed biochemical differentiation of both groups and species within groups. Selective isotopic labeling has allowed identification of glycoproteins and estimation of molecular weight for at least 19 rickettsial proteins. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 197 Rickettsial Genetics

Investigators.

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Description.

The genetic mechanisms of rickettsia are being investigated to develop an understanding of the genetic basis of antigenic variation, metabolic processes, and virulence. The approach includes biochemical analysis of structural components and attempts to produce mutant organisms. Methodology is developed with fast growing rickettsiae and later transferred to slow growing organisms such as R. tsutsugamushi.

Progress.

I. Growth of rickettsiae in cultured cells.

A. Survey of growth in cells particularly suitable for either vaccine development or the study of rickettsiae-host cell interaction.

The growth of spotted fever group rickettsiae in cultured cells has been studied by other investigators using R. rickettsi or R. akari to infect Vero, WI-38, chicken embryo, L-929, duck embryo and rat fibroblast cells. These investigations were directed toward studying the metabolism of rickettsiae in host cells, or were qualitative in nature with emphasis on the production of immunological reagents and quantitation was performed only on the final product. Growth kinetics of other major groups of rickettsiae, such as scrub typhus and Q fever have been studied in tissue culture systems, but there is a noticeable lack of this information for the spotted fever group rickettsiae.

The purpose of this investigation was to study the growth kinetics of R. conori in cells which are particularly suitable for either vaccine development or the study of the molecular biology of rickettsiae-host cell interaction. The WI-38 and DBS-FRHL-2 cells are candidate substrates for virus vaccine production and were logical choices for inclusion in this work. HeLa and L-929 cells have been the subject of extensive biochemical studies, making them a valuable substrate for molecular studies of rickettsial interaction with mammalian cells.

The growth pattern of *R. conori* in irradiated (3,000 rad  $\gamma$  - irradiation) and cycloheximide (1-2  $\mu\text{g}/\text{ml}$ ) inhibited cells is shown in Fig. 1a. Growth was clearly superior in irradiated cells since rickettsiae entered the logarithmic phase of growth more rapidly and achieved greater numbers in irradiated diploid cells than in similar drug treated ones. In continuous cells, maximum growth also occurred in irradiated populations, but the rickettsial lag period was less effected by cycloheximide.

Once rickettsiae entered the logarithmic phase of growth, their rate of multiplication was similar in each host cell, although there was a difference in growth rate depending on the method employed to inhibit cellular division. The doubling times of *R. conori* in each cell type were calculated from the semi-logarithmic data format shown in Fig. 1b and are presented in Table 1 for comparison. Rickettsiae in drug inhibited cells generally evidenced a greater doubling time than seen in irradiated cells, but L-929 cells were an exception, supporting rickettsial growth in a similar manner regardless of the method of cellular inhibition.

The multiplicity of infection of 5 plaque forming units/ml resulted in approximately 50-60% of the cells becoming initially infected, as shown in Fig. 1c. As the average number of rickettsiae per infected cell increased, a parallel increase in percent cells infected was observed which gradually approached 100%. Data collection was normally terminated prior to a significant number of cells exhibiting more than 200 rickettsiae each, but continued incubation invariably led to continued increase in rickettsial number and cell detachment or lysis. The increase in percent cells infected, paralleling logarithmic growth, could be the result of non-lytic release of rickettsiae from infected cells, or the early lysis of cells initially infected by multiple clumped rickettsiae. During the lag and early logarithmic phase of infection no cells were observed which contained rickettsiae too numerous to count, suggesting a non-lytic release of rickettsiae, but it is possible that rare, heavily infected cells were not included in our counting sample.

In order to obtain quantitative data, it was necessary to study growth kinetics in non-proliferating host cells produced by treatment with  $\gamma$ -irradiation or cycloheximide. Rickettsial growth was more rapid and consistent in all cell types when cellular division was arrested by  $\gamma$ -irradiation. Specialized use of cycloheximide to inhibit cellular metabolism for observation of radioactive precursor uptake by rickettsiae has been reported by Weiss, *et al.*, 1972, and their selection of L-929 cells for use with the inhibitor seems appropriate in view of our comparative growth kinetics.

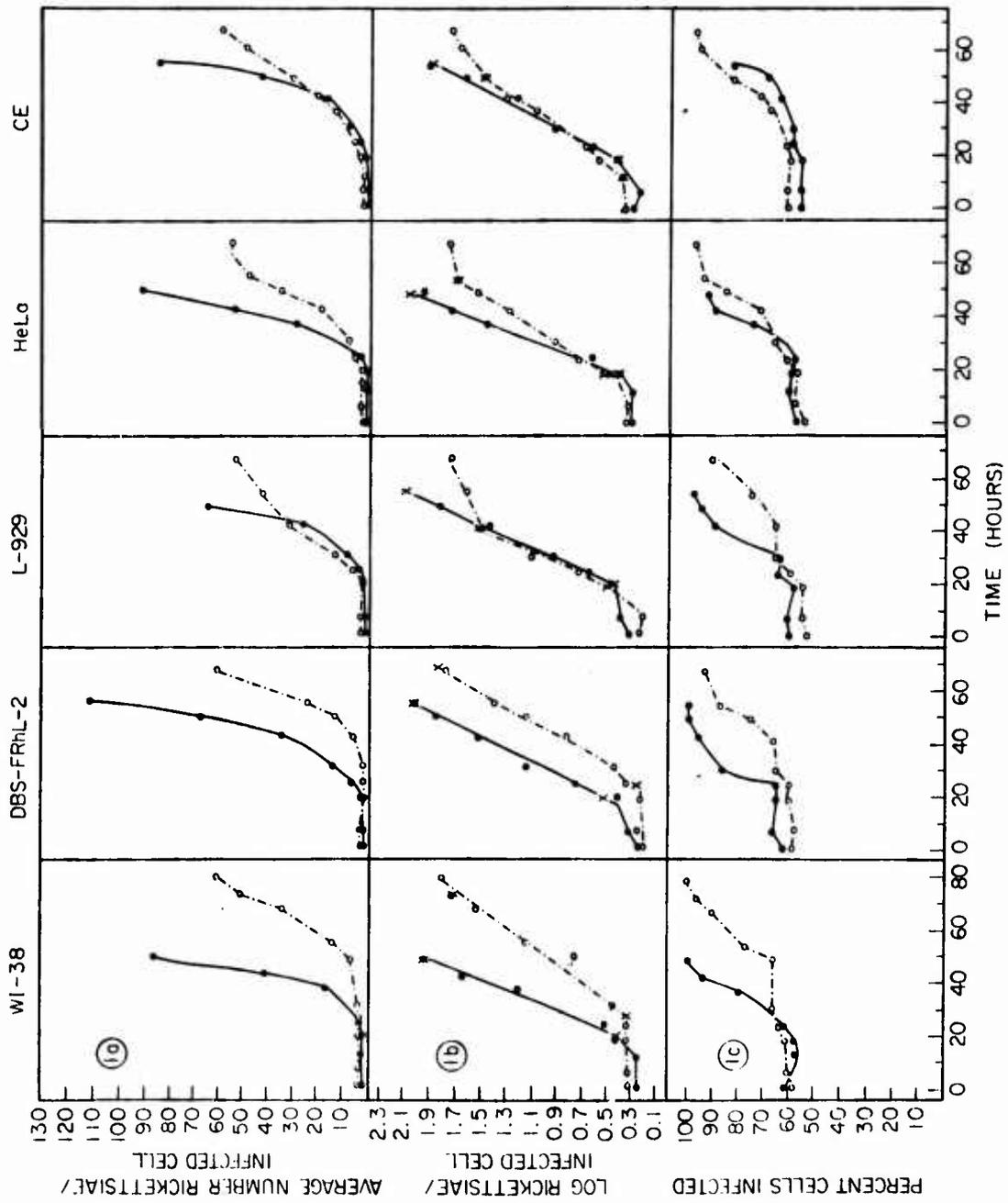


Fig. 1a Growth of *R. conori* in cells arrested by  $\gamma$ -irradiation or cycloheximide. Two hundred cells were observed at each time point and the rickettsiae counted in each infected cell.  $\gamma$ -irradiated cells, ●—●; cycloheximide inhibited cells, ○—○.

Fig. 1b Growth kinetics in cells arrested by  $\gamma$ -irradiation or cycloheximide. The (X—X) indicate line segments derived by least squares linear regression and utilized for computation of rickettsial doubling time in each cell type.  $\gamma$ -irradiated cells, ●—●; cycloheximide inhibited cells, ○—○.

Fig. 1c Percentage of cells infected by *R. conori*. Two hundred cells were inspected at each time point for the presence or absence of rickettsiae.  $\gamma$ -irradiated cells, ●—●, cycloheximide inhibited cells, ○—○.

Table 1. Doubling times of *R. conori* in irradiated and cycloheximide inhibited cells.

Cell type	Rickettsial doubling time (hrs.)	
	Irradiation	Cycloheximide
WI-38	5.7	10.3
DBS-FRHL-2	6.8	8.6
L-929	6.5	7.0
HeLa	5.6	9.1
CE	7.7	10.0

The range of doubling times observed for R. conori in irradiated cells was 5.6 - 7.7 hr, which is compatible with the time required for division of C. burneti in L cells (Roberts, et al, 1959) and R. rickettsi in rat fibroblast cells (Schaechter, et al, 1957), but substantially lower than observed for the growth of R. tsutsugamushi in MBIII or L-929 cells (Hopps, et al, 1959). The rate of growth of R. conori in all cells tested was similar, a phenomenon also observed with R. tsutsugamushi in MBIII and L-929 cells.

B. Determination of optimum growth conditions in representative continuous and diploid cell types.

Since growth rate was found to be similar in all cells tested, WI-38 and L-929 cells were selected for further studies to determine the optimum temperature and pH for rickettsial multiplication. These experiments were performed in suspension culture utilizing irradiated cells infected with R. conori at a multiplicity of infection of 1 PFU/cell. Water-jacketed spinner flasks were utilized in conjunction with constant temperature water circulators to insure optimal temperature control over the range of 26-40C. Infected cells were suspended in MEM with 10% fetal calf serum to a final concentration of  $1 \times 10^5$  cells/ml. Samples were withdrawn immediately and at 6 hr intervals. Fig. 2 shows the results of temperature experiments in irradiated L-929 cells. Calculation of the doubling time of the organism at each temperature evidenced a range from 5.5 hr at 38C to 13 hr at 26C. Growth at 40C was considered insignificant when compared to any other temperature tested. In the range of 32-38C, very little difference in growth rate was observed. The doubling time at 32C in suspension culture was 6 hr, a figure which compares very favorably with the 6.5 hr doubling time found previously in coverslip culture at the same temperature.

pH studies were also performed in irradiated spinner cultures at 34C, with the pH controlled through use of various combinations of organic buffers. Maintenance of pH in the infected cell suspensions was checked at 6 hr intervals. The results of representative cultures at different pH values, utilizing both bicarbonate and organic buffering systems are shown in Fig. 3. There is very little change in the pH of the medium when employing either bicarbonate or organic buffers. The bicarbonate and one organic buffered medium were poised at the same pH to allow comparison of growth kinetics as an index of possible toxicity of the organic buffer on rickettsial growth.

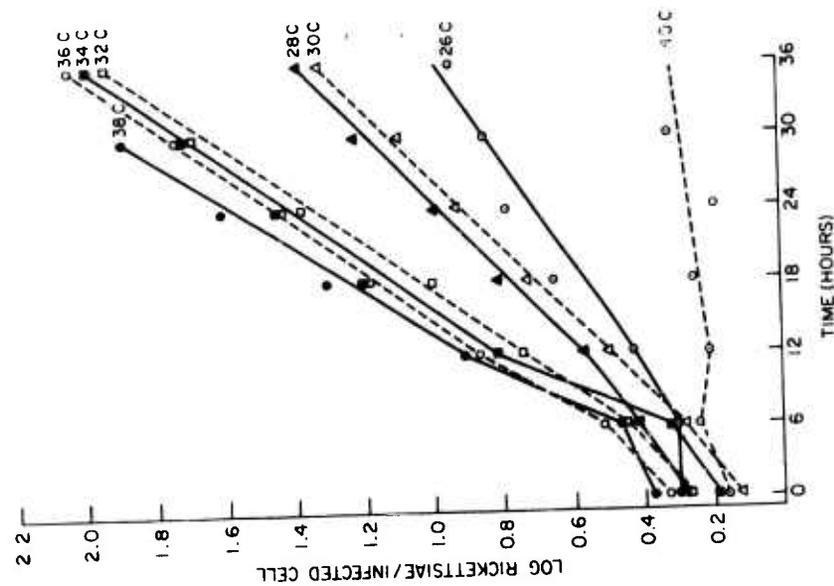


Fig. 2 Growth kinetics of *P. conorii* in irradiated L-929 cells maintained in spinner culture at various temperatures.

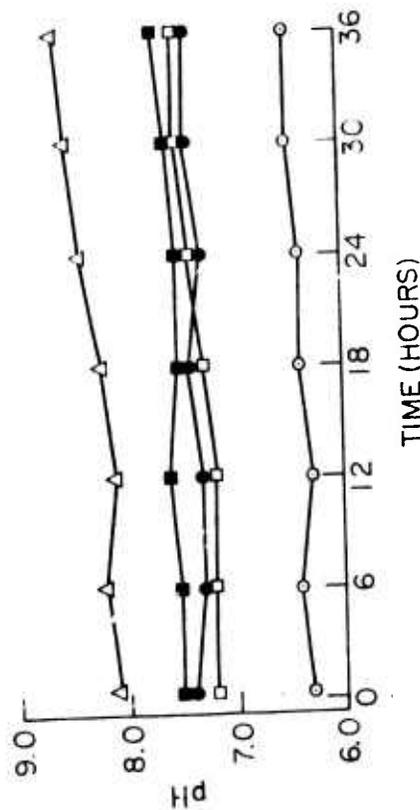


Fig. 3 Stability of pH in irradiated L-929 cell spinner cultures infected with *P. conorii*. All cultures were maintained in minimal Essential Medium containing standard phosphate buffer (solid symbols); in addition, other media contained the following supplemental buffers:

- Δ - 10mM TES + 15 mM HEPES + 10mM PIPES;
- - 10mM PIPES + 10mM BPS + 10mM HEPES + 5mM NaH<sub>2</sub>PO<sub>4</sub>;
- - 10mM BIS-TRIS + 10mM PIPES + 10mM MES + 15mM NaH<sub>2</sub>PO<sub>4</sub>

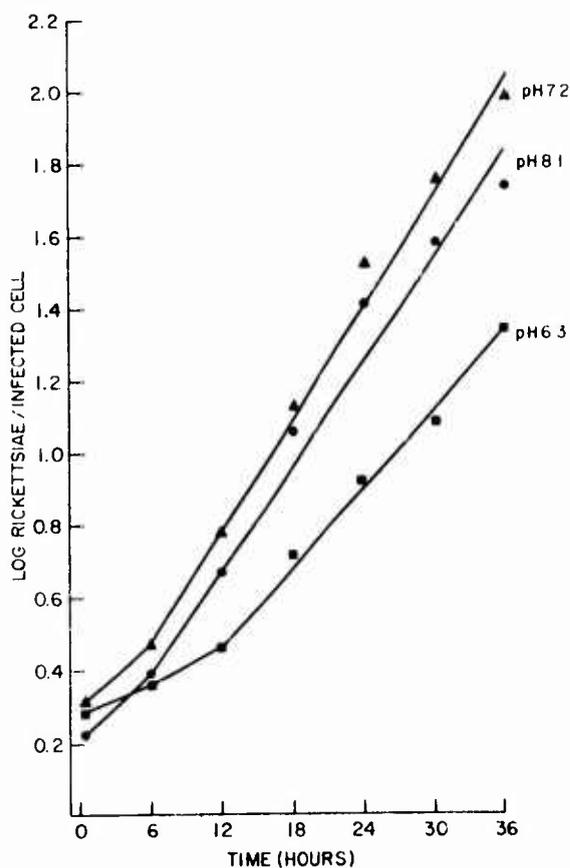


Fig. 4 Growth kinetics of *R. conorii* in irradiated L-929 cells maintained in spinner culture at various pH values.

The results shown in Fig. 4 suggest that rickettsial growth rate is very similar at neutral and slightly alkaline pH values, with a doubling time of 6.0 hr. Growth is depressed at acidic pH and the doubling time increases to 8.1 hr. There was essentially no difference in the calculated doubling times for the rickettsiae grown at 34C in bicarbonate buffered medium as compared to growth in pH 7.2 organic buffered medium at the same temperature, suggesting that the organic buffers produce no toxic effects on rickettsiae.

Preliminary experiments of a similar nature have also been conducted with irradiated WI-38 cells in spinner culture, although some difficulty was encountered due to clumping of cells. Fig. 5 indicates that of the temperatures tested, the most satisfactory growth occurred at 34C with a doubling time of 6 hr. At 38C the

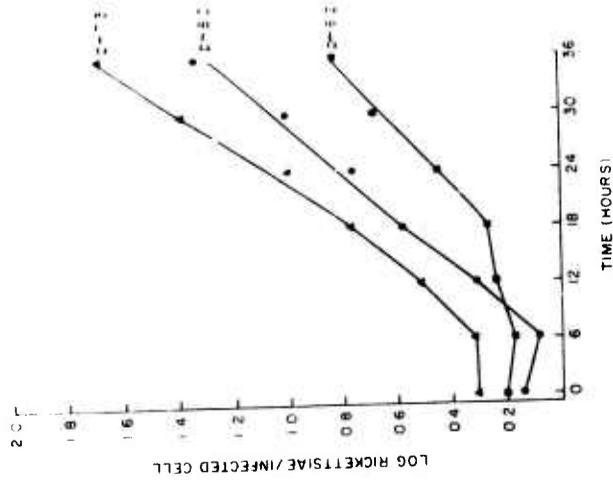


Fig. 6 Growth kinetics of R. conorii in irradiated WI-38 cells maintained in spinner culture at various pH values.

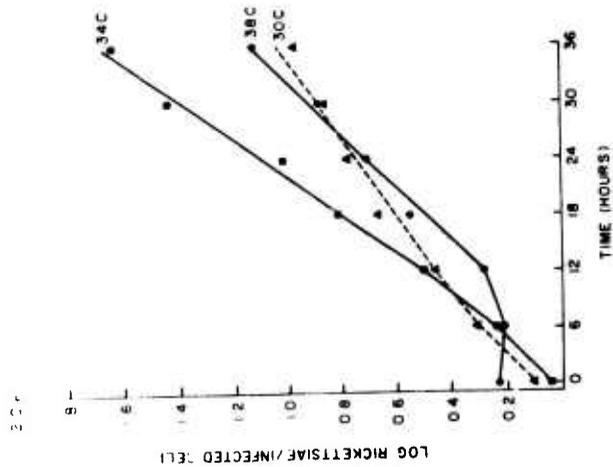


Fig. 5 Growth kinetics of R. conorii in irradiated WI-38 cells maintained in spinner culture at various temperatures.

doubling time was increased to 7.5 hr and further increased to 15 hr at 30C. No growth of rickettsiae was observed at either 26C or 40C, but WI-38 cells underwent gradual autolysis at these temperatures. A comparison of growth rate at 34C in WI-38 and L-929 cells shows an identical doubling time of 6.0 hrs.

The pH studies presented in Fig. 6 tend to parallel those seen in L-929 cells, with superior growth at neutral and alkaline values and reduced growth under acidic conditions. The doubling time at pH 7.3 was 6.0 hrs; pH 8.2 - 7.5 hrs; and pH 6.2 was 10 hrs. As with the temperature experiments, the doubling time of R. conori in both L-929 and WI-38 cells at neutral pH was essentially identical.

The overall results of our studies on the growth of rickettsiae in cultured cells suggests that rickettsial growth may be independent of the cell type used as a substrate and opens the possibility that molecular studies in continuous cell lines and vaccine studies in diploid cell types may both contribute to a unitary concept of rickettsial interaction with host cells.

## II. Selection of conditional lethal temperature sensitive mutants of rickettsiae.

### A. Development of plaque assay and plaque transfer techniques.

Previous studies in this laboratory (Osterman and Parr, 1974) demonstrated plaque formation by R. conori in L-929 cells and indicated the potential usefulness of these cells in the study of the molecular biology of rickettsiae. Technical difficulties previously experienced with this cell line have been resolved by procedural modifications incorporating the use of  $\gamma$ -irradiation to inhibit cell division.

L-929 cells were propagated as previously described, trypsinized, adjusted to  $1 \times 10^6$  cells/ml and subjected to 3,000 rads  $\gamma$ -irradiation, a dose that produces non-dividing multinucleate giant cells capable of supporting rickettsial growth. Culture dishes were seeded with  $2.5 \times 10^6$  cells suspended in 5 ml of M199 with 10% fetal calf serum and incubated overnight at 32C in an atmosphere of 5% CO<sub>2</sub> in air. The resulting monolayers were used for plaque assay employing procedures described previously except M199 was used as growth medium. Plaque transfer was accomplished by plunging a sterile wooden applicator stick through the agarose overlay of a stained plaque assay, lightly touching the infected cell monolayer at the position of the plaque, then withdrawing the stick and plunging it through the agarose overlay of one or more similarly prepared but uninfected cell monolayers. Recipient culture dishes were incubated for 7 or 13 days and overlaid with

neutral red stain as in the normal plaque assay procedure. Fig. 7 indicates the clarity of plaque formation with several different rickettsial agents. It is particularly interesting that plaques were observed with R. tsutsugamushi, strain Gilliam, an agent previously plaqued only in chick embryo cells. Fig. 8 shows plaque formation by R. conori at both 28C and 37C. Plaque size was readily increased by extending the period of incubation, thus providing greater ease and reproducibility in plaque transfer. Other workers have reported rickettsial plaque formation at these temperatures in chick embryo cells (Wike, et al, 1972) and Vero cells (Corv, et al, 1974), but only with substantial reduction in plaque size or loss of plaque definition; factors that seriously hinder effective plaque transfer. The temperature differential of 9C was considered adequate for the isolation of conditional lethal temperature sensitive mutants and establishment of a non-permissive temperature of 37C has important ancillary benefits when considering the possible utilization of temperature sensitive mutants as vaccine agents. Fig. 9 shows the successful transfer of R. conori plaques from one monolayer incubated at the permissive temperature to another monolayer incubated at the non-permissive temperature by use of the applicator stick method. In this instance, none of the plaques nicked from the permissive temperature evidenced conditional lethal temperature mutation and thus all transfers replicated at the non-permissive temperature. Multiple transfers may be accomplished from a single donor plaque to several recipient monolayers.

The use of  $\gamma$ -irradiated L-929 cells in rickettsial plaque formation simplifies and broadens their utilization for molecular studies with rickettsiae. The cell line may be passaged indefinitely and provides an opportunity for field laboratories to establish a self-sustaining system for plaque assay, particularly in geographical areas where there is limited access to pathogen-free eggs normally employed in chick embryo tissue culture. Irradiated mouse fibroblasts support rickettsial plaque formation over a temperature range shown to be adequate for isolation of virus temperature sensitive mutants (Charnure, et al, 1969) and their adaptation to plaque transfer by the applicator stick method provides a rapid, inexpensive, method for transfer of potentially mutated rickettsiae from a single plaque grown at the permissive temperature to duplicate monolayers incubated at both permissive and non-permissive temperatures.

#### B. Mutagenesis of rickettsiae.

The selection of an appropriate mutagen to use with rickettsiae is complicated by the fact that rickettsiae incorporate thymidine very poorly. Most base analogs employed in bacterial studies are analogs of thymidine and it is anticipated on theoretical grounds that they would be inefficient in promoting single site mutations in the rickettsial genome. Therefore, preliminary studies in

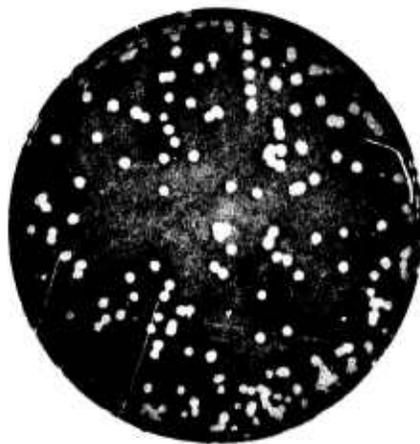


Fig. 7 (A)



Fig. 7 (B)

Fig. 7 Rickettsial plaque formation in irradiated L-929 cells. All monolayers were maintained in M199 with 10% fetal calf serum and stained with neutral red the day preceding photography. (A) R. conori, 8 days post infection; (B) R. tsutsugamushi, 17 days post infection (an additional feeding overlay was provided on day 10 for this plaque assay).

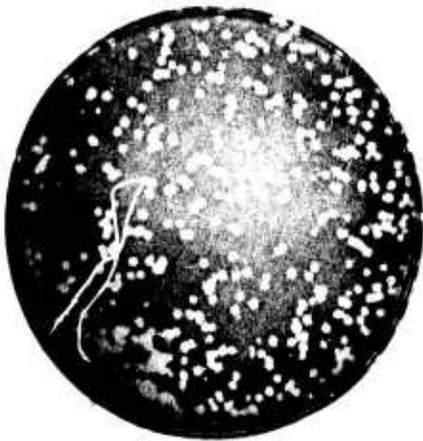


Fig. 8 (C)



Fig. 8 (B)

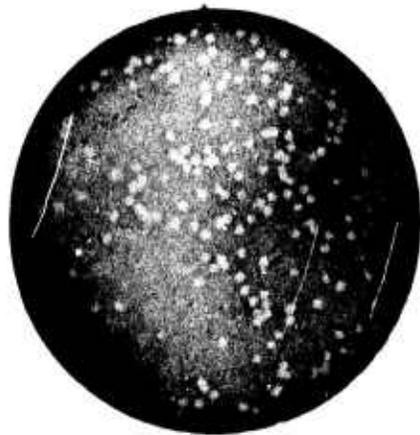


Fig. 8 (A)

Fig. 8 Plaque formation by R. conori at different temperatures. (A) 37C, stained with neutral red 7 days post infection and photographed the following day; (B) 28C, stained with neutral red 7 days post infection and photographed the following day; (C) 28C, stained with neutral red 7 days post infection and photographed at 13 days post infection.

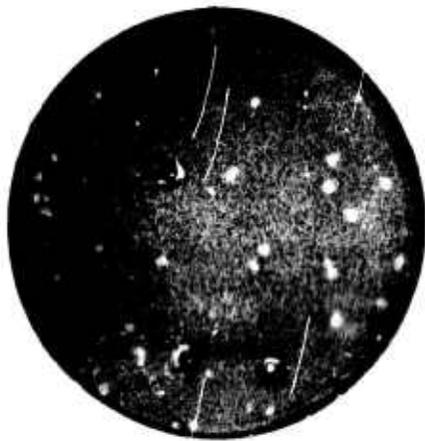


Fig. 9 (A)



Fig. 9 (B)

Fig. 9 Plaque transfer of R. conori. (A) Stained plaque assay, 13 days post infection at 28C, showing transfer from 3 well separated donor plaques. (B) Stained recipient monolayer 8 days post infection at 37C, showing irregular plaques and satellite formation at 10 different positions on the monolayer. The plaques at each position are the result of plaque transfer from a separate and distinct donor plaque. Similar results were achieved with recipient plates incubated at 28C for 13 days.

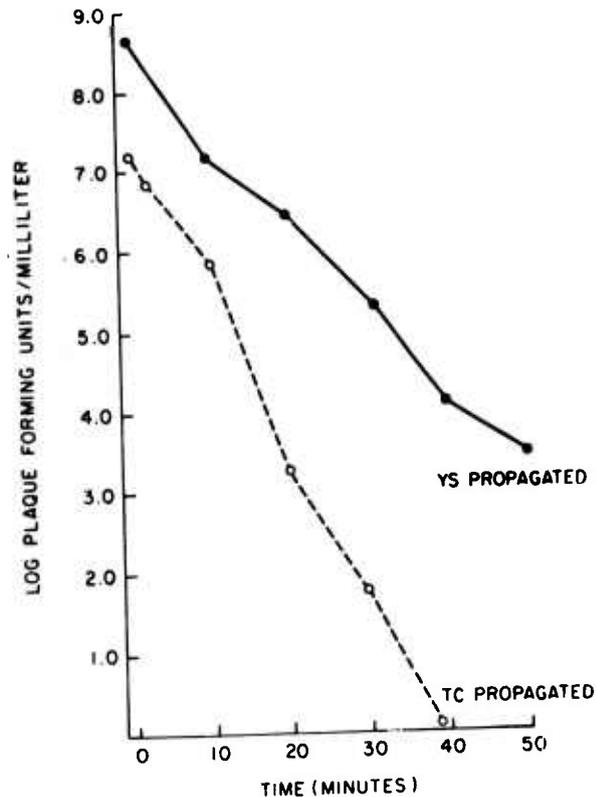


Fig. 10 Inactivation of *R. conori* grown in yolk sac (YS) or tissue culture (TC) by nitrous acid.

rickettsial mutagenesis have focused on nitrous acid, which acts directly on nucleic acids by oxidatively deaminating their bases. Fig. 10 illustrates nitrous acid inactivation curves for both egg grown and tissue culture grown *R. conori*. The exposure of egg grown rickettsiae to 0.1 M  $\text{HNO}_2$  for 40 mins causes a reduction in titer from  $5 \times 10^8$  PFU/ml to  $1 \times 10^4$  PFU/ml. Survivors from this treatment are likely to evidence approximately 1% conditional lethal mutants. Similar inactivation is observed with tissue culture grown organisms, although the time of exposure is reduced to 20 minutes, probably because of reduced cellular contamination. The tissue culture grown rickettsiae are routinely propagated at 37C (non-permissive temperature) to ensure that selected mutants arise from deliberate mutagenesis, thus increasing the probability of obtaining point mutants.

### III. Biochemical and Immunological Studies of Rickettsial Macromolecules.

#### A. Polyacrylamide gel electrophoresis of whole rickettsiae.

When whole rickettsiae are dissociated with 2-mercaptoethanol and sodium dodecyl sulfate and electrophoresed in gels containing SDS, individual proteins may be identified and their molecular weights estimated by reference to the mobilities of appropriate standards. This separation and identification of proteins from intact organisms provides a sensitive method for comparing various rickettsial species and also provides a framework of data which is essential for the analysis of subcellular fractions derived from these microorganisms. Previous studies employing SDS gels to analyze rickettsial proteins (Obijeski, *et al*, 1974) have required large quantities of rickettsiae grown in embryonated eggs and necessarily harvested at poorly defined stages of growth. This study, utilizing radioisotopes and cell culture techniques has allowed the analysis of rickettsial proteins with a greatly reduced number of organisms which were grown under standardized conditions. Specific radioisotope precursors were used to selectively label rickettsial proteins and glycoproteins. In addition, the use of radioisotopes allowed for direct comparison of two counter-labeled rickettsial preparations in a single gel and for the use of internal markers to assist in molecular weight determinations.

Rickettsiae were grown in spinner cultures of  $\gamma$ -irradiated L-929 cells. Cycloheximide was added at 1-2  $\mu\text{g}/\text{ml}$  to inhibit host cell metabolism and to enhance the radiolabeling of rickettsiae. Organisms were routinely harvested during late logarithmic growth. Rickettsial purification was accomplished by homogenizing infected cultures, centrifuging at low speed to remove unbroken host cells, and pelleting rickettsiae from the supernatant by subsequent high speed centrifugation. Rickettsiae were resuspended in a small volume of Brain Heart Infusion broth, layered over a 5-30% sucrose gradient and centrifuged at 2,600  $g$  for 35 mins. Fractions were collected by piercing the bottom of tube and aliquots counted in a liquid scintillation counter. Fig. 11 shows the results of combining  $^{14}\text{C}$  amino acid labeled *R. prowazeki* infected cells with  $^3\text{H}$  amino acid labeled uninfected L-929 cells and subjecting the mixture to the purification scheme. The rickettsiae band uniformly in the gradient with no detectable contamination by host cell protein.

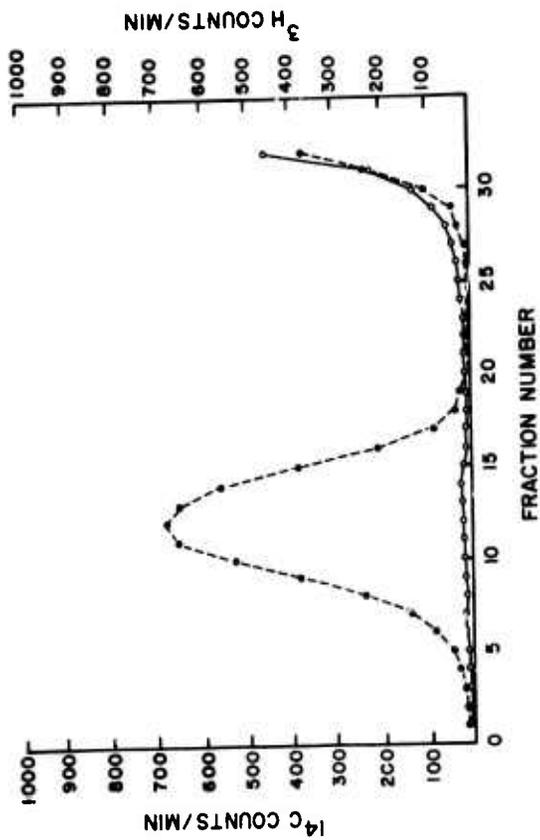


Fig. 11 Sedimentation of *R. prowazekii* in 5-30% sucrose gradient. Centrifugation was at 2,600 g for 35 mins at 4°C; ●—● <sup>14</sup>C amino acid labeled rickettsiae; ○—○ <sup>3</sup>H amino acid labeled uninfected L-929 cells.



Fig. 12 Polyacrylamide gel electrophoresis of *R. typhi*. 7 cm gel stained with Coomassie Blue.

Rickettsial samples were prepared for electrophoresis by boiling in the presence of 2-mercaptoethanol and sodium dodecyl sulfate and were run on 8% SDS-acrylamide gels, either 7 or 12 cm long. After electrophoresis, gels were either stained with Coomassie Blue or sliced into 1 mm segments, digested and counted. Fig. 12 shows a representative stained typhus gel, demonstrating the separation of at least 24 rickettsial proteins. Stained gels were routinely scanned on a densitometer, but in these tracings some of the minor bands are obscured and appear only as shoulders on the major peaks. Fig. 13 shows a densitometer tracing of a 7 cm. stained gel of R. prowazeki superimposed on the radioactive profile of a similar gel which has been sliced. The correlation of radioactive label with stained bands for the 6 major proteins and for 6 minor proteins demonstrates the validity of the radiolabeling technique. Some minor bands in the high molecular region of the gel are too closely spaced to be clearly resolved by our 1 mm slicing procedures. Fig. 14 shows an electropherogram comparing the proteins of  $^{14}\text{C}$  amino acid labeled R. prowazeki and  $^3\text{H}$  amino acid labeled R. typhi co-electrophoresed in the same gel. There is a distinctive divergence in peak pattern occurring between fractions 30-36. Co-electrophoresis of these rickettsiae on a longer 12 cm gel shown in Fig. 15 demonstrates this difference even more dramatically. Similar experiments were performed with spotted fever group organisms. Fig. 16 shows a densitometer tracing of a 7 cm gel of R. rickettsi superimposed on the radioactive profile of a similar gel which has been sliced. There is, again, good correlation of radioactive label with stained bands for the 8 major proteins and for 3 minor proteins. Variations in gel patterns are also observed when representatives of different groups of rickettsiae are contrasted. Fig. 17 shows an electropherogram comparing epidemic typhus organisms and Rocky Mountain spotted fever organisms co-electrophoresed in the same gel. Some differences are discernible in the areas of fractions 4-5 and 30-36. These differences are reinforced and more clearly demonstrated on the 12 cm electropherogram seen in Fig. 18. A divergent gel pattern is obvious at fractions 5-7 and 50-65.

The utilization of radioactive galactose to label R. prowazeki resulted in the distinctive gel pattern shown in Fig. 19.  $^{14}\text{C}$  amino acid labeled organisms are co-electrophoresed with  $^3\text{H}$  galactose labeled rickettsiae. Co-banding of significant quantities of sugar with only specific rickettsial proteins suggest that one high molecular weight and two low molecular weight proteins may be glycoproteins.

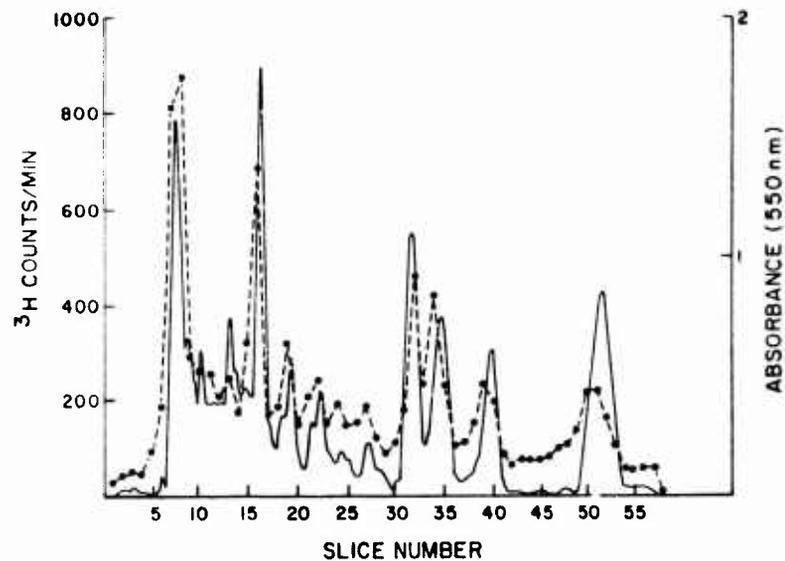


Fig. 13 Polyacrylamide gel electrophoresis of *P. rowazeki*. The solid line is a densitometer tracing of a 7 cm gel stained with Coomassie Blue. The broken line shows the radioactive profile of a similar preparation labeled with  $^3\text{H}$  amino acids and sliced into 1 mm segments prior to counting.

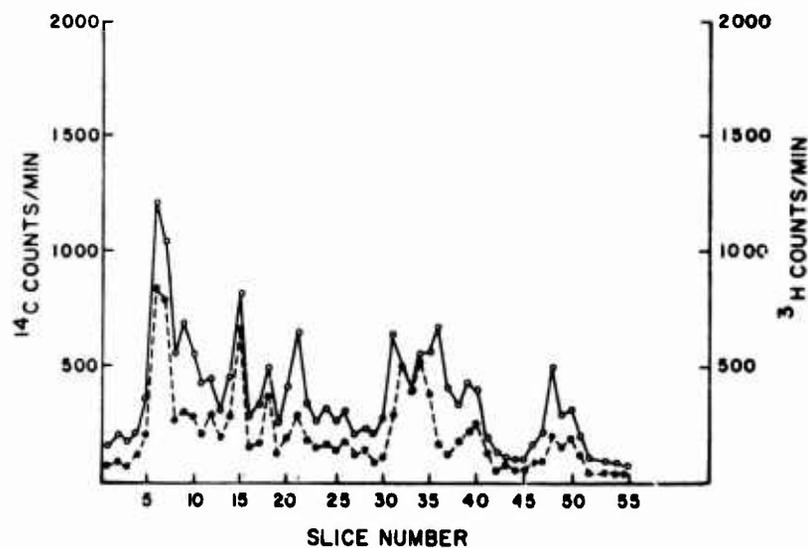


Fig. 14 Co-electrophoresis of  $^{14}\text{C}$  amino acid labeled *P. rowazeki* and  $^3\text{H}$  amino acid labeled *P. tvphi*; 7 cm gel ●-----●  $^{14}\text{C}$  *P. rowazeki*; ○-----○  $^3\text{H}$  *P. tvphi*.

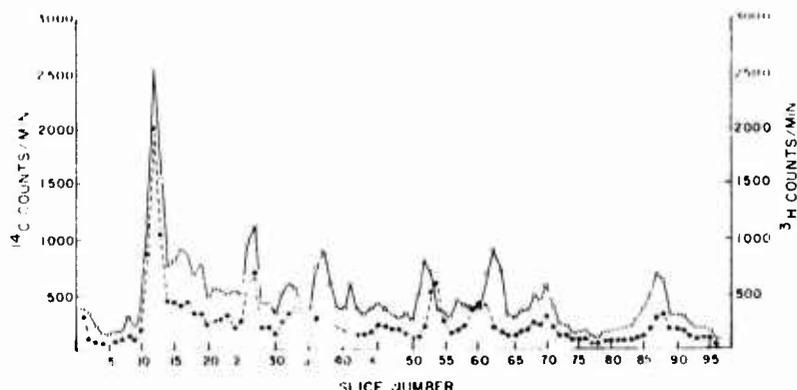


Fig. 15 Co-electrophoresis of  $^{14}\text{C}$  amino acid labeled R. prowazekii and  $^3\text{H}$  amino acid labeled R. typhi:  
 12 cm gel  $\bullet$ - - -  $^{14}\text{C}$  R. prowazekii;  $\circ$ - - -  $^3\text{H}$  R. typhi.

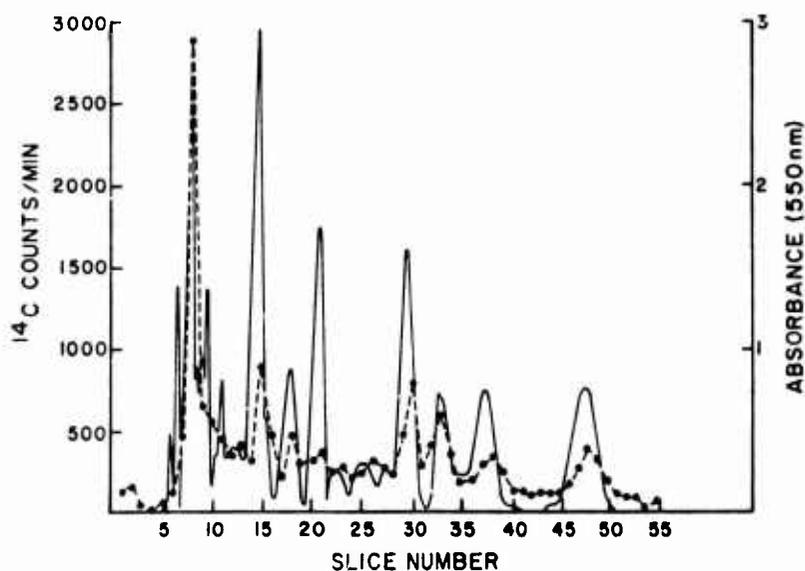


Fig. 16 Polyacrylamide gel electrophoresis of R. rickettsii. The solid line is a densitometer tracing of a 7 cm gel stained with Coomassie Blue. The broken line shows the radioactive profile of a similar preparation labeled with  $^{14}\text{C}$  amino acids and sliced into 1 mm segments prior to counting.

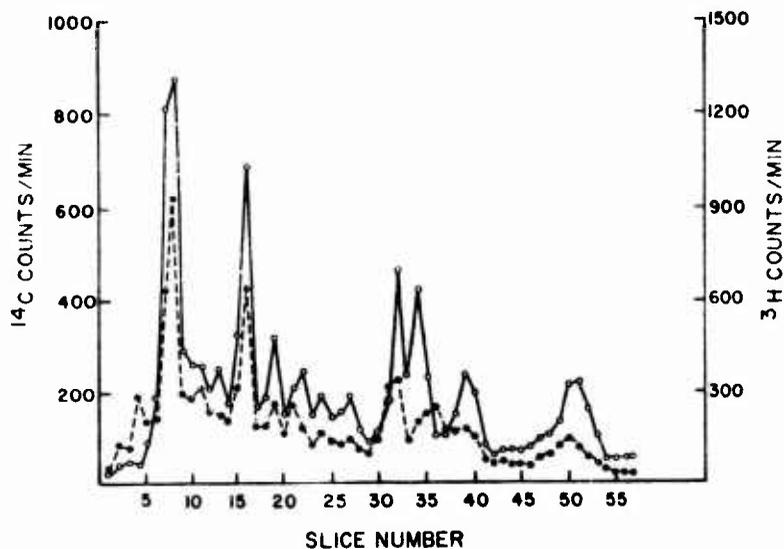


Fig. 17 Co-electrophoresis of  $^{14}\text{C}$  amino acid labeled *R. rickettsi* and  $^3\text{H}$  amino acid labeled *R. prowazeki*; 7 cm gel ●-----●  $^{14}\text{C}$  *R. rickettsi*; ○-----○  $^3\text{H}$  *R. prowazeki*.

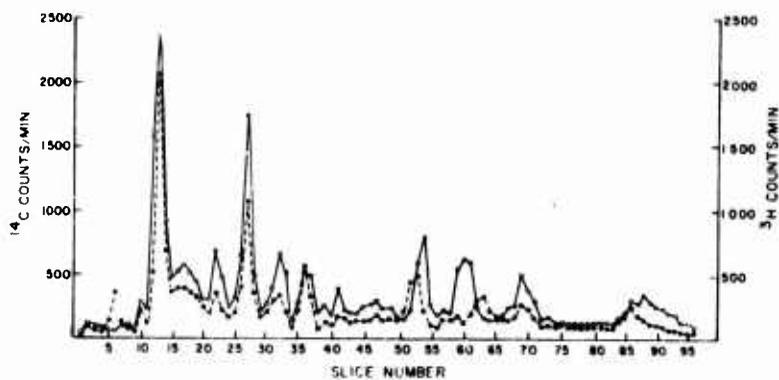


Fig. 18 Co-electrophoresis of  $^{14}\text{C}$  amino acid labeled *R. rickettsi* and  $^3\text{H}$  amino acid labeled *R. prowazeki*; 12 cm gel ●-----●  $^{14}\text{C}$  *R. rickettsi*; ○-----○  $^3\text{H}$  *R. prowazeki*.

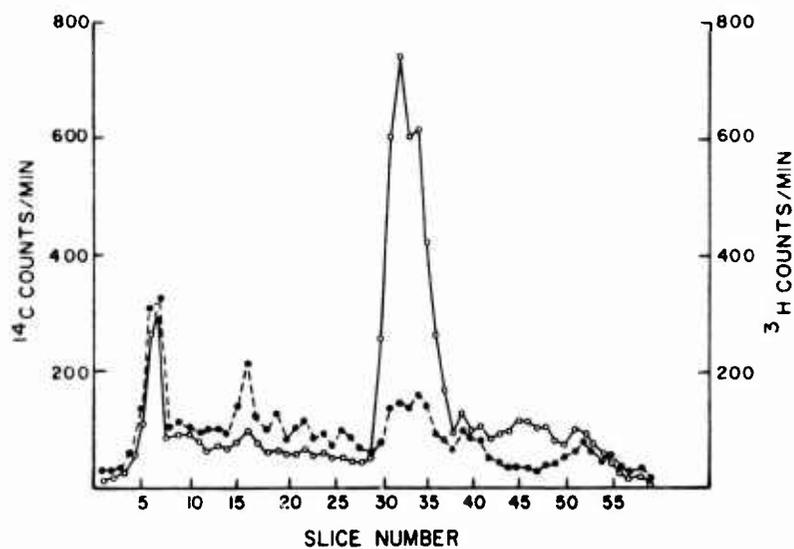


Fig. 19 Polyacrylamide gel electrophoresis of *R. prowazeki*; 7 cm gel ●-----●  $^{14}\text{C}$  amino acid; o---o  $^3\text{H}$  galactose.

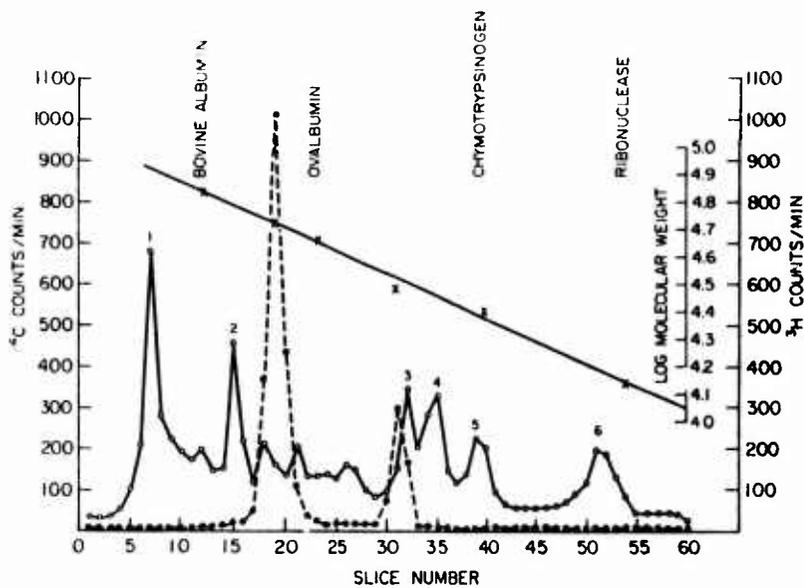


Fig. 20 Co-electrophoresis of  $^3\text{H}$  amino acid labeled *R. prowazeki* and  $^{14}\text{C}$  amino acid labeled Sindbis virus; 7 cm gel. The linear relationship of log molecular weight to relative movement in the gel was established by reference to both external markers (electrophoresed in a similar, but separate gel) and internal markers (Sindbis virus proteins of known molecular weight).

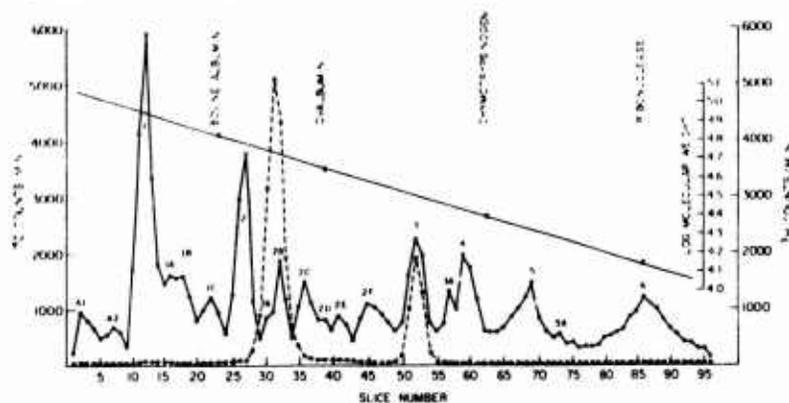


Fig. 21 Co-electrophoresis of  $^3\text{H}$  amino acid labeled R. prowazeki and  $^{14}\text{C}$  amino acid labeled Sindbis virus; 12 cm gel. The linear relationship of log molecular weight to relative movement in the gel was established by reference to both external markers (electrophoresed in a similar, but separate gel) and internal markers (Sindbis virus proteins of known molecular weight).

The molecular weights of rickettsial proteins were estimated with the aid of both internal and external standards. Electrophoresis of molecular weight standards with radioactive Sindbis virus proteins established a linear relationship of relative mobility to molecular weight. Subsequent co-electrophoresis of counter-labeled Sindbis virus and rickettsial protein provided an internal molecular weight marker. Fig. 20 indicates the linearity of log molecular weight of these standards plotted against their relative movement, and also indicates the movement of Sindbis virus proteins relative to R. prowazeki proteins when electrophoresed in a single gel. This has allowed computation of molecular weights for the six major proteins and estimation of molecular weights for additional proteins observed in the stained gels but not clearly separated in the radioactive preparations. Fig. 21 shows a similar molecular weight determination performed on a 12 cm gel and illustrates the separation of minor proteins not seen in the shorter gel. Rickettsial proteins vary in molecular weight from approximately 13,000 to 112,000 daltons. Table 2 shows the molecular weights for the six major proteins and some additional minor proteins of R. prowazeki. Molecular weights of individual proteins are essentially identical when calculated from either 7 or 12 cm gels. The 24 proteins resolved by these techniques represent only a part of the full complement of rickettsial proteins, however a comparison of these proteins does provide a sensitive method for distinguishing between rickettsial species. In addition, the profiles established for whole organisms provides a solid framework for comparison of various subcellular fractions and ultimately for determining the role of these fractions in the immunological response of the host.

Table 2. Molecular weight of *R. prowazeki* proteins

Major Proteins	7 cm gel		12 cm gel	
	Fraction Number	Molecular Weight	Fraction Number	Molecular Weight
1	7	83,000	12	87,000
2	15	62,000	27	60,000
3	32	32,000	52	32,000
4	35	29,000	59	27,000
5	39	25,000	69	21,000
6	51	15,000	86	13,000
Minor Proteins				
A1	-	-	2	112,000
A2	-	-	7	100,000
1A	-	-	16	79,000
1B	-	-	18	76,000
1C	12	68,000	22	68,000
2A	18	55,000	30	56,000
2B	-	-	32	54,000
2C	21	49,000	36	48,000
2D	24	44,000	39	45,000
2E	-	-	41	43,000
2F	26	40,000	45	38,000
3A	-	-	57	28,000
5A	-	-	73	19,000

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 197 Rickettsial Genetics

Literature Cited.

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1. Osterman, J. V., and Parr, R. P. Plaque formation by Rickettsia conori in WI-38, DBS-FRHL-2, L-929, HeLa, and chicken embryo cells. Infect. Immunity 10: 1152-1155, 1974.

Presentations:

1. Oaks, S.C., Jr. Growth of Rickettsia conori in mammalian cells. By Osterman, J.V., Oaks, S.C., Jr., and Parr, R.P. Annual meeting of American Society of Microbiology, New York City, 28 Apr-2 May 1975.
2. Simon, C.M. Analysis of rickettsial proteins by polyacrylamide gel electrophoresis. By Simon, C.M., and Osterman, J.V. Annual meeting of American Society of Microbiology, New York City, 28 Apr-2 May 1975.
3. Osterman, J.V. Growth and plaquing of Rickettsia conori in mammalian cells. Workshop on Rocky Mountain Spotted Fever, Atlanta, Georgia, Communicable Disease Center, 5-6 December 1974.
4. Osterman, J.V. Methods in Molecular Virology. Johns Hopkins University, Baltimore, Md., 31 Jan 1975.
5. Osterman, J. V. Molecular Aspects of Rickettsiology. University of Kansas, Lawrence, Kansas, 18 June 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
				DA OB 6514	75 07 01		
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8A. DDD'S INSTR <sup>f</sup>	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM <sup>g</sup>
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: <sup>h</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		61101A		3A161101A91C		00	
b. CONTRIBUTING						19B	
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>i</sup>							
(U) Spin Labeling of Biomolecules							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>j</sup>							
002300 Biochemistry 008300 Inorganic Chemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				b. PREVIOUS		c. FUNDS (in thousands)	
d. NUMBER <sup>k</sup>				FISCAL YEAR		75	
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f. AMOUNT:				76		1	
g. KIND OF AWARD:				8. CUM. AMT.		53	
20. RESPONSIBLE SPOB ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Punish MAN if U.S. Academic institution)			
NAME: Buescher, COL E. L.				NAME: Beach, LTC D. J.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2211			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Doctor, B. P. PhD			
				NAME: Copeland, E. S. PhD			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) ESR; (U) Spin Label; (U) Spectrometry; (U) Drugs of Abuse; (U) Receptor Binding							
23. TECHNICAL OBJECTIVE <sup>l</sup> 24. APPROACH. 25. PROGRAM (Punish individual paragraphs identified by number. Precede last of each with Security Classification Code.)							
23. (U) The technical objective of this work unit is to develop methods for analysis of important biochemicals and to study mechanisms by use of spin labeling and electron spin resonance (ESR) spectrometry for use in military medicine.							
24. (U) Spin labeling and ESR techniques will be applied to the study of biological systems to study distribution, binding and metabolism of drugs of abuse and other drugs of military importance. In vitro systems and spin labeled drug analogs will be used to define drug binding kinetics. These methods will be combined with immunological techniques to improve drug and drug metabolite analyses.							
25. (U) 74 07 - 75 06 Major advances in the chemistry of spin labels have been reported. Several spin labeled analogs of morphine have been synthesized and tested for biological activity. ESR studies have shown that in drugs that produce clinical signs of phototoxicity, the UV threshold for the production of a triplet state is significantly lower than in drugs devoid of phototoxicity. This threshold is consistent with energies involved in phototoxic responses suggesting a possible mechanism for phototoxicity in drugs of military importance such as phenothiazines and antimalarials. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

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DD FORM 1498  
1 MAR 68

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 198 Spin labelling of biomolecules

Investigators.

Principal: LTC (P) Douglas J. Beach, MSC; LTC Gale E. Demaree, MSC;  
CPT James A. Cella, MSC; Edmund S. Copeland, Ph.D.;  
Bhupendra P. Doctor, Ph.D.

Associate: Billy G. Bass, M.S.; SP5 James P. McGrath, B.S.;  
SP5 Edward F. Kennehan, B.S.; CPT James A. Kelley, MSC.

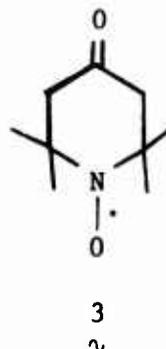
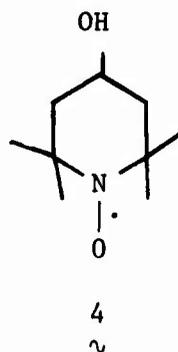
The technical objective of this work unit is to develop methods and materials for the exploitation of paramagnetic resonance for biochemical and other biomedical applications. Work during this fiscal year was concentrated on these studies:

1. Chemistry and applications of nitroxide free radicals.
2. Correlation of ESR studies of drugs with phototoxicity and its modification.
3. Development of spin immunoassays.

#### 1. CHEMISTRY AND APPLICATIONS OF NITROXIDE RADICALS

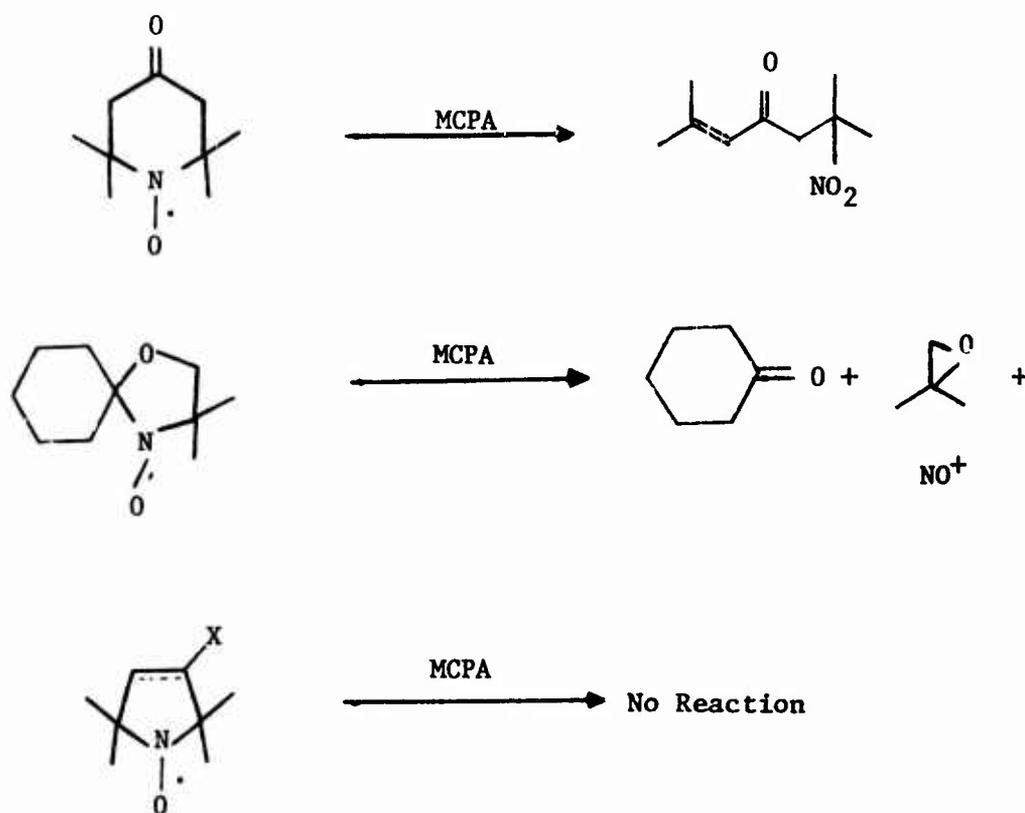
In the course of work on the spin labeling of opiates, a number of aspects of the chemistry of the nitroxides used as spin labels was investigated. From these investigations were derived several applications in the areas of preparative and analytical organic chemistry.

The discovery that keto nitroxide, 3, was produced on attempted generation of alcohol-nitroxide 4 by reaction of the corresponding amine with *m*-chloroperbenzoic acid (MCPA) led to a general method for the oxidation of alcohols. Application of this mild oxidation to the oxidation of olefinic alcohols produces the corresponding epoxy-ketones in excellent yield. Currently, efforts are directed toward combining the alcohol oxidation with the Baeyer-Villiger cleavage of ketones.



Since this sequence would result in cleavage of a carbon-carbon bond  $\alpha$  to an alcohol, application to those antimalarials bearing a secondary alcohol function should provide a means for degradation to derivatives suitable for analysis by gas chromatography.

The chemistry of a number of nitroxides in the peracid system was investigated in an effort to learn more about the processes described above. It has been determined from this study that the interaction of nitroxides with MCPA produces oxoimmonium ions. These ions are presumably responsible for the oxidation of alcohols in this system. Depending on the functional groups attached to the nitroxide the ions generated by this reaction undergo a number of fragmentation reactions to non-paramagnetic products. Some of these fragmentations are illustrated in Scheme 1. Interestingly, nitroxides of the pyrrolidine series do not appear to react with MCPA.



SCHEME 1

## 2. CORRELATION OF ESR STUDIES OF DRUGS WITH PHOTOTOXICITY AND ITS MODIFICATION.

We have previously reported the reduction of the phototoxicity of chlorpromazine by pretreatment with an antiradiation drug, WR 2721. WR 2721 was devoid of any protective actions when it was given after exposure to ultraviolet light. This suggests the hypothesis that WR 2721 prevents phototoxicity through its interaction with some active form of the phenothiazine. Since WR 2721 is a potential mercaptan, it may act in its role as a radical scavenger. If this is the case, some drugs that are phototoxic such as quinoline methanol antimalarials, WR 7930 and WR 7936, should have an activated form with a well-localized energy state. To test this hypothesis, the activation states of drugs having high potential for phototoxicity were compared with several drugs having low phototoxic potential.

ESR was used to look for various singlet and triplet states that might result from photoactivation by exposure to UV radiation that is known to induce phototoxicity in mice treated with these drugs. A series of quinoline methanol antimalarials and a series of phenothiazines were studied. The drugs under study were chosen to give a wide range of potencies within each drug class for producing phototoxicity in mice.

It was found that the triplet state could be induced in the drugs with greater potency for phototoxicity at lower energies than in the drugs with lesser potency for phototoxicity. Generally, the more phototoxic compounds required 3.6 electron volts of energy or less for the induction of the triplet state while the less phototoxic compounds required more than 3.8 electron volts for the induction of the triplet state.

It is planned to extend these studies to the use of more efficient radical scavengers than WR 2721 and to extend the hypothesis to the study of mechanism of retinopathy induction in the case of phenothiazines and other drugs. If this relationship holds, this system might serve as a useful screen for phototoxicity and drug-induced retinopathy. These studies have ignored the possible roles of drug metabolites which must also be considered for full interpretation of the data.

## 3. DEVELOPMENT OF SPIN IMMUNOASSAYS.

In addition to extending the scope of spin labeling for drugs by conjugation with nitroxide radicals, a greater sensitivity has been achieved for spin immunoassays in collaboration with DCD&I. The principle involves the amplification of freed spin label by combining the assay with complement fixation.

This was accomplished by immobilizing the spin label by trapping in liposomes. The liposomes have antigen bound to the external membrane. In the presence of antibody and complement, the liposomes are lysed, releasing the trapped spin label. Antigen present in the sample will compete with the membrane-bound antigen and will be expressed as reduced release of spin label.

This method has several advantages:

1. A non-specific spin label such as TEMPO choline bromide can be used for all assays thereby eliminating the need for synthesis of specific spin labeled antigens.
2. The method is especially suited for lipophilic compounds which cause serious technical difficulties with conventional methods.
3. This method gives a range of sensitivity for spin immunoassays not previously attainable.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 198 Spin labeling of biomolecules

Literature Cited.

Publications:

1. Cella, J.A., Kelley, J.A., and Kenehan, E.F.: Unexpected oxidation of a nitroxide alcohol with m-chloroperbenzoic acid. Chem. Comm. 943: 1974.
2. Cella, J.A., Kelley, J.A., and Kenehan, E.F.: Nitroxide catalyzed oxidation of alcohols using m-chloroperbenzoic acid. A new method. J. Org. Chem. 40: 1860, 1975.
3. Cella, J.A., Kelley, J.A., and Kenehan, E.F.: Nitroxide catalyzed oxidation of alcohols using m-chloroperbenzoic acid. A new method. Abstracts, 169th National Meeting of the American Chemical Society, Philadelphia, PA., April 1975.
4. Copeland, E.S.: Characterization of secondary radical reactions in irradiated ribonuclease. Radiat. Res. 61: 63-79, 1975.
5. Copeland, E.S., Grenan, M.M., and Yang, G.C.: An in vivo investigation of the phototoxic effect and its amelioration with radio-protective compounds. J. Invest. Derm. 64: May 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	3. REPORT CONTROL SYMBOL	
				DA OB 6518	75 06 30	DD-DR&E(AR)1636	
4. DATE PREV. SUMMARY	5. KIND OF SUMMARY	6. SUMMARY ACTY <sup>b</sup>	7. WORK SECURITY <sup>c</sup>	8. REGRADING <sup>d</sup>	9. DRDP <sup>e</sup> NETA <sup>f</sup> H	10. SPECIFIC DATA - CONTRACTOR ACCESS	
74 07 01	K. Compl.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES <sup>g</sup>		12. PROGRAM ELEMENT	13. PROJECT NUMBER	14. TASK AREA NUMBER	15. WORK UNIT NUMBER		
a. PRIMARY		61101A	3A161101A91C	00	199		
b. CONTRIBUTING							
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Membrane Transport in Gut							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>i</sup>							
012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		75 06		DA		C. T <sub>1</sub> -house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE EFFECTIVE: NA				b. PRECEDING		c. FUNDS (in thousands)	
d. NUMBER: 0				74		1	
e. TYPE:				75		35	
f. KIND OF AWARD:				1		35	
g. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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TELEPHONE: 202 576-3551				TELEPHONE: 202 576-3284			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEYWORDS (Precede with Security Classification Code)							
(U) Membrane Transport; (U) Intestinal Mucosa;							
(U) Membrane Potential							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To evaluate the effects of ischemia, hypoxia, and catecholamines on intestinal mucosal electrolyte fluxes and potential difference. These studies provide data that relate to the gastrointestinal mucosal responses seen in severely wounded soldiers.							
24 (U) An in vitro system was used to measure colon membrane electrolyte fluxes, short-circuit current (SCC), and electrical potential difference (PD). To evaluate small bowel viability, transmural ileal PD was measured in vivo comparing control and ischemic intestinal segments.							
24 (U) 74 07 - 75 06. The catecholamines epinephrine and norepinephrine caused a dose-dependent decrease in PD and SCC - an effect blocked by phenoxybenzamine, an alpha adrenergic antagonist. Isoproterenol, a beta adrenergic agonist, had no effect. These changes were associated with an increase in sodium absorption and a decrease in bicarbonate secretion. These data support the idea of an alpha adrenergic receptor mechanism which influences electrolyte transport and may be of importance in the gastrointestinal response to trauma characterized by elevated plasma catecholamine levels. Transmural PD decreased in response to ischemia due to strangulation obstruction or superior mesenteric artery occlusion. The return of PD correlated with survival of the intestine and may be used intraoperatively to predict intestinal viability following ischemic injury. Work has been completed on this work unit.							
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.							

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DD FORM 1498  
1 MAR 66

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 199 Membrane transport in gut

Investigator.

Principal: MAJ Nelson J. Gurll, MC

Background and Problem. Much is known about the basic physiology of water and electrolyte secretion and absorption in the intestine. Recent attention has focused on the pathophysiological alteration of these transport processes in certain diarrheal diseases. We have investigated these transport processes in a variety of clinical conditions important to the surgeon such as shock, mesenteric ischemia, and strangulation obstruction of the intestine.

Experimental Approach. In vitro studies of rabbit colonic mucosa were performed using modified Ussing chambers. The effects of various catecholamines on ion transport were determined using radioisotopes under short circuit current conditions. Transmural electrical potential difference (PD) was measured in vivo in rabbit small intestine. Responses in the PD to mesenteric ischemia and strangulation bowel obstruction were noted in loops of control and ischemic intestine.

Results and Discussion. The alpha-adrenergic agonists, epinephrine and norepinephrine, caused a dose-dependent decrease in short circuit current (equals net ionic fluxes) and PD. These changes were associated with an increase in sodium absorption and a decrease in bicarbonate secretion. The effects of epinephrine and norepinephrine were independent of substrate but were dependent on the presence of CO<sub>2</sub> and bicarbonate on the mucosal surface. These alterations in ion transport were blocked by the alpha-adrenergic antagonist phenoxybenzamine. We concluded that there exists an alpha adrenergic mechanism which influences sodium and bicarbonate transport in the colon and may be important in the gastrointestinal response to shock in which the circulating level of catecholamines are quite high.

Transmural potential differences across the small intestine fell in response to both occlusion of the superior mesenteric artery and clamping of the small bowel (a model of strangulated intestinal obstruction). Potential difference was significantly lower in ischemic compared to control loops of rabbit ileum and was lower in those loops ultimately developing intestinal infarction. Mucosal potential difference could, therefore, be used as an indicator of intestinal viability.

Project 3A161J01A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 199 Membrane transport in gut

Literature Cited.

Publications:

1. Gurll, N.: Colonic adrenergic receptors for electrolyte fluxes. Gastroenterology 66: 705, 1974.
2. Gurll, N., and Zinner, M.J.: Intestinal water and electrolyte absorption. Amer. Surg. 41: 189-192, 1975.
3. Fromm, D., Schwartz, J.H., and Quijano, R.: Transport of H<sup>+</sup> and other electrolytes across isolated gastric mucosa of the rabbit. Amer. J. Physiol. 228: 166-171, 1975.
4. Gurll, N. and Braxton, G.: Potential difference as a predictor of intestinal viability. J. Surg. Res. 18: 611-613, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL DD DR&E(AR)63A	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	DA OC 6431	75 07 01		
74 07 01	D. Change	U	U	7. REGRADING <sup>e</sup>	8A. DDB'S INSTN <sup>f</sup>	8B. SPECIFIC DATA- CONTRACTOR ACCESS	8. LEVEL OF SUM
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a. PRIMARY	61101A	3A161101A91C	00	200			
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c. CONTRIBUTING							
11. TITLE (Precede with security Classification Code) <sup>h</sup>							
(U) Cardiac Performance in By-pass Surgery							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING			
b. NUMBER: NA				FISCAL YEAR		b. FUNDS (in thousands)	
c. TYPE:				75		1	
d. KIND OF AWARD:				76		91	
18. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME:			
				NAME: DA			
22. KEYWORDS (Precede EACH with security Classification Code) <sup>j</sup>							
(U) Myocardial Metabolism; (U) Cardiac Dynamics; (U) Extracorporeal Circulation							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security Classification Code)							
23 (U) To evaluate various methods of protecting the myocardium and achieving cardiac stasis during extracorporeal circulation for cardiac surgery. The results of these studies will provide guidelines for military cardiac surgeons concerning the most efficacious methods of establishing cardiac stasis.							
24 (U) Surgical bypass procedures will be employed to place experimental animals on total cardiopulmonary bypass to study alterations in cardiac dynamics and metabolism created by the following: (1) metabolic and respiratory changes in the coronary perfusate; (2) various degrees of myocardial hypothermia; and (3) use of ventricular fibrillation, cardioplegic agents, and/or anoxic arrest.							
25 (U) 74 07 - 75 06. Myocardial respiratory function and total coronary blood flow were evaluated during cardiopulmonary bypass in 18 dogs. The fibrillating heart was found to be associated with an increase in myocardial oxygen utilization and metabolic rate which was compensated for by a corresponding increase in total coronary blood flow. Following anoxic arrest of the heart, there appears to be an initial impairment to oxygen utilization. Oxygen consumption does not return to normal after 15 minutes of restoring coronary blood flow.							
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.							
<sup>k</sup> Available to contractors upon contractor's request.							

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1 MAR 68

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 200 Cardiac performance in bypass surgery

Investigator.

Principal: LTC Arthur W. Fleming, MC

Alterations in Myocardial Metabolism, Function, and Structure in the Beating Heart while on Cardiopulmonary Bypass during Electrical (AC) Ventricular Fibrillation and during Anoxic Arrest

A. Statement of the Problem. To determine the degree of alterations in myocardial function, metabolism, and structure created by techniques used during cardiopulmonary bypass to permit meticulous intracardiac or coronary artery bypass surgical procedures.

B. Background. Soon after the first successful use of clinical cardiopulmonary bypass, attempts were made in experimental models to develop methods for obtaining a motionless, relaxed or bloodless heart to facilitate intracardiac surgical repairs. The use of electrical ventricular fibrillation and anoxic arrest have both been evaluated as means of obtaining the above goals. However, there is still disagreement as to which of these two techniques results in the least metabolic, functional, and structural changes in the myocardium; and under what circumstances these alterations occur.

Previous studies have yielded neither uniform results nor uniform interpretations. The primary difficulty with studies performed on cardiopulmonary bypass appears to be the necessity for measuring and controlling multiple parameters which, in turn, may result in a progressive loss of accuracy and significance. However, cardiopulmonary bypass is such a formidable intrusion into the mechanism of homeostasis, that any attempt to measure isolated parameters without being aware of related changes may constitute a worse pitfall. Thus, our initial studies have been designed basically to develop a model in which multiple variables could be either controlled or eliminated, and the remaining variables could be accurately measured and recorded.

C. Experimental Approach. Thirty-eight dogs of either sex, weighing between 18 to 36 kg. were bled up to 100% of their estimated blood volume over a period of 17 to 19 days. This blood was maintained at 4° C until the morning of surgery. The intraoperative technique was basically the same as that used for clinical cardiopulmonary bypass except for the following additions: The total coronary venous return is diverted by a cannula into the right atrium and ventricle. This cannula is in turn passed through the chest wall and connected in series to (1) a sampling stopcock, (2) an extracorporeal flowmeter, and (3) a graduated cylinder. This blood is then returned to the cardiotomy reservoir; the pulmonary

artery is occluded to prevent escape of blood; and a cannula is inserted into the left atrial appendage for measuring coronary blood flow in the working heart.

After placing the dogs on total cardiopulmonary bypass, the coronary blood flow, systemic blood flow, arterial and venous pressures, and EKG were recorded. Paired arterial and coronary venous samples were obtained for pH, PO<sub>2</sub>, PCO<sub>2</sub>, and electrolytes. The circumstances under which the initial measurements were made were identical for all animals and served as the baseline period. Animals were then randomly divided into 3 groups: (1) normothermic perfusion in the beating nonworking heart; (2) electrical ventricular fibrillation, and (3) anoxic arrest.

D. Results and Discussion. Measurement of the coronary venous return established the following: (1) Baseline flow was approximately 5.7% of the total systemic flow and was  $148 \pm 12$  ml/min.; (2) animals which had electrical ventricular fibrillation with an AC current always had an increase in flow when compared to flow in the beating, empty heart in the same animal; and (3) the reactive hyperemia that occurred following anoxic arrest allowed up to 43% of the total systemic flow to go through the myocardium (mean of 25%). The hearts which were fibrillated showed a greater cardiac output during the recovery period. There was also less evidence of pathological changes, such as endocardial and sub-endocardial hemorrhages, less intracellular edema, less myocardial bleeding, and less fragmentation of myofibrils and loss of cell detail.

E. Conclusion. Initiation of electrical ventricular fibrillation with an alternating current (AC) and then allowing the fibrillatory pattern to persist in a normal canine heart on cardiopulmonary bypass creates less metabolic, functional, and structural changes than anoxic arrest.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 200 Cardiac performance in bypass surgery

Literature Cited.

Publications:

1. Fleming, A. W., and Green, D. C.: Traumatic aneurysms of the thoracic aorta. Ann. Thorac. Surg. 18: 91-101, 1974.
2. Fleming, A. W., Etheridge, M. L., and Jenkins, E. B.: Total coronary blood flow measurements and myocardial metabolism during cardiopulmonary bypass in a canine model. Am. Surg. 11: 214-220, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)16.5a	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	DA OC 6432	75 07 01		
74 07 01	D. Change	U	U	7. REGRADING <sup>a</sup>	8a. DIS'N INSTR <sup>a</sup>	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM A. WORK UNIT
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b. CONTRIBUTING							
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Fibrinolysis in Peripheral Blood Vessels							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003500 Clinical Medicine							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD			
73 07	CONT	DA		C. In-House			
17. CONTRACT/GRAANT				18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (in thousands)	
a. DATE/EFFECTIVE:	NA	EXPIRATION:		PRECEDING			
b. NUMBER: <sup>a</sup>				FISCAL YEAR	75	2	50
c. TYPE:		4. AMOUNT:		CURRENT	76	1	37
d. KIND OF AWARD:		f. CUM. AMT.					
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: <sup>a</sup> Walter Reed Army Institute of Research				NAME: <sup>a</sup> Walter Reed Army Institute of Research			
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				ADDRESS: <sup>a</sup> Washington, DC 20012			
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NAME: Buescher, COL E. L.				NAME: <sup>a</sup> Gervin, MAJ A. S.			
TELEPHONE:				TELEPHONE: 202 576-3794			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Buckman, CPT R. F.			
				NAME: DA			
22. KEYWORDS (Precede each with Security Classification Code) (U) Fibrinolysis; (U) Thrombosis; (U) Blood Vessels							
23. TECHNICAL OBJECTIVE, <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To evaluate fibrinolytic activator mechanisms in vascular and microvascular systems; to determine the effects of chemical, ischemic and mechanical trauma on this system as it relates pathogenetically to thrombosis and adhesion formation, and to explore techniques for restoration of lost fibrinolytic activity. Conditions of decreased fibrinolysis which occur after combat trauma might contribute significantly to increased mortality and morbidity by affecting vascular patency and wound healing conditions.							
24 (U) The presence of fibrinolytic activator activity in peritoneum and GI tissue, the effect of ischemic, mechanical and chemical trauma on this activity, the time course of return and the first identification of an inhibitor to fibrinolytic activity were described by use of an in vitro fibrin plate assay technique.							
25 (U) 74 07 - 75 06. Fibrinolytic activity is present within microscopic arteries and veins and normal GI tissue. The system is responsible for removal of fibrin deposition and the molding of fibrin deposits in the early stages of wound healing. Mechanical, chemical and ischemic trauma significantly reduces this activity and is related to microvascular thrombosis and abnormal wound healing. Exogenously administered activators of this system (urokinase or streptokinase) can restore lost activity and prevent complications of hypofibrinolysis. Preparation of tissue for autologous transplantation (vein grafts) may be also associated with decreased fibrinolytic activity and affect surgical results. Treatment of this tissue prior to grafting with activators of the fibrinolytic system can restore fibrinolytic activity and may avoid these complications.							
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.							

PII Redacted

<sup>a</sup> Available to contractors upon contractor's approval

DD FORM 1498  
1 MAR 68

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 201 Fibrinolysis in peripheral blood vessels

Investigators.

Principal: MAJ Alfred S. Gervin, MC

Associate: CPT Robert F. Buckman, Jr., MC

I. Microaggregates

A. Background and Statement of the Problem. Microaggregates composed of platelets and white blood cells in a fibrin matrix are formed during the storage of human blood. The infusion of large volumes of these microaggregates can produce pathologic changes in pulmonary structure and function and may be significant in the etiology of post-traumatic pulmonary insufficiency. In the experimental animal, the removal of microaggregates from aged blood can prevent these pathologic alterations. As demonstrated by Blaisdale, Mosely and MacNamara, post-transfusion pulmonary insufficiency is a significant cause of morbidity and mortality during combat trauma. Research has been directed toward elucidating the factors involved in microaggregate formation and in evaluating multiple techniques for the prevention of microaggregate formation or for the removal of microaggregates from aged blood.

B. Experimental Approach. Evaluation of microaggregates was undertaken using 3 techniques: a model T multichannel particle size analyzer, the screen filtration pressure (SFP) apparatus of Swank, and the weighing of filter grids before and after the passage of known volumes of blood. A comparison of these techniques revealed the Coulter counter to be the only reproducible quantitative method for evaluating microaggregates. The SFP and weighing of filters were unreliable, unreproducible and subject to multiple factors not involved with microaggregate volumes.

C. Results and Discussion.

1. Microaggregates in Stored Human Blood

Strict quantitation of microaggregates and the time course of the appearance of microaggregates in human blood anticoagulated in both ACD and CPD was undertaken. No microaggregate formation was noted for the first 7 days. Between 7 and 21 days a great volume of microaggregate formation occurred. From 21 to 28 days of storage smaller increases occurred. The volume of microaggregates in both anticoagulants after 28 days of storage were equal, the value being approximately  $1500 \pm 100 \mu^3 \times 10^3/\text{mm}^3$ .

The volume of microaggregates was noted to occur only in the buffy coat, with plasma and packed red blood cell fractions being virtually free of microaggregate concentrations. Additionally, packed red blood cells,

glycerol frozen red blood cells, and commercially prepared plasma and albumin fractions were all noted to be low in microaggregate content.

2. Evaluation of Blood Filters. Filters with the capability for removing microaggregates from stored blood are now commercially available. The following studies were undertaken to determine the microaggregate removing capacities of these filters under various situations of volume and flow. The 5 filters tested were a 40 micron grid filter, a packed dacron wool filter, a polyurethane mesh filter, a combination urethane mesh nylon wool filter, and a new complex grid dacron wool filter of composite design. Microaggregates were quantified in blood before and after passage through each of these filters so that the removal of microaggregates of different diameters could be calculated. Additionally, the microaggregate removing ability of these filters was tested when multiple units of blood were administered through each filter at high and low transfusion rates. The results of these studies suggest that the 40 micron grid filter effectively removes microaggregates for a full 5 unit transfusion. The dacron wool filters were noted, after approximately 1000 cc of transfusion, to release particles (unload) and therefore not be safe for use beyond this point. The polyurethane filter was noted to occlude routinely after approximately 1 unit of blood. The most effective filter was the composite grid dacron wool filter which effectively removed greater than 90% of particles for a full 5 unit transfusion at all flow rates.

3. Evaluation of Factors Involved in Microaggregate Formation. These studies suggest that the removal of platelets and white blood cells from fresh blood prior to storage prevents microaggregate formation. Additionally, the in vitro or in vivo treatment of blood with aspirin reduces by approximately 50% but does not prevent microaggregate formation. Treatment of blood, before or after storage, with activators of the fibrinolytic system (urokinase or streptokinase) likewise reduce but does not totally prevent microaggregate formation. The constant agitation of blood during storage reduces microaggregate formation by approximately 40% but causes significant red blood cell hemolysis and damage to blood components. After microaggregates have been formed, treatment with Arvin followed by routine filtration reduces microaggregate levels to immeasurable levels. Likewise, the centrifugation of blood for 5 minutes at 5000 rpm after storage followed by routine filtration completely removes all microaggregate volumes. A study is now being undertaken to determine the effect of fibrinogen on microaggregate formation. The blood from patients before and after treatment with Arvin is being stored for 21 days. Arvin treatment reduces blood fibrinogen levels to 0 without altering other known blood coagulation or fibrinolytic components. The results of this study are incomplete as the storage is still in effect.

## II. Fibrinolysis

A. Background and Statement of the Problem. The fibrinolytic activator system is a cascading enzyme system which is found in tissues of mesothelial origin. Plasminogen, an inactive precursor molecule, is converted by activators contained within tissue and blood to plasmin, an active lytic endopeptidase which attacks and degrades fibrin and fibrinogen. This system is important in (1) maintenance of vascular patency by the removal of fibrin depositions which occur throughout daily activity and (2) in wound healing by molding and structuring the original fibrin deposits so that they can be absorbed and replaced by normal tissue. The absence of fibrinolysis in the vascular system is known to predispose to vascular thrombosis. Decreased levels of tissue activator may lead to hypertrophic wound healing and significant adhesion formation.

B. Experimental Approach. Fibrinolysis has been strictly quantitated in this laboratory by the perfection of the fibrin slide and plate techniques of Astrup and associates. Fibrinolytic activator is quantitated by the placement of tissues or euglobulin extracts of tissues on fibrin plates, with determination of the subsequent zones of lysis after 18 hours incubation at 37°C. Plasminogen is differentiated from plasmin activity by utilizing plates which had been heated at 80° for 15 minutes. Fibrin slides are utilized with a semi-quantitative localization of fibrinolytic activator activity. For the first time, a technique for the identification of inhibitors of the fibrinolytic system has been developed in this laboratory. The technique consists of placing biopsies of tissues suspected of possessing fibrinolytic inhibitors around a tissue biopsy possessing a known activator activity and measuring resultant zones of inhibition and lysis.

### C. Results and Discussion.

1. Blood Vessels. In previous work, fibrinolysis activity has been demonstrated in arteries and veins and to be decreased following traumatic injury of vessels. The time course of the return of fibrinolysis activity in blood vessels has also been reported. In this study, the effect of chemical trauma on fibrinolytic activity was determined. Numerous drugs (potassium chloride, Keflin) associated with clinical thrombophlebitis were placed in venous segments and were observed to significantly decrease vascular fibrinolytic activity. Thus, the intravenous infusion of these medications with subsequent decrease in fibrinolytic mechanisms may be a cause of thrombophlebitis.

2. Autologous saphenous vein is now a common graft material for bridging vascular defects. During the preparation of these grafts, distension is used to determine vascular integrity. In this study, the distension of veins during preparation for grafting significantly decreased fibrinolytic activity and may predispose to loss of vascular patency. The treatment of distended veins possessing low levels of fibrinolytic activity with urokinase restores vascular fibrinolytic activity and may prevent these complications.

3. Tissue Fibrinolytic Mechanisms. The abrasion and crushing of the rat cecum significantly decreased fibrinolytic activity. Values had returned to control level by 96 hours. The creation of ischemic patches within peritoneum significantly decreases fibrinolytic activity for greater than 96 hours. The periods during which fibrinolytic activity is depressed are significant intervals during which predisposition to adhesion formation occurs. Creation of peritoneal defects with suturing of the peritoneum was noted to decrease fibrinolytic activity. Peritoneal defects which are not closed maintained normal levels of fibrinolytic activity. This study firmly demonstrates that tight suturing of peritoneal defects causes significant decreases of fibrinolytic activity with subsequent adhesion formation. Leaving peritoneal defects open is associated with normal levels of fibrinolytic activity and no adhesion formation. Thus, the surgical principle that all peritoneal defects should be closed is subject to question from the point of view of adhesion formation.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 201 Fibrinolysis in peripheral blood vessels

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)336	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY DCTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8A. DISEASE INSTN <sup>f</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS	
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
9. NO./CODES <sup>g</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	61101A	3A161101A91C	00	202			
10. PRIMARY							
11. CONTRIBUTING							
12. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Antigenic Composition of Trypanosomes							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
002600 Biology 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER:				FISCAL YEAR		75	
C. TYPE:				CURRENCY		2.9	
D. KIND OF AWARD:				76		4.0	
E. AMOUNT:						90	
F. CUM. AMT.						90	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DOD # U.S. Academic Institution)			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				[REDACTED]			
23. KEYWORDS (Precede EACH with Security Classification Code) <sup>j</sup>				24. ASSOCIATE INVESTIGATORS			
(U) African trypanosomiasis; (U) Immunity; (U) Antigens;				NAME:			
(U) Protozoa; (U) Tropical Medicine; (U) Antibodies				NAME:			
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) The objective of this work unit is to characterize the immune mechanisms involved in the host response to the trypanosomes, to characterize the antigens responsible for induction of immunity, and to investigate the feasibility of immunoprophylaxis against these militarily important diseases.							
24 (U) The approach is to develop systems in experimental animals and in culture for measurement of the effects of immune stimulation and effectors on the development of these diseases. Emphasis is on an analysis of the composition of the antigens involved in the induction of immunity.							
25 (U) 74 07 - 75 06 Study of cytotoxic antibody from rats immunized with irradiated African trypanosomes by an in vitro method has continued. It has been shown that the cytotoxic activity requires a heat labile serum factor or factors (probably complement components), but apparently not complement component 4. Antigen can absorb the cytotoxic activity from serum. The activity rises promptly after immunization but also promptly falls to lower levels. Cell transfer of immunity to murine trypanosomes has been accomplished and is apparently associated primarily with B lymphocytes. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1974 to 30 June 1975.							

PII Redacted

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1 MAR 68

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 202 Antigenic composition of trypanosomes

Investigators.

Principal: LTC Carter L. Diggs, MC, M.D.

Associate: John Barbaro, Ph.D.; John Bussey; CPT Gary H. Campbell, MSC, Ph.D.; James Dillon, B.S.; SP5 Klaus Esser, B.S.; Barbara J. Flemmings, B.S.; Mary B. Gibbs, Ph.D.; LTC S.M. Phillips, MC, M.D.; D.T.O. Wong, Ph.D.

1. Complement requirement for immune serum mediated cytotoxicity to Trypanosoma rhodesiense in vitro.

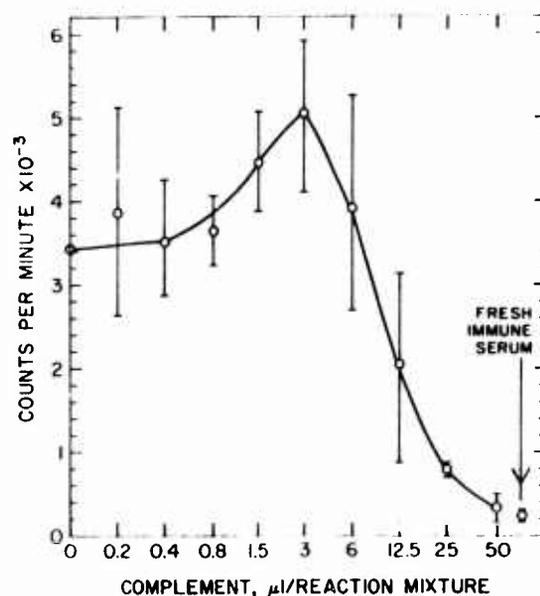
Objective. This project aims at an elucidation of the requirement for complement components in antibody mediated killing of trypanosomes.

Description. The basic technique used in these studies is the in vitro assay of serum mediated cytotoxicity as described previously. (See WRAIR Annual Report FY 1974). Although not proven, it is assumed that this reaction is mediated by antibody. The procedure consists of incubation of isolated organisms with immune or normal serum followed by pulsing with radiolabelling leucine. After an additional period of incubation, the amount of protein synthesized is estimated by scintillation counting of the incorporated leucine. Immune serum obtained from rats immunized with gamma irradiated parasites can abolish protein synthesis as measured in this assay. However, heating at 56°C for 30 min reverses this inhibition so that heated immune serum allows protein synthesis to proceed as well as it does in normal serum. The present studies were undertaken to elucidate the basis of this heat lability.

Progress. Fresh normal serum can completely restore the cytotoxic activity of heated immune serum. Figure 1 illustrates protein associated counts as a function of added fresh normal serum. There is a slight but reproducible enhancement of synthesis on addition of small amounts of fresh serum, followed by marked inhibition with increasing amounts. This inhibition culminates in counts indistinguishable from those observed with fresh immune serum. Either rat serum or guinea pig serum can be used as a source of the heat labile factors(s).

Additional studies have been conducted using serum from C4 deficient guinea pigs in an attempt to determine what serum constituents are required for the cytotoxic reaction. For these studies, immune serum was de complemented by absorption with sheep erythrocyte stromata sensitized with rabbit antibody. In preliminary studies, C4 deficient

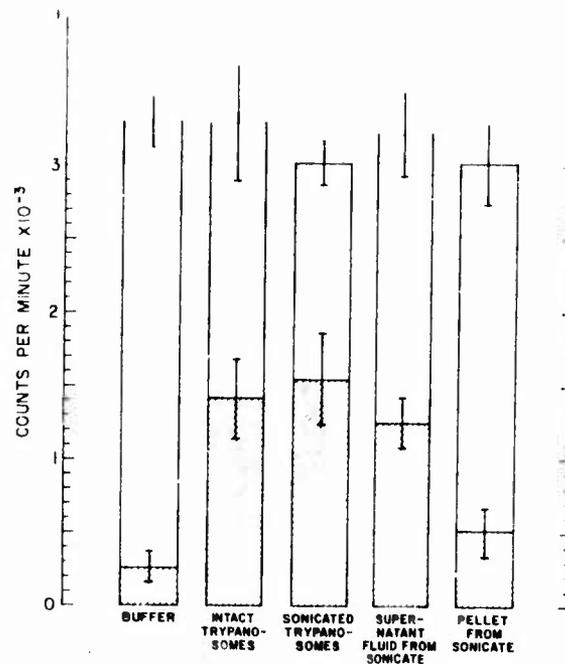
serum supported the cytotoxic reaction as well as did normal serum.



EFFECT OF SERUM COMPLEMENT ON IMMUNE SERUM  
MEDIATED INHIBITION OF T. RHODESIENSE PROTEIN SYNTHESIS

Fig. 1

Discussion. These studies indicate that the serum mediated cytotoxic reaction against trypanosomes requires heat labile normal serum constituents, probably components of the complement system. The activity of C4 deficient serum suggests that an alternate pathway of complement mediated cell damage is operative. Studies currently underway are aimed towards a confirmation of the C4 independence of the reaction and an exploration of the role of other serum factors, especially C1.



ABSORPTION OF ANTI-TRYPANOSOME IMMUNE SERUM ACTIVITY BY TRYPANOSOMES OR FRACTIONS THEREOF (SHADED BARS). CONTROL REACTIONS (UNSHADED BARS) PERFORMED WITH NONIMMUNE SERUM.

Fig. 2

## 2. Absorption by antigen of anti-T. rhodesiense cytotoxic activity.

Objective. These studies were designed to explore the ability of trypanosomes and extracts thereof to absorb cytotoxic activity from immune serum and to investigate the physical properties of the antigen(s) using absorptive capacity as a marker.

Description. Immune sera were incubated with intact or sonicated trypanosomes and separately with the supernatant fluid or pellet obtained after centrifugation of sonicated organisms. The serum was assayed for in vitro cytotoxicity as described in the previous section and in the WRAIR Annual Report, FY 1974. In other experiments, extracts of purified, lyophilized trypanosomes were used in a similar way.

Progress. Cytotoxic activity against trypanosomes was removed from immune serum by intact or sonicated organisms and by the supernatant fluid obtained after centrifugation of the sonicate (Figure 2); The pellet had no detectable ability to remove cytotoxic activity. The dose response relationship between amount of crude antigen used and protein synthesis was complex; some preparations were toxic in that they inhibited protein synthesis in the presence of normal serum. However in these cases, dilution of antigen resulted in loss of direct toxicity while the ability to absorb immune serum activity was retained (Table 1). Similarly, some preparations exhibited slight anti-complementary activity but only at concentrations greater than those at which absorption of serum activity could be demonstrated. Preliminary G-200 Sephadex chromatographic observations with two separate batches of antigen indicate that the protective antigen is retained to some extent with an elution pattern corresponding to a molecular weight of 100,000-250,000. Polyacrylamide gel electrophoresis of the crude antigen was reproducible from batch to batch and indicated a highly heterogeneous mixture of at least several dozen components.

Discussion. These results indicate that the in vitro trypanosome cytotoxicity assay can be used to monitor antigens. The antigen(s) concerned are not readily sedimentable and are thus probably not membrane bound. Alternatively, membrane fragments small enough to remain in suspension during centrifugation are created during sonication. This, however, seems unlikely since the antigenically active materials are not voided on G-200 sephadex. Presently, efforts are being made to improve absorption efficiency through the use of solid phase immunoadsorbents. Purification of the antigens by isoelectric focussing is envisaged.

### 3. Time course of production of anti-T. rhodesiense serum cytotoxic activity.

Objective: These experiments were designed to study the kinetics of production of anti-T. rhodesiense activity in the serum of rats immunized with gamma irradiated T. rhodesiense.

Description. Inbred CDF rats (The Charles River Breeding Laboratories) were injected with  $3 \times 10^8$  T. rhodesiense. (Wellcome strain) which had been irradiated with 70 krad from a  $^{60}\text{Co}$  source. The animals were bled at one week and a sub-group given a second infection of irradiated organisms. In a second experiment additional injections of immunogen were given. The sera were assayed for cytotoxic activity by the in vitro assay described above and in the WRAIR Annual report, FY 1974.

Progress. With a single immunizing dose, the cytotoxic antibody

Table 1

Dose dependence of absorption of anti-T. rhodesiense immune serum activity with antigen:

Microliters of Antigen	CPM + 95% confidence limits	
	In normal Serum	In Immune Serum
0	5880 <sub>±</sub> 720	1210 <sub>±</sub> 160
0.05	6260 <sub>±</sub> 500	1760 <sub>±</sub> 750
0.1	5860 <sub>±</sub> 760	1760 <sub>±</sub> 400
0.2	5890 <sub>±</sub> 320	2010 <sub>±</sub> 320
0.4	5660 <sub>±</sub> 810	2730 <sub>±</sub> 355
0.8	5720 <sub>±</sub> 560	5900 <sub>±</sub> 1490
1.6	5410 <sub>±</sub> 470	5580 <sub>±</sub> 1410
3.1	4490 <sub>±</sub> 540	4730 <sub>±</sub> 1040
6.3	3520 <sub>±</sub> 200	3850 <sub>±</sub> 920
12.5	2600 <sub>±</sub> 80	2260 <sub>±</sub> 310
25	1560 <sub>±</sub> 220	1610 <sub>±</sub> 250

Table 2

Cytotoxic Antibody Response in Rats Immunized With Irradiated *T. rhodesiense*: one and two dose regimens

Days after immunization	Relative potency, mean $\pm$ 95% confidence limits	
	Single dose regimen	Two dose regimen
7	1.2 $\pm$ 0.3	
16	0.5 $\pm$ 0.2	1.2 $\pm$ 0.3
29	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
36	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1
43	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
50	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
57	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1
73	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1
86	0.06 $\pm$ 0.04	0.04 $\pm$ 0.03

response rises quickly to a maximum at one week (Table 2) and falls thereafter. A second injection sustains the activity temporarily, but sera from animals so treated also has relatively lower activities by the third week after immunization. Further, additional booster injections (Table 3) do not appear to sustain the activity. Activity is also apparently unaffected by challenge with virulent organisms.

Discussion. Although a fall in activity occurs in both series presented, it should be emphasized that the minimal residual activity observed represents very effective anti-trypanosomal activity. The reference serum was obtained after prolonged immunization, but with a rest period for several months prior to the final immunizing infection; it is possible that the large amount of antigen given over a relatively short time interval to the donors of the serum studied here was supra-optimal. Equally likely are changes in immunoglobulin class and anti-

Table 3

Cytotoxic Antibody Response in Rats Immunized With Four Doses of Irradiated T. rhodesiense: Lack of Effect of Challenge.

Relative potency, mean  $\pm$  95% confidence limits

Days after immunization	Immunized only	Immunized and challenged on day 35
6	0.5 $\pm$ 0.1	_____
14	0.8 $\pm$ 0.5	_____
21	0.4 $\pm$ 0.2	_____
28	0.3 $\pm$ 0.2	_____
35	0.2 $\pm$ 0.2	_____
42	0.2 $\pm$ 0.2	0.3 $\pm$ 0.2
49	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2
82	0.02 $\pm$ 0.04	0.2 $\pm$ 0.3

body affinity as well as in actual antibody concentration which influence the potency of the serum.

#### 4. Immediate Hypersensitivity reactions in Trypanosomiasis.

Immediate hypersensitivity reactions have been demonstrated in helminthic infections by many investigators, whereas to date, no one has demonstrated the production of IgE-like immunoglobulin from protozoan infections. Studies have started in collaboration with Medical Zoology, to determine of rabbits infected with Trypanosoma rhodesiense are capable of evolving an immediate hypersensitivity reaction.

Previous investigation with Schistosoma mansoni established the

Table 4

Rabbit No.	days after Infection	Release from Leukocytes				Basophil Counts				Release from platelets		
		Total $\mu$ g Hist	Hist Released $\mu$ g	% Released	Before H. Release	After H. Release	% Hist	Total $\mu$ g Released	Hist Released $\mu$ g	% Released		
477	7	0.40	0.05	13.0	575,000	550,000	4.4	1.16	0	0		
	20	0.14	0.14	100.0	140,000	13,500	90.4	2.53	0.18	7.1		
	27	0.26	0.09	33.3	395,000	170,000	57.0	1.56	0.09	5.6		
	37	0.23	0.11	50.0	390,000	235,000	40.0	1.41	0	0		
482	Died											
	14	0.32	0	0	505,000	525,000	+3.9	2.50	0	0		
	22	0.25	0.02	7.7	457,500	397,500	13.1	2.29	0.02	<1.0		
	30	0.50	0.07	14.8	540,000	615,000	+13.9	1.24	0	0		
	Died											

condition in rabbits for demonstrating IgE-like immunoglobulin by the leukocyte-dependent histamine release reaction. Briefly, the reaction involved the reaction of specific antigen with basophil bound IgE immunoglobulin causing the basophil to degranulate and release its histamine. In addition, the basophils produced a platelet activating factor (PAF) which was capable of aggregating and releasing histamine from normal rabbit platelets. The in vitro histamine release was found to be a more reliable assay for the production of IgE antibody than the passive cutaneous anaphylactic reaction.

Three rabbits infected with 10,000 T. rhodesiense, strain 1886 were tested four weeks after infection by the in vitro histamine release reaction. Two of the three rabbits released better than 50% of the available histamine while a normal control rabbits released only 7%. This preliminary result suggested the feasibility of the method for detection of immediate-type hypersensitivity reaction from trypanosome infected rabbits.

The first series of experiments were planned to determine the optimal time for demonstrating the in vitro histamine release reaction. Two rabbits were infected with 20,000 T. rhodesiense, strain 1886 and tested periodically until the animals died from the infection. Table 4 lists the results obtained from this experiment. It is apparent that the amount of PAF produced, as demonstrated by histamine release from normal platelets is limited. However, the little release that did occur was found approximately 20-27 days post infection. The results also demonstrate that histamine release and degranulation of basophils occurs and tends to persist until the death of the rabbit.

The problems associated with the demonstration of IgE sensitization of basophil is rendered difficult by the antigenic variation known to occur. One is never quite sure that the antigen used is specific for the immunoglobulin and that negative results indicate no antibody. Future experiments planned are the sensitizing of rabbits with irradiated variants, determining whether in vitro leucocyte dependent release does occur and then establishing that the reaction is mediated by IgE immunoglobulin.

##### 5. Antigenic variation in Trypanosoma rhodesiense.

Objective: These studies were designed to investigate the biological relationship of serological variants of T. rhodesiense to each other as well as to the host during infection.

Description: Infection of Rhesus monkeys with T. rhodesiense results in a series of peaks of parasitemia which have their counterpart in human infection. The immune response of the animal seems frustrated because when a high parasitemia is brought under control by immune pres-

sure, presumably antibody, then another peak of parasitemia arises. This next peak appears to be resistant to any antibody that has preceded it. When new antibody arises and controls the second peak, then a third variant population begins to peak. This process continues until the animal dies through damage by sequelae of the immunological process itself.

This antigenic variation has also frustrated attempts at vaccination. Various products of trypanosomes have been successfully used to immunize animals against a particular variant. Although immunization is not successful, against a heterologous variant, the possibility of multivalent vaccine does exist. To assess the potential of a multipotent vaccine more must be known about the number, appearance and serological relationship between trypanosome variants appearing during the course of infection.

This study was designed to provide information about the variant specific response of a Rhesus monkey to infection with 10,000 *T. rhodesiense* organisms of the Wellcome strain. Blood was collected and frozen in 10% glycerol at -70C to preserve variant trypanosome populations. Sera for antibody determinations was frozen at -70C until use. Antibody titers were determined against the various variant populations by the *in vitro* neutralization test and the antibody mediated cytotoxicity assay as described in the 1974 annual report. Organisms were recovered from monkey blood by infecting ICR mice. Organisms were separated from the mouse red blood cells on DEAE 52 columns. Mice were never infected for more than three days. When populations of only one variant type were needed, organisms were cloned by microscopically examining small drops of infected blood. Drops containing only one organism were injected into a mouse. After three days the blood was injected into two more mice. After an additional three days infected mice were sacrificed and the trypanosomes were frozen as cloned stabiliates.

Progress: Figure 3 shows the course of parasitemia in an infected Rhesus monkey. Organisms were isolated from peaks of parasitemia on days 7, 13, 21, and 27 after infection. Serum samples were taken on days 9, 14, 17, 24, and 28 for use in serological assays. All serum samples were tested against organisms isolated at various times after infection. The results of the *in vitro* neutralization test are shown in table 5. The general trend observed was that serum taken after the appearance of a variant population would neutralize that population. Serum obtained the appearance of a given population did not neutralize that population of antigenic variants. The only exception to this rule was the lack of neutralization of peak 2 organisms by serum 2. Serum 2 was not tested at a concentration higher than a 1/8 dilution.

The results of the antibody mediated cytotoxicity assay are shown

in table 6. The general trend again is seen that serum taken after the appearance of a variant population would neutralize that population. In contrast to the *in vitro* neutralization test, peak 2 organisms were neutralized by serum ?

### IN VITRO NEUTRALIZATION TEST

	SERUM SAMPLES			
	NS <sup>0</sup> (d0)	S1 (d10)	S2 (d14+17)	S3 (d24)
Wellcome (d0)	0 <sup>b</sup>	5	5	5
P1 (d7)	0	5	5	5
P2 (d13)	0	0	0	5
P3 (d21)	0	0	0	5

a Results From Assay of 1/8 Dilution of Serum  
 b Number of Mice Living 10 Days or Longer

Table 5. In vitro neutralization test.

Antibody Mediated Cytotoxicity Test

		SERUM SAMPLES					
		S1 (d10)	S2 (d14+17)	S3 (d24)	S4 (d28)	NS (d0)	RAT anti- Wellcome
Trypanosome isolates	Wellcome (d0)	+ <sup>a</sup>	+	+	+	0	+
	P1 (d7)	+	+	+	+	0	+
	P2 (d13)	0 <sup>b</sup>	+	+	+	0	0
	P3 (d21)	0	0	+	+	0	0
	P4 (d27)	0	0	0	+	0	0

+<sup>a</sup> Inhibition of Uptake of H<sup>3</sup> Leucine  
 0<sup>b</sup> No Inhibition of Uptake of H<sup>3</sup> Leucine

Table 6. Antibody mediated cytotoxicity test.

Parasitemia in a Rhesus Monkey  
 Infected with *T. rhodesiense*

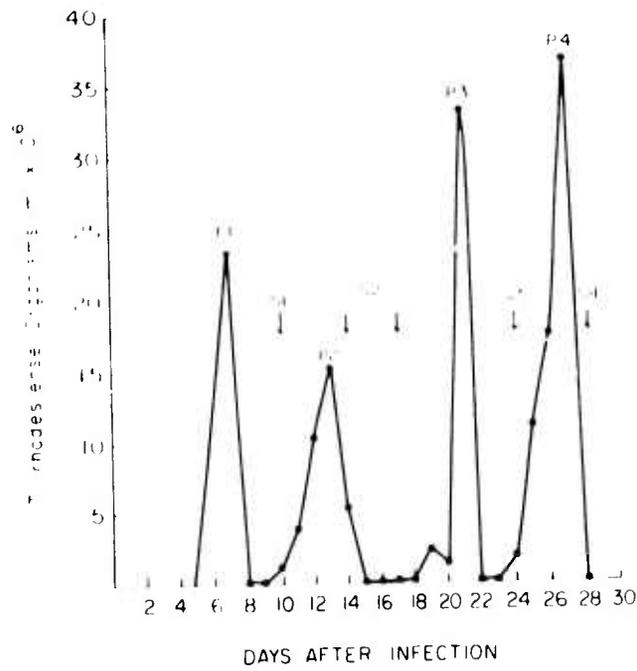


Figure 3. Course of parasitemia in a Rhesus monkey infected with *Trypanosoma rhodesiense*. (P) indicates collection of parasites for stabulates. (S) indicates collection of sera.

The antibody mediated cytotoxicity assay was used to determine the amounts of antibody occurring against the specific variants at different times during the infection. Figure 4 shows the time course of antibody against the variant populations. The animal was able to mount an antibody response to each new variant populations even several days after the first parasitemic peak. The antibody against each separate population of organisms fell with time but its rate did not appear to be affected by subsequent peaks of parasitemia.

These results stress the serological autonomy of the variant populations of organisms. To further investigate the autonomy of the parasitic peaks, mice were immunized with  $10^7$  irradiated organisms as shown by Duxbury in the 1974 annual report. The immunized mice were then challenged with 1,000 organisms from homologous or heterologous parasitemic isolates. Table 7 shows the day of 50% mortality in groups of 6 mice when challenged 12 or 43 days after single dose immunization.

Irradiated Wellcome and irradiated P4 protected very well against homologous challenge. Irradiated P2 and P3 showed slight protection against homologous challenge. In most instances heterologous challenge showed an unaltered rate of killing in immunized animals. The exception to this was the protection elicited in mice immunized with peak 3 organisms. Wellcome immunization did not protect against peak 3 challenge. Two explanations for this one way protection seemed likely. The possibility exists that both peak 3 and Wellcome organisms were presented in peak 3 immunization material. The second possibility exists that a population of organisms exists which contain two antigen (Wellcome and 3) which are important in inducing immunological protection.

To distinguish between the two possibilities, organisms from peak 3 were cloned by raising populations of organisms in three day mouse passage from microscopically selected single organisms. Three clones of organisms were obtained from twenty inoculations. Animals were immunized with irradiated clones and were challenged as before with homologous or heterologous populations of organisms. The results are seen in Table 8. From these results it is clear that the population of organisms originally isolated from the third peak of parasitemia in the Rhesus monkey contained organisms with both serological identity and non-identity with the original Wellcome inoculum. Two of the three classes from peak 3 were identical with the Wellcome organism.

The results of the immunization experiments not only stress serological autonomy of the variant organisms but indicate that populations may be made up of more than one serological variant.

Discussion: Infection with *I. rhodesiense* in a Rhesus monkey results in a series of well defined peaks of parasitemia. These variant populations of organisms are serologically distinct. The response of the host to each variant population also seemed to be distinct. These

VARIANT SPECIFIC ANTIBODY TITERS DURING  
T RHODESIENSE INFECTION

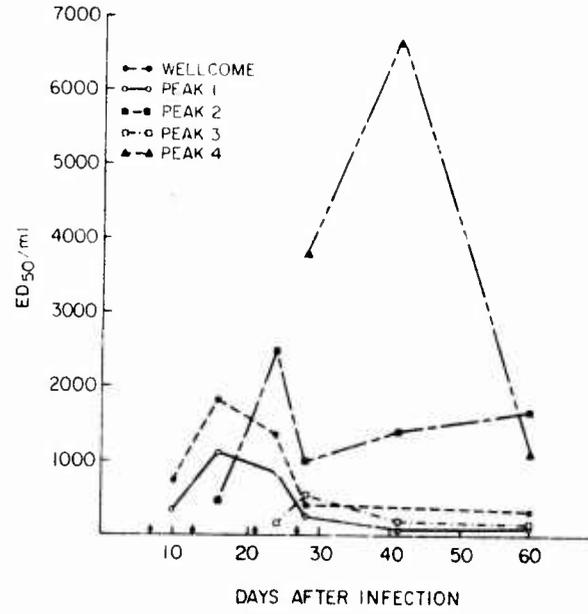


Figure 4. Time course of variant specific antibody titers using the antibody mediated cytotoxicity test.

Table 7

Variant Specific Immunity After Single Dose Immunization

Immunizing Treatment	Wellcome		Challenge Population (10 <sup>3</sup> organisms)			
	d12	d43	d12	d43	d12	d43
Normal (No Immunization)	5'	4.5	5	4.5	5.5	4.5
Wellcome	>30	>30	5	4.5	5	4.5
γP2	5	4.5	7	7	5	4.5
γP3	>30	>30	5	4.5	13	6
γP4	4.5	ND	4.5	ND	4.5	>30

<sup>1</sup> Values given are time in days after challenge for 50% mortality.

Table 8

Immunizing Treatment	Wellcome	P2	P3	CP3A <sup>2</sup>	CP3B	CP3C
Normal (No Immunization)	4.5 <sup>1</sup>	4.5	4.5	4.5	4.5	4.5
γWe11	>30	4.5	4.5	>30	>30	5
γP2	5	5.5	4.5	4.5	4.5	4.5
γP3	>30	3.5	>30	>30	>30	5
γCP3 A	>30	4.5	4.5	>30	>30	6
γCP3 B	>30	4.5	4.5	>30	>30	6
γCP3 C	4.5	5	4.5	4.5	4.5	>30

<sup>1</sup> Values given are time in days after challenge for 50% mortality

<sup>2</sup> CP3A, CP3B and CP3C are cloned variant populations.

findings emphasize the need for a multipotent vaccine. They only begin to provide answers to questions that would be useful to evaluating the potential success of such a vaccine.

Wellcome type organisms were found from blood taken during the third peak of parasitemia. This was unexpected since the serum of the monkey at that time contained anti-Wellcome antibodies. The possibility occurs that the Wellcome organism appeared during the few three day passages that were made to prepare the peak 3 organisms as immunization and challenge inocula.

The likelihood that this might occur is emphasized in a recent publication on new techniques for studying antigenic variation (1). These authors found spontaneous occurrence of variant organisms during three day passages in mice using fluorescent labeled monospecific antisera prepared against clones isolates of organisms of different antigenic types. The findings in the article emphasize the necessity for using clones populations or monospecific antisera when studying antigenic variation.

New information about the numbers and occurrence of antigenic variants is necessary to evaluate the potential of a multipotent vaccine. Future efforts to study antigenic variation in experimental animals or in nature must make use of newly described techniques to provide the best possible interpretation of the findings.

#### 6. Mechanisms of immunity involved in protection against Trypanosoma rhodesiense.

Objective. These studies will attempt to define mechanisms of immunity induced by injections of irradiated Trypanosome rhodesiense organisms.

Description. Immunity to T. rhodesiense can be induced in experimental animals by injection of irradiated organisms. The mechanisms of immunity elicited by this procedure may provide insight into its potential use as a vaccine. Experiments were begun to describe the populations of cells involved in the immunity. An in vitro correlate of cell mediated immunity was first studied. The ability of a soluble antigen of T. rhodesiense to cause normal or immune spleen cells to undergo blastogenesis and incorporate tritiated thymidine was studied.

Progress. C57B1/6 mice were immunized with 10 irradiated trypanosomes of the Wellcome strain. Protection against challenge with 10<sup>9</sup> trypanosomes of a homologous antigenic type was present at three days and lasted for several weeks. Immunization with other variant populations was also possible.

Spleen cells from animals immunized against three variant populations of organisms, Wellcome, P2 and P3, (see above, this Annual Report), responded to soluble antigen of both homologous and heterologous origin. Normal spleen cells did not respond to similar concentrations of antigen. The blastogenic response was present by day 5 after immunization and lasted as long as 22 days. The peak response occurred between day 9 & 12 (Table 9). Subpopulations analysis using anti-theta and anti-light chain antisera revealed both a B cell and a T cell component involved in the antigen specific blastogenic response.

Table 9

Time Course of Blastogenic Response of Spleen Cells from Mice Immunized With Wellcome Organisms and Tested Against Antigens from Other Variant Populations.

Wellcome immune spleen cells tested with antigen from.	<u>Days after Immunization</u>						
	5	9	12	15	19	22	25
Wellcome <sup>1</sup>	9.6 <sup>2</sup>	19.	9.4	4.0	6.3	2.0	1.3
P2	11	13	11	2.6	4.2	2.4	1.3
P3	9.6	13	8.3	2.9	4.9	2.1	2.3

Normal spleen cells tested with antigen from:

Wellcome	1.7	0.9	1.0	1.4	2.5	1.3	1.7
----------	-----	-----	-----	-----	-----	-----	-----

1 a 100 µg/ml final concentration of each antigen was used (Lowry protein determination)

2 Stimulation Index =  $\frac{\text{CPM cells + Antigen}}{\text{CPM cells alone}}$

Discussion. The blastogenic response of immune spleen cells to soluble antigen of T. rhodesiense probably represents the response of immune spleen cells to many different antigens of the trypanosome. This is emphasized by the lack of variant specificity in the blastogenic response. A variant specific response could have been covered by the response to many other antigens contained in the organisms. A variant specific response is presumably a response to the antigens directly involved in induction of immunity in vivo. The variant specific response was shown in vivo using these variant populations of organisms (see this annual report).

Future effort will look for a variant specific blastogenic response using purified preparations of variant antigens. Additional approaches to mechanisms of immunity are the characterization of models of immune cell or serum transfer of immunity.

#### 7. Study of the volume heterogeneity of African trypanosome populations during the course of the disease.

Objective: To determine whether there is modification of trypanosome cell volume during the course of infection in Rhesus monkeys, and if so to obtain information relevant to its association with remission and antigenic variation.

Three morphological forms of Trypanosoma rhodesiense ("slender", "intermediate" and "stumpy") have been observed in the blood of infected mammals which appear to be related to the remissions and relapse characteristic of the disease. The cause of this polymorphism has not been established, however, it correlates with the antigenic variability of the trypanosomes. The theory has been advanced (2) that the "slender" forms are variants against which antibodies have not yet been produced by the host. These forms are responsible for multiplication of the parasite. "Stumpy" trypanosomes arise from the "slender" forms by the action of antibody. The findings of Duxbury, et al (3) of the Department of Medical Zoology, WRAIR, with the Wellcome strain of T. rhodesiense, monomorphic in rats, does not support this theory. The Wellcome strain of organisms produced the same cyclic relapsing disease and antigenic variants in monkeys as observed with polymorphic strains of the parasite. However, the Wellcome strain while monomorphic in rats, may be polymorphic in the monkey. On the other hand, morphologic changes may occur more rapidly with this strain than with other strains of T. rhodesiense. The current study using a combination of visual and electronic sizing technics has been designed to detect such changes should they occur in the Rhesus monkey.

Description: A female Rhesus monkey (P091) was inoculated intravenously with 10,000 T. rhodesiense (Wellcome strain) organisms obtained

from the stock rat strain. The course of the infection was monitored as follows. Beginning on the third day after infection, wet blood smears were prepared daily from ear pricks and examined microscopically for trypanosomes. Upon onset of parasitemia, 1 ml EDTA and 1 ml heparin anti-coagulated blood were obtained by venipuncture daily up to 29 days post-infection. An additional 1.5 ml of blood was collected for serum on Monday, Wednesday and Friday of each week. Serum samples were stored frozen and tested at the end of the collection period for antibodies to the original and antigenic variants which arose in the course of the infection (3). The battery of tests performed daily on each sample of EDTA blood was as follows: (1) Wet mount examined microscopically for the presence of the parasite; (2) Blood smear stained with Wright's for microscopic comparisons of trypanosome sizes; (3) Nile blue preparations made in duplicate for enumeration of trypanosomes in the blood by hemocytometry; (4) Hematocrit to monitor the anemia of the monkey. Whenever the parasitemia exceeded  $1.6 \times 10^6$  per ml blood, the heparinized blood sample was diluted with an equal volume of a phosphate-glucose-saline solution buffered at pH 8 and placed on a small cellulose column. The trypanosomes recovered in 0.5 ml of the effluent from the column were further diluted in a modified Eagle's solution (ISOTON) for volume size distribution analyses. The size distribution of trypanosome populations were determined using the Coulter ZBI Electronic Cell Counter and Channalyzer. Size distribution patterns displayed on the Channalyzer were recovered on an X - Y Plotter.

A second female Rhesus monkey (P225) was inoculated with 10,000 T. rhodesiense (EATRO 1886), a strain known to be polymorphic in rats and mice. No organisms were seen in wet mounts made from blood obtained by ear prick up to 15 days post-infection. Subsequently, a third female Rhesus (P931) infected with 50,000 T. rhodesiense (EATRO 1886) died from an overwhelming parasitemia 20 days after inoculation. Rhesus P225 was then reinfected with 20,000 of these organisms. In each case, the procedure used to monitor the course of the infection was essentially the same as that described for the first Rhesus (P091) monkey.

Progress: The concentration of T. rhodesiense (Wellcome strain) in the blood of Rhesus P091 after infection with 10,000 organisms is shown in Table 10 Figure 3. Four distinct waves of parasitemia were noted during the 28 day sampling period. The mode and range of the size distribution analyses in Table 1 indicate that there was no marked change in the volume size distribution of these trypanosome populations. This was confirmed by microscopic comparisons of the shapes and size of trypanosomes in stained blood smear preparations where no morphologic alterations were observed.

Table 11 shows the concentration and volume size distribution of T. rhodesiense, EATRO 1886 strain in the blood of rhesus P931 following

infection with 50,000 organisms. This high concentration of organisms was used because an earlier attempt to produce the disease in Rhesus P225 with 10,000 organisms of this strain had failed. As can be seen, the parasitemia was overwhelming in P931 causing death 20 days after infection during the second wave of parasitemia. Considerable aggregation of platelets was noted in the heparinized samples of blood collected from Rhesus P931. As these aggregates passed through the cellulose columns, there was considerable overlap in the platelet and trypanosome size distributions. For this reason, the range of trypanosome sizes is not presented in Table 11. However, the modes for the trypanosome populations in Table 11 are comparable to those for the Wellcome strain of the organism in Table 10. A striking difference between the EATRO 1886 and Wellcome strains is the plateau in volume sizes observed on the 10th and 16th days post-inoculation in Table 11 which is not seen in Table 10. Comparisons of the sizes of trypanosomes in stained blood smears did not reveal any marked differences in the morphology of the organisms in the 9 to 19 post-infection samples.

Table 12 shows the results of the re-infection of Rhesus monkey P225 with 20,000 *T. rhodesiense*, EATRO 1886 strain. These results were also disappointing in that only two cycles of parasitemia were realized before the demise of the monkey at 25 days post-infection. The mode for the initial populations in Table 3 suggests that between the 5th and 8th days post-infection, the predominating size was smaller than that of the organisms of the same strain in Rhesus P931 (Table 11) or of the Wellcome strain in Rhesus P091 (Table 1). As was seen with the EATRO 1886 strain of trypanosomes in Table 2, a plateau in volume size occurred on the 6th to 8th day and the 14th day post-infection in monkey P225 (Table 12). There was no discernible difference in sizes or shapes of organisms observed in the 5 to 24 day post-inoculation stained blood smear preparations.

Discussion: The results of the present study have shown that modification of trypanosome cell volume during the course of infection in the Rhesus monkey does not occur with the Wellcome strain. Had any rapid morphological changes occurred, it is not likely they would have gone undetected since observations were made every 24 hours during the first three cycles of parasitemia. Neutralization tests in mice using the sera collected 2 or 3 days after each remission have shown that four antibodies arose during the course of the infection in Rhesus P091. Since remissions did occur following production of antibody and without morphologic change in the organisms, it must be concluded that the morphological forms of *T. rhodesiense* reported in the literature are not caused by the presence of antibody in the host nor are they associated with the remission of this disease.

The results obtained with two Rhesus monkeys in Tables 11 and 12 suggests that the EATRO 1886 strain of trypanosomes may be more viru-

lent in the monkey than the Wellcome strain. Parasites appeared earlier and the levels of parasitemia were much higher in EATRO 1886 infected animals than was expected. The EATRO 1886 strain employed is polymorphic in mice and rats; however, no such changes were observed in the monkey. Although the sera from the two monkeys infected with EATRO 1886 strain trypanosomes have not as yet been tested for antibodies, it can be assumed that such antibodies were elicited. The absence of any morphological change and presence of antibody would lend support to the conclusion cited earlier that morphological alterations in trypanosomes are not the result of the action of antibody. The underlying cause of this phenomenon must lie elsewhere.

Table 10

Concentration and Volume Size Distribution\* of *T. rhodesiense* (Wellcome Strain) in the Blood.\*\*\* of Rhesus Monkey 091 following Infection With 10,000 Organisms.

Day after infection	Organisms/ml blood ( $\times 10^6$ )	Volume ( $\mu^3$ ) Size Distribution	
		Mode	Ranges
4-6	None seen***		
7	23	16.1	(9.3-41)
8	None seen		
9	None seen		
10	1	****	****
11	2.7	****	****
12	10.5	18.5	(10-41)
13	16.0	17.6	(10-41)
14	5.2	15.9	(10-41)
15	None seen		
16	None seen		
17	<0.01	****	****
18	0.11	****	****
19	2.4	****	****
20	1.3	****	****
21	3.3	18.8	(10-41)
22	None seen		
23	None seen		

\*= Size analysis made of trypanosomes separated from whole blood by cellulose column fractionation.

\*\*= The preinfection hematocrit was 35%. By 28 - days post-infection the hematocrit had declined to 22%.

\*\*\*= None seen microscopically in 70 hpf of wet blood mount.

\*\*\*\*= Concentration of trypanosomes recovered from cellulose column too low for size analysis.

Table 10 Continued

Day after infection	Organisms/ml blood (x10 <sup>6</sup> )	Volume ( $\mu^3$ ) Size Distribution	
		Mode	Ranges
24	1.5	****	****
25	11.9	20.2	(11-47)
26	18.0	16.4	(9.4-38)
27	37.0	18.8	(10-39)
28	None seen	_____	_____

Table 11

Concentration and Volume Size Distribution of *T. rhodesiense* (EATRO 1886) in the Blood\*\* of Rhesus Monkey P931 Following Infected With 50,000 Organisms.

Day after infection	Organisms/ml Blood (X10 <sup>6</sup> )	Volume ( $\mu^3$ ) Size Distribution****	
		Mode	Ranges
4	None seen***		
5	2	Done	_____
6	16	20.54	(7-50 $\mu$ )
7	38	19.2	
8	82	20.5	
9	152	15.6	
10	202	15.5	(Plateau from 5.9 - 19.3)
11	108	20.1	
12	73	17.6 - 22.9	(Bi-model)
13	88	17.6	
14	83	20	
15	Not done		
16	92	1.7	(plateau from 12.7 - 20.0)
17	128	16.8	
18	285	15.9	
19	2,300	14.7	

Deceased

- \*= Size analysis made of Trypanosomes as cited in table 1.  
 \*\*= The preinfection hematocrit was 38% and declined to 15% 19-days post infection.  
 \*\*\*= See Table 1  
 \*\*\*\*= Platelet size distribution overlapped the size distribution of the smaller trypanosomes in the population. The volume of the largest trypanosomes varied from 23-28 $\mu^3$ .

Table 12

Concentration and Volume Size Distribution\* of *T. rhodesiense* (EATRO 1886) in Blood\*\* of Rhesus Monkey P225 following Infection With 20,000 Organisms\*\*\*

Day after infection	Organisms/ml Blood ( $\times 10^6$ )	Volume ( $\mu^3$ ) Size Distribution	
		Mode	Range
1-4	Not done		
5	92	12.6	41
6	106	113.5 Plateau (10.6 - 15.6)	41
7	142	11.9 (10.3 - 15)	41
8	145	15.1	41
9	14	****	****
10	Not done		
11	0.6	****	****
12	1.8	****	****
13	4.7	****	****
14	44.0	14.5 (10.6 - 16.5)	
15	64.0	21.4	(11 - 41)
16	86.0	22.0	(11 - 42)
17	6.2	****	****
18	4.2	****	****
19	20.0	Not done	
20 $\nabla$	Not done		
21	113	19.9	(10.2 - 42)
22 $\nabla\nabla$	167	20.7	(10.6 - 44)
23	130	Not done	
24	240	Not done	
25	Deceased		

- \*= Size analysis made as in Table I  
 \*\*= Preinfection that was 41% and declined to 14% at time of death  
 \*\*\*= Frozen stock organism passed twice in mice before inoculation  
 \*\*\*\*= See footnote Table I  
 $\nabla$ = Monkey dehydrated; 400 ml Ringer's lactate administered  
 $\nabla\nabla$ = Monkey infused with 25ml normal heparized normal Rhesus blood

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 202 Antigenic composition of trypanosomes

Literature Cited.

References:

1. Meirvenne, N.V., Janssens, P.G., and Magnus, E.: Antigenic Variation In Syringe Passaged Populations Of Trypanosoma (Trypanozoon) Brucei Ann Soc. belge Med trop., 55, 1-23, 1975.
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Publications:

1. Diggs, C., Dillon, J. and Flemmings, B.: Antibody Mediated Cytotoxicity to Trypanosoma rhodesiense; Requirement for a Heat Labile Serum Factor. Fed. Proc. 34: 1026, 1975.
2. Diggs, C.L., Langbehn, H.R., Schoenbechler, M.J., Wellde, B.T.: Trypanosoma rhodesiense: Variant Specificity of Immunity Induced by Irradiated Parasites. Exp. Parasitology 34: 125, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OC 6436	2. DATE OF SUMMARY 75 06 30	REPORT CONTROL SYMBOL DD-DR&R(A)434	
3. DATE PREV SUMMARY 74 07 01	4. KIND OF SUMMARY H. Term	5. SUMMARY SCTY U	6. WORK SECURITY U	7. REGRADING NA	8. DDDP'S INSTR' NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: a. PRIMARY 61101A		b. CONTRIBUTING 3A161101A91C		c. CONTRIBUTING 00		d. LEVEL OF SW A. WORK UNIT 203	
11. TITLE (Precede with Security Classification Code) (U) ADP Applications in Cardio-Vascular Stress							
12. SCIENTIFIC AND TECHNOLOGICAL AREA 016200 Stress Physiology							
13. START DATE 74 07		14. ESTIMATED COMPLETION DATE 75 06		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House	
17. CONTRACT/GRANT a. DATES/EFFECTIVE: NA b. NUMBER: c. TYPE: d. KIND OF AWARD:				18. RESOURCES ESTIMATE PREVIOUS FISCAL YEAR 74 75		19. PROFESSIONAL MAN YRS 0 2	
20. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research ADDRESS: Washington, DC 20012 RESPONSIBLE INDIVIDUAL NAME: Buescher, COL E.L. TELEPHONE: 202-576-3551				21. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Div of Medicine ADDRESS: Washington, DC 20012 PRINCIPAL INVESTIGATOR (Punish DOD if U.S. A candidate for Honorary) NAME: Olsson, COL R.A. TELEPHONE: 202-427-5123 SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:			
22. GENERAL USE Foreign intelligence not considered				23. PERFORMING ORGANIZATION NAME: DA			
24. KEYWORDS (Precede EACH with Security Classification Code) (U) Exercise; (U) Shock (U) Cardiovascular System; (U) Military Stresses; (U) Computer Processing							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Punish individual paragraphs identified by number. Precede rest of each with Security Classification Code.) 23. (U) Research is directed toward: (1) establishment of ADP systems for analysis of cardiovascular parameters, such as phasic blood pressure and flow and descriptions of cardiovascular function derived therefrom; (2) development of on-line analysis of selected variables for process control during studies of cardiovascular adjustments to exercise, shock and other stress of military importance. 24. (U) Interfacing PDP-8e computer with 5 Hewlett-Packard Physiological Data Acquisition systems, programming control module to digitize and tape signals simultaneously being recorded on photographic film, design of experiments to define events in the cardiac cycle and use of results in programming CDC 2600 for definitive data analysis. 25. (U) 74 07 - 75 06 After 9 months of efforts to program this computer to handle widely diversified physiological signal input, it is clear that its memory is too small for even adequate preprocessing. This project is terminated and the computer is to be laterally transferred to another Division of the Institute. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

PII Redacted

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1 MAR 68

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Project 3A16110(A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 203 ADP Applications in cardio-vascular stress

Investigators.

Principal: COL Ray A. Olsson, MC

Associate: CPT Clifford D. Stirba, MC

Description.

The work of the Department of Cardiorespiratory Diseases is largely studies of the hemodynamic responses to stress and disease in conscious instrumented experimental animals. Experimental data is usually collected as oscillographic recordings of instantaneous blood flow and blood pressure on photographic film. Analysis of these records is extremely time consuming; the records from 1 hour of experimentation require 15-20 hours to analyze "by hand," i.e. with ruler and polar planimeter. Even so, only about a quarter of the potentially useful information in these records is ever extracted, simply because data processing is so laborious. Studies in chronic instrumented preparations entail daily experiments for one to several months, this slow workup of data forces investigators to proceed with their experiments without clearly knowing the results of what has gone before. Automatic data processing seemed a natural solution to this problem, so several years ago we began planning a system for ADP of cardiovascular signals with the Division of Biometrics and Information Processing. On the basis of a defined need for a limited amount of on-line analysis and the requirement for extensive off-line detailed analysis, Division of Biometrics recommended pre-processing on a PDP-8/e and definitive reduction on the WRAIR CDC-3500.

Progress and Results.

A PDP-8/e was obtained and during this reporting period one officer and one enlisted man worked on developing the necessary program. It soon became apparent that the instrument lacked several expensive peripherals to facilitate program development, and procurement of these peripherals was halted by the mid-year fund curtailment. As a result of the requirement for extensive program interrupts, which was driven by the need to operate in real time, and the configuration of the CPU, the programming became unmanageably complex. The prospect of continued funding restrictions finally led to the decision to abandon this effort. The computer will be laterally transferred within the Institute.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL: DD-DR&E(AR)6J6	
3. DATE PREV JUMPR <sup>c</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>d</sup>	6. WORK SECURITY <sup>e</sup>	7. REGRADING <sup>f</sup>	8. DES'N INSTR <sup>g</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS		10. LEVEL OF SUM A. WORK UNIT
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		
10. NO./CODES: <sup>h</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER
A. PRIMARY		61101A		3A161101A91C		00		204
B. CONTRIBUTING								
C. CONTRIBUTING								
11. TITLE (Precede with Security Classification Code) <sup>i</sup>								
(U) Selective Breeding of Rats for Response to Drugs and Other Agents								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>j</sup>								
012600 Pharmacology 012900 Physiology 016200 Stress Physiology 016800 Toxicology								
13. START DATE <sup>k</sup>			14. ESTIMATED COMPLETION DATE <sup>l</sup>		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 07			CONT		DA		C. In-House	
17. CONTRACT/GRANT					18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:		NA			PRECEDENCE			
B. NUMBER <sup>m</sup>		EXPIRATION			FISCAL YEAR		25	
C. TYPE		4. AMOUNT			CURRENCY		0.1	
D. KIND OF AWARD:		F. CUM. AMT.			76		.2	
20. RESPONSIBLE DOD ORGANIZATION					21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research					NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012					Div of Neuropsychiatry			
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					SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE					ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered					NAME: Hawkins, T.D. DA			
					NAME:			
23. KEYWORDS (Precede EACH with Security Classification Code) <sup>n</sup> (U) Pharmacogenetics, (U) Alcohol, (U) Drugs, (U) Dependency, (U) Behavior, (U) Breeding, (U) Genetics, (U) Rats								
23. TECHNICAL OBJECTIVE, <sup>o</sup> 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23. (U) Experimental examination of the interrelationship between genetic, developmental, and environmental variables in determining behavioral response to drugs and other CNS active agents in order to elaborate biological mechanisms underlying military bioeffects problems.								
24. (U) Development of selectively bred lines of rats which exhibit differential response to alcohol and drugs of abuse and other CNS active agents. Concurrent development of behavioral methodology by which different breeding lines can be compared with one another and can be utilized in providing animal models of pharmacological effects of abuse and models of physical dependence.								
25. (U) 74 07 - 75 06. Previous title was as follows: Production of Alcohol Dependent Rats. Selective breeding of rat family lines was performed in three separate areas: selection for differential alcohol intakes, selection for differential barbital intakes, and selection for sensitivity to audiogenic seizure. Two different inbred lines of high-alcohol drinkers became more firmly established. Selection for barbital intakes resulted in two consistent filial generations of high and low drinkers. Audiogenic-seizure prone lines were developed successfully and were utilized in microwave bioeffect studies. Investigations of audiogenic-seizure sensitivity during alcohol and barbital withdrawal were performed in collaboration with the neurochemistry group, Division of Neuropsychiatry. Alcohol-addicted rats exhibited maximum seizure sensitivity at 8 hr into withdrawal. Barbital-addicted animals showed greatest sensitivity at 48 hr into withdrawal. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.								

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DD FORM 1498

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PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 204 Selective breeding of rats for response to drugs and other agents

Investigators.

Principal: Edward L. Hunt, B.A.

Associate: T. Daryl Hawkins, M.S.

Description.

Selective breeding of rat family lines continued during the FY 75 reporting period in three separate areas: selection for differential alcohol intakes, selection for differential barbital intakes, and selection for sensitivity to audiogenic seizure. Concurrently, additional studies were made of seizure sensitivity during alcohol and barbiturate withdrawal. These studies extended earlier observations of physical dependence in random bred rats.

Progress.

1. Selective breeding of specialized rat lines.

Work designed to produce rats having preference for alcohol has continued with the primary breeding colony at George Washington University. Earlier in the year, due to a major change in management at their animal facility, only moderate progress occurred, but breeding and testing rates increased considerably during the third and fourth quarters. Two different inbred lines of high-alcohol drinkers are now established. The progeny of one line now displays a marked preference for alcohol. Progeny of the second line exhibits a slightly lower preference for alcohol but appears to have greater fertility (larger litter sizes). Consideration is being given to crossing these lines for selection of animals having both characteristics.

The selection for breeding of animals having high and low barbiturate intake has resulted in two consistent filial generations. These rats will be particularly valuable in studies of the general acceptability of a variety of different drugs to determine if such animals, in turn, exhibit differential alcohol intakes.

Selective breeding for audiogenic-seizure proneness has resulted in the production of two filial generations of animals which exhibit

sensitivity. Parental stock was chosen from earlier alcohol addiction studies in which large individual differences in sensitivity to sound were noted. In contrast with litters from stock Walter Reed rats, which show extremely low sensitivity to sound, some litters from the current breeding lines exhibited a 50% incidence of wild running attacks and clonic-tonic seizures upon being subjected to repeated, 105 db, sound challenges, with testing started at three weeks of age. These rats will have general utility in studies of drugs of abuse and other CNS active agents. They are being employed currently in microwave bioeffects studies (see report for Work Unit 057).

## 2. Seizure sensitivity during barbital and alcohol withdrawal.

In collaboration with the Neurochemistry group of the Department of Neuroendocrinology, Division of Neuropsychiatry, further experiments were performed investigating the time course of audiogenic-seizure proneness during both barbital and alcohol withdrawal. In contrast with the earlier work which employed repeated testing of the same subjects, the current work was performed with independent animal groups, each of which was tested at a different time in the withdrawal period. Alcohol addicted rats exhibited maximum seizure sensitivity at 8 hr following removal of the alcohol drinking solution. Barbital addicted animals however, exhibited maximum sensitivity at 48 hr. Also, the threshold sound intensity for the most sensitive group of barbital rats was approximately 5 db lower than that for the most sensitive group of alcohol addicted rats. These observations should be of use in designing experiments to investigate similarities and differences in neurological mechanisms involved in physical dependence on alcohol and barbiturates.

**PROJECT 3A161102B71P**  
**BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE**

**Task 01**  
**Biomedical Sciences**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OA 6431	75 07 01	DD-DR&E(AR)836	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8. DRG'S INSTN <sup>d</sup>	9a. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>e</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
b. PRIMARY	61102A	3A161102B71P	01	025			
d. CONTRIBUTING							
RESEARCHER	CARDS 114F						
11. TITLE (Precede with security Classification Code) <sup>b</sup>							
(U) Ecology and Control of Disease Vectors and Reservoirs							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>b</sup>							
002600 Biology 005900 Environmental Biology 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
54 09		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PERCENTAGE		b. FUNDS (in thousands)	
b. NUMBER: NA				FISCAL YEAR		75	
c. TYPE:				CURRENCY		5	
d. KIND OF AWARD:				76		60	
18. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Div of CD&I			
				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: Eldridge, LTC B.F.			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Ward, Dr. R.A.			
				NAME: Reinert, MAJ J.F.			
22. KEYWORDS (Precede each with security Classification Code)							
(U) Arboviruses; (U) Ecology; (U) Mosquitoes; (U) Disease Vectors; (U) Control; (U) Taxonomy							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Provide individual paragraphs identified by number. Precede text of each with security Classification Code.)							
23. (U) Studies emphasize control of vectors of arbovirus and parasitic diseases of military significance. Objectives are incrimination of vectors and understanding of host-parasite relationships initially, understanding of vector biology and disease transmission mechanisms ultimately in order to develop more effective control procedures							
24. (U) Invertebrate vectors and vertebrate reservoirs and hosts are collected in areas of known disease activity. Infection rates are determined, as are flight ranges, biological processes, such as pathogen transmission, flight physiology, and diapause are studied in the laboratory.							
25. (U) 74 07 - 75 06. The ecology of vertebrates which are potential hosts of California encephalitis viruses (Keystone and Jamestown Canyon subtypes) was studied. The eastern gray squirrel appears to be involved in Keystone subtype virus transmission by Aedes atlanticus mosquitoes, but direct evidence of the infection of mosquitoes by viremic vertebrates was not obtained. Parity studies of Aedes atlanticus females indicate that second blood meals are uncommon, thus limiting the role of vertebrates in providing virus infections to mosquitoes. Diapause in Aedes canadensis has been shown to be controlled by photoperiod. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

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DD FORM 1498 1 MAR 66

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Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 025 Ecology and control of disease vectors and reservoirs

#### Investigators

Principal: LTC Bruce F. Eldridge, MSC  
Associate: Ronald A. Ward, Ph.D.; Douglas M. Watts, Ph.D.;  
MAJ John F. Reinert, MSC; CPT John F. Burger, MSC;  
CPT James W. LeDuc, MSC;  
CPT John W. Taylor, MSC; Robert R. Pinger, Ph.D.;  
William Suyemoto, B.S.; Leroy Bell, B.S.; David E. Hayes;  
SSG Timothy C. Pugh; SP4 Ralph W. Martin; SP4 Michael E.  
Ferriss; SP4 Marjorie J. Quitania

#### Description

This task involves field and laboratory studies of the relationship between selected arthropods and various aspects of their natural environment, especially those aspects relating to certain pathogenic organisms, their hosts, and their reservoirs. Included are ecological and physiological studies on arthropods, studies of transmission mechanisms and the development of improved methods of control of arthropods of medical importance.

#### Progress

1. Ecology of arboviruses in the eastern coastal plains of the United States

a. Introduction. Investigations on the ecology of California encephalitis group viruses (CE) were continued. The discovery that KEY was transmitted transovarially in Aedes atlanticus raised the question of the requirement for a vertebrate reservoir and consequently, emphasis during the year was placed on the ecology of vertebrate animals which were potential reservoirs of Jamestown Canyon (JC) and Keystone (KEY) subtype viruses of CE. Studies were also conducted on the gonotrophic cycles of female Aedes atlanticus, the vector of KEY virus in our study area. These studies, plus related laboratory studies, were done to determine the degree of vector-vertebrate contact, and thus the opportunity for infection of the vector population from viremic vertebrates. For a detailed description of the Pocomoke Cypress Swamp Study Area (PCS) refer to Annual Progress Reports for previous years, especially for FY 70. This report covers field and laboratory data accumulated since last year's annual progress report. Virological aspects of this program are reported under Project 3A061102B71Q.

b. Collection of immature Aedes atlanticus. Aedes atlanticus larvae and pupae were collected from several different breeding sites in the upland area west of the Pocomoke Cypress Swamp (PCS) study area

during June, August and September of 1974. Larvae were also obtained from a breeding site located in southern Delaware on 9 August. Specimens were transported to the laboratory where they were identified and pooled for virus isolation studies. The number of specimens per pool ranged from 1 to 50; however, most pools consisted of 25 specimens. Larvae and pupae were stored at  $-70^{\circ}\text{C}$  for virus isolation studies. The collections of immature Aedes atlanticus are summarized in Table 1.

c. Collection of adult Aedes atlanticus. Adult female A. atlanticus were trapped with CDC miniature light traps, supplemented with dry ice, each day and night from 18 August through 7 September, 1974. Collections were made at 9 different sites in and adjacent to the PCS study area. Mosquitoes were identified on wet ice and stored in pools of 1 to 50 mosquitoes at  $-70^{\circ}\text{C}$  for virus isolation attempts. Ten females from each day's collection from 18 August through 1 September were saved and frozen in order to determine parity status of the mosquito population. These females were later thawed and dissected in normal saline. Ovaries were gently teased apart so that individual ovarioles could be examined under a compound microscope and the condition of parity determined. This was done as described in Detinova<sup>1</sup>. The results of the examination of ovaries of A. atlanticus females are shown in Fig. 2. All females trapped during the first four days after emergence of the first females were nulliparous. Uniparous females appeared on the 5th day and by the 13th day, all females dissected were uniparous. No multiparous individuals were identified. No females were available for dissection after the 13th day of collection, although a relatively small number of females were trapped on the 14th -- 19th day of the period of adult activity. Some of the females trapped during this period may have been multiparous.

d. Virus isolations from A. atlanticus. Except for slight modifications adult and immature mosquitoes were assayed for virus as described in the WRAIR Annual Progress Report for FY 1974. The volume of 4.0% bovine albumin phosphate buffered saline (baps used for pools containing 10 or more specimens was 2.0 ml, while pools containing fewer than 10 specimens received 1.0 ml. After trituration of specimens in Ten Broeck tissue grinders, they were centrifuged for 30 minutes at 3000 rpm at  $4^{\circ}\text{C}$ . Supernatant from each suspension was inoculated intracerebrally into 3 -- 5 day old mice, 0.03 ml per mouse. All mice showing signs of illness or that died during a 10 -- 12 day observation period were stored at  $-70^{\circ}\text{C}$  for virus isolation studies. After all adult and immature mosquitoes were assayed for virus, brain tissue was aspirated from mice that were saved during the study. The brain tissue was suspended in 4.0% baps such that the final concentration was 20%. This suspension was passaged intracerebrally into a litter of mice by members of the Department of Virology, WRAIR. Brain-baps suspensions from the second mouse passage are being employed in virus identification studies utilizing plaque reduction neutralization tests with KEY and JC virus hyperimmune mouse ascitic fluid. The neutralization tests are being performed by members of the Department of Virology according to methods described in FY 1974 WRAIR Annual Progress Report. The validity of virus isolates was considered by reinoculating

the original mosquito suspensions intracerebrally into mice, 0.03 ml per mouse. Attempts to isolate virus from 142 fourth stage larvae that were obtained in Delaware were unsuccessful. Also, virus was not isolated from 58 pools that contained 996 pupae that originated from the upland study area. However, 9 suspected KEY virus isolates were made from 423 pools that contained 9,902 A. atlanticus larvae collected in the upland study area. A list of possible KEY virus isolations obtained from A. atlanticus larvae and pertinent information is presented in Table 2. Although larvae and pupae were obtained from different breeding sites, most were collected from the same general area, thus a valid analysis of the geographical distribution of the virus isolates could not be made.

A total of 26 possible isolates from 339 pools that contained 12,667 female mosquitoes are being characterized and identified by members of the Department of Virology, WRAIR. Adult collection data and presumptive virus isolates are shown in Table 3. Figure 1 summarizes the adult female A. atlanticus trapped during 1974 and the number of KEY isolates from adult mosquitoes.

e. Experimental mosquito transmission of arboviruses. Evidence accumulated on the ecology of arboviruses enzootic in the Del Mar Va peninsula area have implicated the involvement of several mosquito species in natural transmission cycles. Eastern equine encephalitis (EEE) and western equine encephalitis (WEE) viruses have been isolated mainly from Culiseta melanura, and Keystone virus from Aedes atlanticus. The vector status for Jamestown Canyon (JC) virus has remained obscure due to the infrequent isolation of this virus from mosquitoes. While the evidence is strong that C. melanura and A. atlanticus are involved in the natural transmission cycle of EEE and WEE viruses and Key viruses respectively, investigation is needed to fulfill the requirements necessary to fully incriminate them as vectors. One criterion that has not been considered is the demonstration of transmission of these viruses under laboratory conditions. Furthermore, the potential vector capabilities have not been determined for these or other mosquito species that inhabit the Del Mar Va Peninsula. Studies are now in progress to determine the mosquito species capable of horizontal transmission of EEE, WEE, KEY and JC viruses. In addition, attempts will be made to demonstrate vertical transmission in those mosquito species found to be susceptible to infection with these viruses.

(1) Mosquito Colonization. One difficulty frequently encountered in assessing the vector competence of a given mosquito species is that of obtaining a source of mosquitoes. Several approaches have been used by others; however, there are no standard procedures for maintaining species that will not mate in captivity.

Attempts to establish self-mating colonies of A. atlanticus, A. canadensis, and C. melanura have been unsuccessful in our laboratory. A colony of C. melanura has been maintained at Yale University for several years. During November of 1974 larvae from this colony were

brought to the Department of Entomology, WRAIR in an attempt to start a colony. Very few of the larvae pupated and 90% of the adults that emerged were males. Mortality rate in larvae was very high which apparently was caused by fungal infection. A similar problem was experienced during the same time period with the colony maintained at Yale University.

Attempts to establish a colony of C. melanura from specimens collected in the PCS were initiated on 25 October 1974. Twenty-seven blood engorged and/or gravid females were obtained from decayed tree-root cavities located in the upland study area. These females were transferred to our insectary and placed in a 2-foot square screen cage containing an oviposition substrate and sugar water. A chicken was restrained and placed in the cage overnight at frequent intervals to serve as a source of blood. All egg rafts were removed daily and placed in a hatching medium.

Over 1 1/2 month period 20 egg rafts were deposited by the field collected C. melanura. Very few of these mosquitoes took a bloodmeal. Of the eggs laid, most were fertile. Larvae were reared on a mixture of dog chow and liver powder. Adults were placed in a 2-foot square cage that was provided with an oviposition substrate and sugar water. Chickens were made available nightly; however, feeding did not occur until 10 days after emergence of the mosquitoes. All egg rafts were transferred directly to a hatching medium.

A total of 7 egg rafts were laid by progeny originating from eggs of field collected C. melanura. However, only one egg raft was fertile. This egg raft yielded 94 larvae, most of which developed only to the 4th stage. The possibility that larvae were in diapause was considered; however, there was no evidence of a change in photoperiod or other environmental factors that might have terminated development of larvae.

A. atlanticus. -- Attempts to maintain this species in the laboratory by forced mating technique have been unsuccessful. The major problem appears to be due to low or the lack of sperm transfer to females. Of the females that couple with males, no more than 10 to 15% laid fertile eggs. A. atlanticus eggs hatch well in hay infusion broth and the larvae thrive on a mixture of dog chow and liver powder. The time required for development from egg to adults is very rapid as adults can be produced in 4 to 5 days at 80°F. Adults survive well and limited observations suggest them to live for more than 30 days in the laboratory.

A. infirmatus. -- This mosquito species is of interest because of its close resemblance to A. atlanticus. Furthermore, in the southern U.S. numerous isolations of KEY virus and trivittatus virus have been isolated from this species. Eggs of this species were obtained from soil samples taken from the upland study area. They were hatched in a 1:1000 nutrient broth - water mixture and larvae were reared on dog chow and liver powder. Unlike A. atlanticus, this mosquito species was easily inseminated during induced copulation. In some cases up to 90% of the females laid fertile eggs.

(2) Virus donor and recipient animals. Studies aimed at finding a virus source for infecting mosquitoes with KEY virus have been conducted primarily on hamsters. Virus was detected in blood specimens taken on days 2, 3 and 4 following subcutaneous inoculation with  $10^{5.0}$  SMLD<sub>50</sub>/ 0.03 ml of a second passage stock KEY virus. Virus titers averaged  $10^{1.4}$  on day 2,  $10^{2.4}$  on day 3 and  $10^{3.1}$  for day 3 post inoculation.

Suckling mice are being evaluated as an indicator animal for virus transmission by mosquitoes. Preliminary findings indicate that most Aedes species mosquitoes feed readily on mice. This, plus the fact that mice are highly susceptible to infection with these viruses make them suitable as indicator of virus transmission.

(3) Experimental transmission attempts with KEY virus. Attempts to infect A. atlanticus with KEY virus using hamsters as a virus donor have been unsuccessful. In two experiments, approximately 100 mosquitoes that originated from field collected eggs were allowed to feed on hamsters that had been inoculated subcutaneously with KEY virus. Blood specimens taken before and after mosquitoes fed on hamsters failed to produce illness in mice nor was virus recovered from any of the mosquitoes that fed on the hamsters.

The mosquitoes A. canadensis and A. infirmatus became infected after ingesting KEY virus in defibrinated blood from a membrane feeder. Transmission attempts to mice however, have been unsuccessful.

(4) Experimental transmission attempts with JC virus. A. canadensis became infected after ingestion of JC virus in defibrinated blood from a membrane feeder. On ingesting blood containing  $10^{4.0}$  SMLD<sub>50</sub>/0.03 ml of JC virus, 50% of the mosquitoes became infected and 20 - 30% transmitted a lethal dose of virus to mice. Further experiments have demonstrated the extrinsic incubation period for this virus, at 25°C, to be 8-9 days. At lower doses,  $10^{2.5}$  and  $10^{1.5}$  SMLD<sub>50</sub>/0.03 ml, infection rates were reduced considerably and no virus transmission to mice was unsuccessful. Experiments in progress suggest that JC virus is vertically transmitted by A. canadensis.

f. Vertebrate Hosts of KEY Virus. Ecological investigation conducted on the Del Mar Va peninsula suggest whitetail deer and gray squirrels are involved in the natural infectious cycle of KEY and JC viruses. In addition, cottontail rabbits have been implicated in the natural cycle of KEY virus in southern U.S.

Laboratory experiments are in progress to assess the role of gray squirrels as vertebrate host of KEY virus. Squirrels were provided by Dr. Vagn Flyger, Department of Natural Resources, University of Maryland. On arrival in the laboratory each animal was bled in order to determine the presence or absence of KEY virus neutralizing antibodies. Those animals found to be free of antibody were inoculated subcutaneously with  $10^{4.5}$  SMLD<sub>50</sub>/0.03 ml of second mouse brain passage KEY virus. At various

time intervals blood was obtained by intracardiac puncture and subsequently assayed for virus by intracerebral inoculation into suckling mice. The recovery of mouse lethal agents from blood of squirrels was confirmed as KEY virus in mouse neutralization test employing KEY hyperimmune rabbit serum.

As shown in Table 4 all animals inoculated became viremic. Virus was not detected in 1:10 blood-bpa dilutions at 24 hours post inoculation; however, traces of virus was recovered from blood of some animals at 48 hours post inoculation. Virus blood levels increased considerably by 72 and 96 hours post inoculation but had decreased below detectable levels in all but 3 squirrels by 96 hours post inoculation.

## 2. Bionomics of floodwater mosquitoes

a. The effect of photoperiod on the induction of diapause. Laboratory studies were initiated to determine the effects of photoperiod on diapause induction in floodwater mosquitoes. Data so far are available only for Aedes canadensis and Psorophora ferox, since there was insufficient Aedes atlanticus material available for study.

The first set of experiments was designed to determine whether embryonic diapause could be induced by subjecting embryonating eggs to a photoperiod in which the photophase was short (9 hours) and the scotophase, or dark period was long (15 hours). Eggs were obtained from artificially inseminated A. canadensis which had been reared in the laboratory at 25°C under a 15:9 (L:D) photoperiod. Eggs were collected within 24 hours of the time they were laid and divided in half, one half being placed in a 15:9 (L:D) photoperiod, the other in a 9:15 (L:D) photoperiod environment. Both environments were held at 25°C. After 10 days the eggs were flooded with a hatching medium (1:1000 Nutrient Broth), and the number of hatched larvae were counted. After 24 hours, the unhatched eggs were examined to determine the number of viable embryos remaining. The percent hatch was then determined for both treatments based on the number hatching and the total number of viable eggs. Results are presented in Table 5. A majority of the viable eggs which underwent embryonation at the 15:9 photoperiod hatched, whereas only 1 egg from the two lots which were held after 9:15 photoperiod hatched.

This experiment was duplicated with eggs of Psorophora ferox mosquitoes reared in the laboratory under the same conditions as mentioned above. Results of hatching experiments with eggs incubated at the two photoperiod regimes are presented in Table 6. Four replicates were run at 25°C. Photoperiod during embryonation appears to have little effect on the percent hatch.

The next set of experiments was aimed at determining the "critical photoperiod", or that photoperiod in which the photophase is just short enough to induce diapause in Aedes canadensis eggs. Although these experiments are still in progress, it appears that very little hatching

occurs at photoperiods with a light phase of less than 13 hours. This phenomenon is believed to be a safety mechanism which prevents eggs laid in the field in late fall from hatching until spring. Further experiments are being initiated to test this hypothesis.

In the third set of experiments, parental generation mosquitoes are being subjected to varying photoperiods and their eggs tested to determine if embryonic diapause can be induced through the parental generation. These experiments are still in progress. It appears, however, that there is a definite parental effect in the case of Psorophora ferox.

Preliminary Conclusions:

(1) The embryonating egg is a photosensitive stage in Aedes canadensis.

(2) Egg diapause in A. canadensis can be induced by subjecting embryonating eggs to photoperiods of less than 13 hours of light.

(3) Treatment of embryonating eggs with photoperiods with as little as 9 hours of light do not produce diapause in Psorophora ferox.

b. Effect of photoperiod on breaking of diapause of floodwater mosquito embryos. This work is a continuation of studies started and reported last year. Soil samples were collected in the field and divided into 7 approximately equal parts. One part was flooded immediately and the remaining 6 parts are subjected to 2 different photoperiods, 3 parts at L:D 16:8 and 3 at L:D 8:16 (or 15:9 and 9:15). A conditioning temperature of 25°C was used in both cases. One part from each photoperiod regime was flooded 7 days after collected, one part at 14 days, and the remaining part at 21 days. All larvae were removed 3 days after flooding and the samples were sieved for eggs. Results of collections made in March and May 1974 were presented in last year's Annual Progress Report. Since then, collections were made in September, November, and December, 1974. The results of all 5 collections and floodings are shown in Tables 7, 8, and 9 for Aedes atlanticus, A. canadensis, and Psorophora ferox, respectively.

In the case of Aedes atlanticus (Table 7) photoperiod does not appear to influence the hatching of eggs, regardless of when the collections were made. Conditioning at a temperature of 25°C does, however, appear to promote hatching, and the colder the time of year, the longer is the period of warming which seems to be needed to promote hatching. Also, samples collected in September, November, and December yielded eggs which hatched at a rate of 48-72%, even after 28 days of conditioning. This is probably because these eggs had been deposited in 1974, and a period of chilling is needed before hatching.

In the case of Aedes canadensis (Table 8), photoperiod is also of no consequence in promoting hatching. Also, in contrast to A. atlanticus,

a period of warming does not seem to have any effect on hatching. Eggs collected in March hatched immediately upon flooding. No A. canadensis were present in May samples, possibly because the entire brood had hatched. Eggs collected in September and November usually did not hatch even after long periods of conditioning. December eggs hatched at rates of 48-49% after varying periods of warming. September and November samples had not been chilled, whereas December samples had undergone some chilling. This probably accounts for the hatching results.

Psorophora ferox is, like A. atlanticus, a mosquito species with a generally southern geographical distribution. The results (Table 9) are somewhat intermediate between those for A. atlanticus and those for A. canadensis, a northern species. There is no photoperiod effect, and the duration of the warming period did not appear important for most samples, but a period of chilling does appear necessary. The December samples, however, differed from all others. No eggs hatched upon initial flooding, and the hatch rate gradually increased with the length of the conditioning period.

Our preliminary conclusions are:

(1) All 3 species deposit a certain proportion of eggs each year which will not hatch until they have undergone a period of chilling.

(2) Aedes atlanticus eggs must, in addition, undergo a long period of warming.

(3) Photoperiod has no effect on hatching of fully embryonated eggs.

(4) Psorophora ferox will hatch after chilling and a shorter period of warming than A. atlanticus, but longer than that required for A. canadensis.

(5) In the case of A. canadensis, only short photoperiod eggs enter diapause which can be broken only by chilling. Long photoperiod (summer) eggs presumably would hatch if flooded the same season as they were deposited, thus permitting a second generation.

(6) P. ferox eggs appear unresponsive to photoperiod during embryonation. Factors controlling their life cycle remain undiscovered.

3. Biosystematic Studies of Mosquitoes. Manuscripts have been completed for the following 3 subgenera of Aedes: Edwardsaedes, Indusius and Rhinokusea. The subgenus Edwardsaedes and its included species are re-described and completely illustrated. Many new specimens and additional data on distribution and biology were presented. The monotypic subgenus Indusius was redefined and compared to other subgenera of Aedes. The single included species, pulverulentus, has all known stages described and illustrated. This represents the first taxonomic treatment of the subgenus since 1934. Rhinokusea, a subgenus confined primarily to the coastal

regions of the Oriental region, was redefined and the 3 included species completely described and all stages fully illustrated. One new species was described and compared to the other 2 species. All 3 species of the subgenus are morphologically very similar and difficult to separate from each other, however, characters were outlined and keys presented for separation of all life stages.

A 249 page monograph of a new interpretation of the large, primarily Oriental, subgenus Verrallina of genus Aedes was published. All 93 included species were assigned to section and series categories. Seven new species were completely described, lectotypes for 5 species were designated, and 9 species received changes in their taxonomic status. Fifty-eight species received taxonomic description. Sixty-six plates of illustrations of species were included. Considerable new information on biology, distribution and corrections to published species was presented.

Work is continuing on a comparative study of the female genitalia of the 37 subgenera of Aedes. Several large subgenera have proven to be extremely complex and when worked out have increased the value of the system being developed. Approximately 85% of the illustrations have been completed.

Taxonomic studies will be continued on the subgenera Paraedes and Aedimorphus of Aedes.

Identifications were made for several lots of mosquitoes submitted by the USDA.

A 6 week field collecting trip was made to the Amazon region of Brazil. Collections were made in the Belem, Maraba and Altamira areas and resulted in several hundred individually reared mosquitoes with their associated immature skins. The collections represented 14 culicid genera and over 57 species. A number of additional species are represented by species groups and complexes which require additional taxonomic treatment. The resulting specimens from this trip from the nucleus for future taxonomic studies of the area and will be used in providing direct taxonomic support for the US Army Medical Research and Development team stationed in Brazil.

The subgenus Christophersiomyia of Aedes was revised. Descriptions, illustrations and keys were prepared for the adults, pupae and larvae of the 5 included species.

In connection with arbovirus studies conducted by Dr. William Scherer, Cornell University Medical College, a collection of 2,800 mosquitoes were identified from the southeastern Pacific coast of Guatemala. The material submitted included samples collected by CDC light traps with and without CO<sub>2</sub> bait from areas which contained VE endemic and former VE epizootic sites. All specimens were collected during August 1973.

The most abundant species collected were: Culex (Culex) spp. (1,096), C.(C.) no. nigripalpus (408), Mansonia titillans (385), C.(C.) no. corniger

(293), Aedes taeniorhynchus (270), C. (Melanoconion) spp. (173), Aedeomyia squamipennis (74), Coquillettidia nigricans (62), Deinocerites pseudus (16), Uranotaenia lowii (12) and Aedes angustivittatus (19). A further breakdown of the Culex (Culex) species could not be made due to the lack of associated males or immature stages. It is worth noting that the above collections did include several important vectors of VE.

#### Conclusions and recommendations:

1. Although it is possible to maintain mosquitoes in the laboratory by artificial insemination, this technique has not been satisfactory for providing adequate numbers of A. atlanticus for virus transmission experiments. Further studies will be conducted in an attempt to colonize this species. In addition, field collected females will be allowed to oviposit in the laboratory to provide eggs for virus transmission studies.
2. The repeated isolation of KEY virus from A. atlanticus adults and immatures is consistent with previous findings and strengthen evidence that this mosquito species can serve as a year round maintenance host for this virus.
3. The discovery that KEY virus is vertically transmitted in A. atlanticus has prompted several questions that must be addressed in order to understand the role of this transmission route in the ecology of KEY virus. One, and perhaps the most important question that will be considered during the coming years is whether or not this mode of virus transmission above is efficient enough to allow the virus to persist continuously in nature.
4. Ecological investigation indicate that 2 CE group viruses are enzootic in Maryland. This information is based on studies conducted in the Del Mar Va peninsula that have dealt with selected mosquito species. Mosquito species that inhabit other areas of Maryland should be considered in order to better understand the distribution of viruses known to circulate in the Del Mar Va peninsula and to establish whether or not other CE group viruses are enzootic in Maryland.
5. Gray squirrels were shown to be susceptible to infection with KEY virus as indicated by a viremia that was detected in all animals inoculated with the virus. Further investigation will be necessary to determine whether or not the magnitude of viremia is sufficient to infect mosquitoes, especially A. atlanticus.
6. Hamsters develop a viremia following inoculation with KEY virus, however, the variation in response in regard to magnitude and duration of the viremia makes them somewhat of a risk to use to infect mosquitoes. This becomes very critical when dealing with a mosquito species that is difficult to maintain in the laboratory. Consideration will be given to the use of a membrane feeder as the dose of a virus can be controlled and this method has been used effectively for studying other virus-mosquito relationships.

7. The factors responsible for controlling egg hatching in floodwater mosquitoes which are potential CE vectors are complex and variable among the species. Additional research must be conducted to determine:

- a. presence of embryonic diapause
- b. factors inducing diapause
- c. factors breaking diapause

8. Revisions of the subgenera of the important mosquito genus Aedes have been nearly completed for Southeast Asia.

TABLE 1

Aedes atlanticus larvae and pupae collected in the Pocomoke Cypress Swamp  
and in Delaware, 1974

No. of Pools for Virus Isolation	Life Cycle Stage	No. of Specimens
92	2nd instars	2050
15	2nd and 3rd instars	305
96	3rd instars	2198
102	3rd and 4th instars	2526
118	4th instars	2823
<u>58</u>	pupae	<u>996</u>
471		10,898

TABLE 2

KEY virus isolates from Aedes atlanticus larvae collected  
Pocomoke Cypress Swamp, 1974

Isolate No.	Life Cycle Stage	No/pool	Reisolation
L-104	4th stage larva	25	+
L-106	4th stage larva	14	+
L-110	3rd stage larva	30	-
L-115	3rd stage larva	25	+
L-117	3rd stage larva	16	-
L-138	2nd stage larva	25	+
L-146	2nd stage larva	25	+
L-156	2nd stage larva	25	+
L-160	2nd stage larva	25	+
L-263	4th stage larva	25	+
L-404	3rd & 4th stage larva	25	+

TABLE 3

1974 Aedes atlanticus light trap collections

Date	Traps	No. Females	Females/Trap	KEY isolates	Females/ Isolate
20 Aug	16	348	21.75		
21 "	16	661	41.31		
22 "	16	2484	155.25	6	414
23 "	16	1840	115.00	4	460
24 "	16	1304	81.50	1	1304
25 "	8	805	100.63	1	805
26 "	16	1009	63.06	4	252
27 "	16	891	55.69	3	297
28 "	16	607	37.94		
29 "	16	385	24.06		
30 "	0				
31 "	16	534	33.38	1	534
1 Sep	16	548	34.25	2	274
2 "	8	207	25.88		
3 "	16	310	19.38	1	310
4 "	16	166	10.38	1	166
5 "	16	129	8.06		
6 "	24	325	13.54	2	162
7 "	14	72	5.14		
Totals	264	12625	47.82	26	486

TABLE 4

Magnitude & duration of viremia in gray squirrel after subcutaneous inoculation with  $10^{4.5}$  SMLD<sub>50</sub>/0.03 ml of KEY virus

Gray Squirrel No.	Hours Post Inoculation				
	24	48	72	96	120
1	<u>1/</u>	-	1.9 <sup>2/</sup>	1.4	-
2	-	-	2.3	2.4	1.0
3	-	-	1.5	2.0	1.6
4	-	+ <sup>3/</sup>	1.5	2.0	-
5	-	+	3.1	2.6	+
6	-	+	+	2.5	1.5
7	-	-	1.7	1.9	-
8	-	+	1.6	1.5	-
9	-	+	-	-	-
10 (Controls)	-	-	-	-	-
11 (Controls)	-	-	-	-	-

1/ Virus not detected at  $10^{-1}$  dilution

2/ SMLD<sub>50</sub>/0.03 ml

3/ Trace of virus, less than half of mice died at  $10^{-1}$  dilution

TABLE 5

Effect of photoperiod exposure of embryonating eggs on hatching, Aedes canadensis

Lot No.	Photoperiod (L:D) 15:9			Photoperiod (L:D) 9:15		
	No. Eggs Viable	No. Eggs Hatching	Per Cent Hatching	No. eggs Viable	No. Eggs Hatching	Per Cent Hatching
1	210	193	91.9%	66	0	0
2	97	64	66.0	116	1	0.9

TABLE 6

Effect of photoperiod exposure of embryonating eggs on hatching, Psorophora ferox

Lot No.	Photoperiod (L:D) 15:9			Photoperiod (L:D) 9:15		
	No. Eggs Viable	No. Eggs Hatching	Per Cent Hatching	No. Eggs Viable	No. Eggs Hatching	Per Cent Hatching
1	161	101	62.7%	224	148	66.1%
2	82	26	31.7	56	20	35.7
3	90	59	65.6	179	140	78.2
4	145	129	89.0	88	78	88.6

TABLE 7

Hatch rate<sup>1</sup> of Aedes atlanticus eggs in soil samples subjected to two photoperiod regimes for various periods of time

Collection Date	Photoperiod (L:D)	Day 7	Day 14	Day 21	Total Eggs
8 Mar 0/15(0) <sup>2</sup>	16:8	43/96(45)	84/93 (90)	69/69 (100)	483
	8:16	34/94	62/64 (97)	66/67 (98)	
2 May 0/9(0)	16:8	21/24(91)	11/11/(100)	8/10(80)	68
	8:16	10/10(100)	7/8 (88)	5/5 (100)	
12 Sep 3/3(100)	16:8	7/21(33)	10/14 (71)	3/5 (60)	65
	8:16	3/8(38)	6/9 (67)	6/8 (75)	
4 Nov 0/23(0)	15:9	10/28 (36)	4/7 (57)	11/21 (52)	129
	9:15	2/24 (8)	9/23 (39)	17/26 (65)	
18 Dec 0/1 (0)	15:9	9/18 (50)	5/7 (71)	14/29 (48)	69
	9:15	0.5 (0)	1/6 (17)	3/4 (75)	

<sup>1</sup>No. Eggs hatching/ No. Eggs viable (% hatching)

<sup>2</sup>Hatch rate at initial flooding

TABLE 8

Hatch rate<sup>1</sup> of Aedes canadensis eggs in soil samples subjected to two photoperiod regimes for various periods of time

Collection Date	Photoperiod (L:D)	Day 7	Day 14	Day 21	Total Eggs
8 Mar 2/2 (100) <sup>2</sup>	16:8	6/6 (100)	30/30 (100)	6/6 (100)	97
	8:16	40/40 (100)	4/5 (80)	10/10 (100)	
2 May -	16:8	-	-	-	0
	8:16	-	-	-	
12 Sep 0/1 (0)	16:8	3/9 (33)	0/3 (0)	-	19
	8:16	0/3 (0)	0.3 (0)	0/1 (0)	
4 Nov -	15:9	1/2 (50)	0/1 (0)	0/1 (0)	13
	9:15	2/8 (25)	0/1 (0)	-	
18 Dec 0/9 (0)	15:9	9/10 (50)	26/36 (72)	14/29 (48)	148
	9:15	13/19 (68)	29/31 (94)	8/15 (53)	

<sup>1</sup>No. eggs hatching/No. eggs viable (% hatching)

<sup>2</sup>Hatch rate at initial flooding

TABLE 9

Hatch rate<sup>1</sup> of Psorophora ferox eggs in soil samples subjected to two photoperiod regimes for various periods of time

Collection Date	Photoperiod (L:D)	Day 7	Day 14	Day 21	Total Eggs
8 Mar	16:8	20/20 (100)	9/9 (100)	12/12 (100)	298
0.5 (0) <sup>2</sup>	8:16	151/155 (97)	45/45 (100)	55/57 (96)	
2 May	16:8	38/38 (100)	22/22 (100)	24/24 (100)	134
1/29 (4)	8:16	27/29 (93)	15/16 (94)	5/5 (100)	
12 Sep	15:9	1/31 (3)	0/8 (0)	2/6 (33)	90
1/2 (50)	9:15	6/16 (37)	12/26 (40)	0/3 (0)	
4 Nov	15:9	4/12 (33)	5/13 (38)	4/12 (33)	88
0/14 (0)	9:15	3/24 (12)	4/20 (20)	3/7 (43)	
18 Dec	15:9	20/56 (36)	69/80 (86)	83/85 (98)	367
0/46 (0)	9:15	17/38 (45)	55/62 (89)	46/46 (100)	

<sup>1</sup>No. eggs hatching/No. eggs viable (% hatching)

<sup>2</sup>Hatch rate at initial flooding

Caption for Figures 1 and 2, following:

Figure 1. (Upper). Number of female Aedes atlanticus captured in light traps supplemented with dry ice, combined day and night collections, and isolations of KEY virus isolations from mosquitoes, Pocomoke Cypress Swamp, 1974.

Figure 2. (Lower). Parity of female Aedes atlanticus mosquitoes captured in light traps, based on sample of 10 mosquitoes from each collection 20 August - 1 September, 1974, Pocomoke Cypress Swamp.

Figure 1

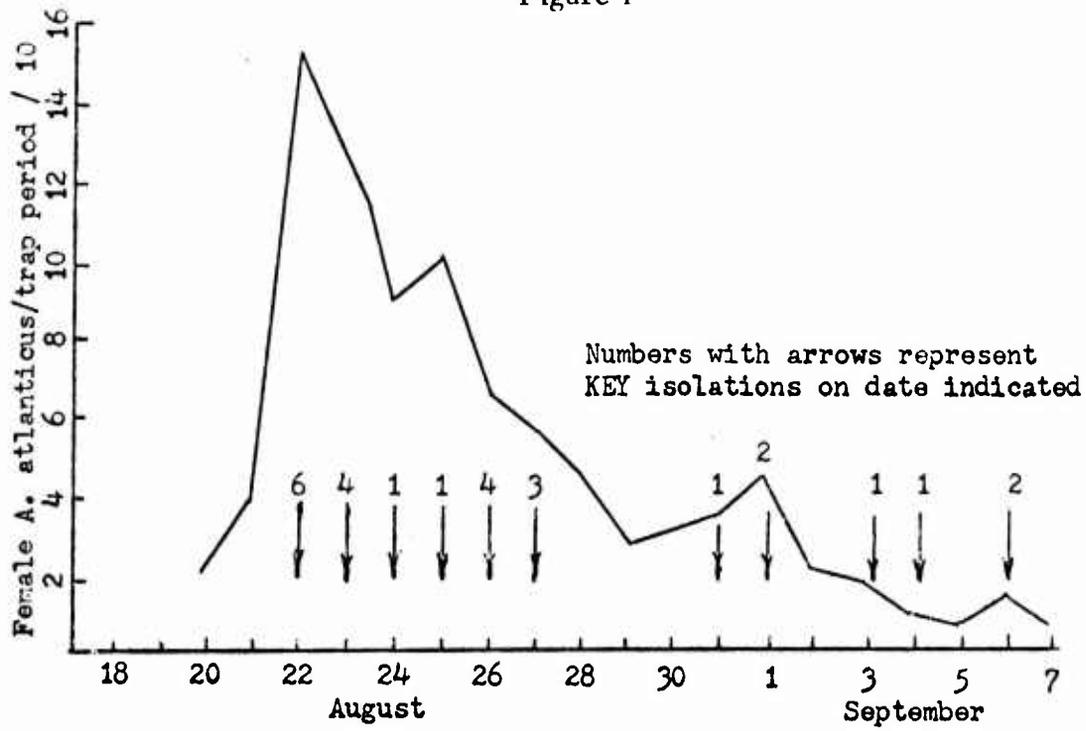
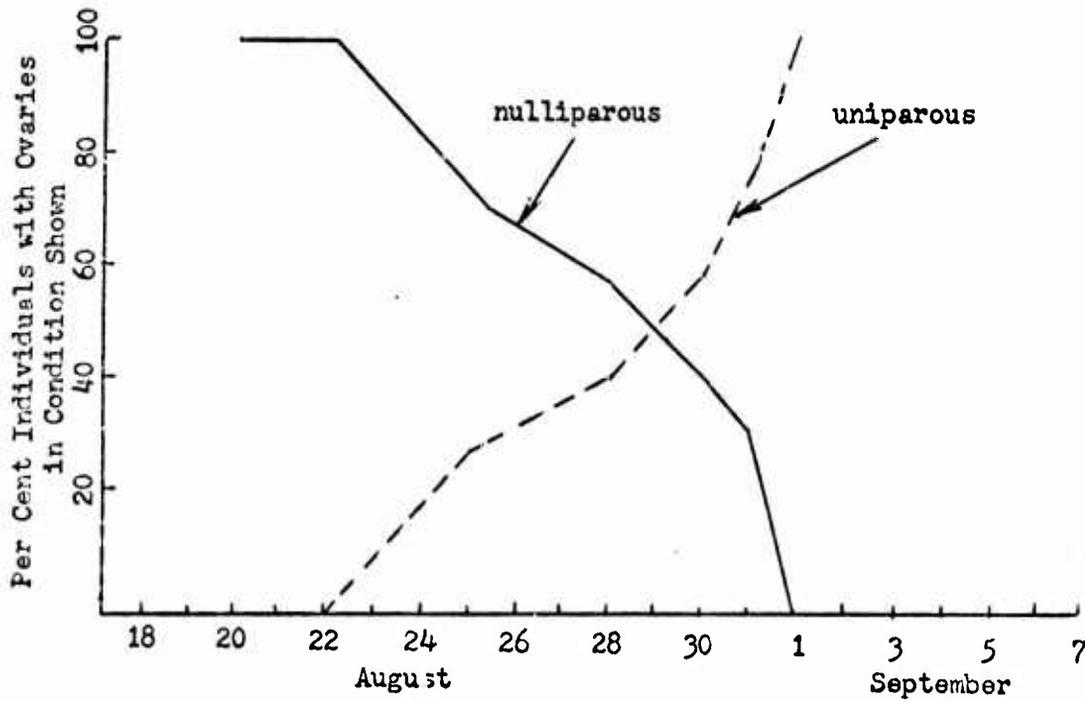


Figure 2



Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 025 Ecology and control of disease vectors and reservoirs

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OB 6453	75 07 01	DD-DR&E(AR)136	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY ACTY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRAS. NO <sup>c</sup>	8A. ORG'S INSTR <sup>c</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>d</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER
A. PRIMARY		61102A		3A161102B71F		01	074
B. CONTRIBUTING							
C. <del>XXXXXXXXXX</del>		CARDS 114F					
11. TITLE (Precede with Security Classification Code) <sup>e</sup>							
(U) Molecular Basis of Biological Regulation and Chemotherapeutic Drug Pharmacology							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>f</sup>							
002300 Biochemistry							
13. ST. FY DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. FUNDS (In \$ thousands)	
A. DATES/EFFECTIVE:		EXPIRATION:		PREVIOUS		PROFESSIONAL MAN YRS	
NA				75		6	
B. NUMBER <sup>g</sup>				FISCAL YEAR		100	
C. TYPE:		D. AMOUNT:		CURRENT		6	
				76		120	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Div of Medicine			
				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish GRA# if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: Hahn, F. E., PhD			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3657			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Krey, Anne K., M.S.			
				NAME: Wolfe, Alan D., PhD			
				DA			
22. KEYWORDS (Precede Each with Security Classification Code)							
(U) R-factors; (U) Bacterial Drug Resistance; (U) DNA; (U) Chemotherapy							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The scientific objective is to study the molecular pharmacology of elimination of R-factor-mediated bacterial multi-resistance to chemotherapeutic drugs. The ultimate objective is the development of clinical anti-R-factor drugs for the treatment of drug-resistant diarrheas and other resistant infections of prime military importance.							
24. (U) Approaches are fundamental laboratory investigations using advanced microbiological, biochemical and biophysical methods and concepts.							
25. (U) 74 07 - 75 06 R-factor R1-mediated ampicillin resistance is intrinsic, i.e. not the direct consequence of hydrolysis of the antibiotic by bacterial penicillinase. Cell-free studies on the replication of R-factor DNA have demonstrated the existence of three different DNA-polymerizing enzymes. Dissociation of the methyl green-DNA complex by R-factor eliminating compounds is proportional to their eliminating potencies. Hence, the nature of the methyl green-DNA complex and of its dissociation by drugs has been studied in detail. The antibiotic, distamycin A, which inhibits the bacterial transmission of R-factors is a template poison for poly dA and poly c <sup>+</sup> in cell-free experiments with RNA polymerase. An archetypical structure for chloramphenicol-type drugs has been recognized. Structural modifications may render such compounds refractory to R-factor-mediated resistance and less prone to give rise to aplastic anemia, a serious complication in chloramphenicol chemotherapy. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.							

PII Redacted

DD FORM 1498  
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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 67 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 074 Molecular basis of biological regulation and chemotherapeutic drug pharmacology

Investigators.

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Description.

Molecular biological and pharmacological as well as microbiological research studies with the objectives (1) of providing fundamental information on the replication and elimination of bacterial plasmids (R-factors) as a basis for developing anti-R-factor compounds for clinical use in man and (2) of elucidating modes of action of selected antimicrobial agents.

Progress and Results.

Attempts to eliminate resistance determinants from R-factor R1 in continuous culture of Escherichia coli R2. All in vitro studies on the chemical elimination of resistance determinants from bacterial R-factors in this laboratory as well as reported over the years in an extensive literature have used the identical experimental design: cultivation of R-factor containing bacteria in broth media overnight in the presence of non-growth-inhibitory concentrations of eliminating compounds, starting with a low bacterial titer ( $\sim 10^4$  bacteria per ml). This limits the experimental conditions arbitrarily to one cultural passage,  $\sim 18$  hrs cultivation time and maximally 20 bacterial doublings. Under these conditions, kinetics of elimination are difficult to determine because of the progressive growth of the test culture. We have made a computer simulation of the rate of R-factor elimination, assuming that per doubling time a constant percentage of R-factors will not be replicated but this does not take into account the possibility of reinfection of R<sup>-</sup> progeny bacteria with R-factor for which the probability will increase with increasing bacterial densities. In contrast, elimination experiments of R-factors from Salmonellae in mice in vivo by others have demonstrated complete restoration of chemotherapeutic drug sensitivity over a period of six days. These studies differ from the typical in vitro experimental design by accommodating a much longer experimental period and using a culture system which is not closed. We have tried, hence, to employ an in vitro system with similar characteristics in elimination studies, using a continuous culture apparatus (Bioflow

Model C 30, New Brunswick Scientific) which can be programmed to remove predetermined volumes of culture continuously and to replace them with fresh growth medium, thereby maintaining a constant bacterial density in the culture vessel.

Our very extensive experiments encountered serious technical difficulties and had to be discontinued without conclusive results. Even with extraordinarily high rates of culture withdrawal and medium replacement, it was not possible to stabilize the bacterial density at low titers which are required for elimination experiments. This remained the case even after redesigning the culture vessel to accommodate a smaller volume and reducing growth rates by lowering the incubation temperature. Elimination experiments with *E. coli* RS2 harboring R1 were, nevertheless, attempted but yielded scattered data from which no clearcut kinetic course could be derived. The scientific conceptualization underlying these experiments remains valid but their technical execution requires a pilot-plant scale fermenter. Others appear to have had similar ideas and perhaps technical difficulties. In 1970, Watanabe, one of the foremost R-factor researchers, announced the undertaking and eventual publication of R-factor elimination kinetic studies in continuous culture but no such paper has come out in print.

"Intrinsic" ampicillin resistance conferred upon *Salmonella typhimurium* by R-factor R1. We reported, in 1974, that *S. typhimurium* R1<sup>+</sup> is sterilized by combinations of 60 µg/ml of ampicillin plus each of several R-factor eliminating DNA intercalants. In serial dilution experiments, the R-factor-carrying *S. typhimurium* grew overnight in the presence of 2500 µg/ml of ampicillin! It is unlikely that this quantity of antibiotic can be hydrolyzed by the amounts of β-lactamase elaborated by (initially) a few thousand bacteria. When the viability of cultures of *S. typhimurium* R1<sup>+</sup> in medium containing 60 µg/ml of ampicillin was followed as a function of time, it decreased progressively by a factor of 40 but, by the sixth hour, growth resumed at the rate of a drug-free control culture. The amounts of ampicillin in the filtered experimental medium were determined by serial dilution bioassay, using a sensitive indicator strain of *Staphylococcus aureus*. The concentration of ampicillin in the medium remained constant for the first 4 hours and at 6 hrs, when exponential growth of *S. typhimurium* R1<sup>+</sup> resumed, was still 30 µg/ml. Only after 13 hrs was no active ampicillin left in the culture. It follows that escape from the bactericidal effect of ampicillin was not primarily a result of the enzymatic hydrolysis of the antibiotic and that the operational ampicillin resistance does not require the destruction of all active antibiotic in the experimental environment. The literature begins to show indications that intrinsic penicillin resistance, not mediated by enzymatic degradation of the antibiotic, is also being observed and studied in other laboratories. We have recently received a unique gift of <sup>35</sup>S-labelled ampicillin which

enables us to study the R-factor mediated ampicillin resistance phenomenon and the molecular events which attend the sterilization of bacteria by combinations of ampicillin and R-factor eliminating drugs.

Molecular biology of R-factor elimination. Short of a few reports that complete elimination of the genetic characteristics of plasmids from bacteria was accompanied by the absence of plasmidic DNA from these organisms, there exists little information on the molecular pharmacology of the elimination phenomenon or on the fate of the R-factors, especially after segregation of genetic determinants occurs. While intact plasmid DNAs of the same base composition as the chromosomal DNAs of their host bacteria can be separated by isopycnic centrifugation in the presence of propidium iodide which converts closed circular plasmid DNA into a more compact and, hence, denser form, any method for the separation of plasmidic and chromosomal DNAs which is based upon such conformational differences between these DNA species could potentially fail for incomplete plasmid DNAs, i.e. for biosynthetic intermediates or for residues after partial elimination of genetic determinants. We elected to study a system in which the chromosomal DNA (*E. coli*) has an AT/GC ratio of 1 and the plasmid DNA (from *Pseudomonas*) an AT/GC ratio of 0.5 so that the two species can be separated by isopycnic centrifugation on the basis of intrinsic density differences, regardless of molecular size, weight or conformation. Such an *E. coli* strain WR 110 R<sup>+</sup> and its isogenic parent R<sup>-</sup> have been acquired. The R-factor contains resistance determinants for carbenicillin, neomycin, kanamycin, ampicillin and tetracycline.

The antibiotic resistance properties of this strain were verified. The presence of the plasmid in this strain (and most importantly, the absence of "cryptic" plasmids) was also demonstrated. The isogenic non-plasmid-containing strain, W 3110, was carried through identical procedures as an R-factor-free control. Cultures grown overnight in Difco Penassay Broth (pH adjusted to 7.6 with additional K<sub>2</sub>HPO<sub>4</sub>) were added to 2 volumes of ice-cold absolute ethanol (to facilitate and make more complete the following lytic procedure), harvested by centrifugation and suspended in 25% sucrose in 0.05 M Tris-HCl at pH 8.0. The cells were gently lysed with lysozyme, ethylenediaminetetraacetic acid and sodium lauryl sulfate. The chromosomal DNA was removed from plasmid DNA by precipitation with 1.0 M sodium chloride. The existence of covalently closed circular plasmid DNA was shown by cesium chloride-ethidium bromide equilibrium density gradient centrifugation of the cleared lysate material. The organisms were also grown in the presence of <sup>14</sup>C-labeled or <sup>3</sup>F-labeled thymidine and the presence of radioactive-labeled plasmid DNA was also shown by assaying fractions of CsCl and/or CsCl-ethidium bromide gradients for radioactivity.

A micro method for plate counting and for replica plating was developed. Diluted suspensions of bacteria are filtered on sterile 0.45  $\mu$ m Millipore 47 mm disc filters which are then placed on a sterile absorbent pad impregnated with liquid culture medium and contained in a 50 X 12 mm Petri dish. After overnight incubation at 35 C, numbers of colonies are counted visually (the Artek bacterial colony counter may be adapted to permit automatic counting of these smaller Petri dishes). The filter disc with bacterial colonies on its surface can be used as a master copy for replica plating of additional 50 X 12 mm Petri dishes containing media-soaked absorbent pads and filter discs. The introductory technical phase of this work is completed and studies on the fate of R-factor DNA in elimination experiments are under way.

R-factor DNA biosynthesis in cell-free systems. A project undertaken to increase our understanding of R-factors by studying their replication in vitro has continued, with emphasis on both the isolation and purification of R-factor DNA, and of the enzymes and "gene products" involved in DNA replication.

In view of the continuous need for large quantities of intact R-factor DNA, a method for the isolation and purification of R-factor DNA was adapted<sup>1</sup> and developed by which milligram quantities of this DNA species are obtained. The method comprises sequential use of: (1) polyethylene glycol 6000 (PEG) to concentrate bacteria, (2) selective release from bacteria of R-factor DNA, a procedure which we have found to also release DNA replicating enzymes, (3) further use of PEG to concentrate the DNA and (4) separation of various DNA species by isopycnic centrifugation in CsCl-ethidium bromide (EBr) solutions. R-factors thus obtained have the property of stimulating DNA synthesis in crude bacterial extracts. (Reference 2) A similar procedure was developed independently and published by a group in England shortly after the completion of our experiments.

At least 12 different gene products have been implicated, e.g. in the replication of bacterial viruses<sup>3</sup> and gel electrophoresis of crude extracts supporting R-factor DNA replication in our experiments have revealed the presence of more than 15 different proteins. These components are now being extensively purified by ammonium sulfate precipitation and DEAE cellulose column chromatography; presently three different polymerizing activities have been observed. This distinction is based upon (1) the presence of polymerizing activity in three different gradient fractions of the effluent from the DEAE columns, and (2) the differential response of these fractions to KCl. Thus, the earliest eluting fraction exhibits relatively minor variation in activity upon variation in the concentration of KCl, while the second eluted activity possessed an absolute requirement for a high concentration of KCl. The third fraction or region replicates DNA optimally in low concentrations of KCl.

E. coli is known to possess three different DNA replicating enzymes which differ in their activity in the presence of KCl.<sup>4</sup>

Interaction of isolated R-factor DNA with intercalative eliminating drugs. While the conformational changes produced by intercalation in circular supercoiled DNA have been studied in several viral DNAs, we consider it necessary to reproduce and refine such studies using isolated pure R-factor DNAs. This requires, firstly a routine method of preparing R-factor DNA which we developed as reported above. Secondly, the usual method of collecting DNA fractions from the pierced bottom of centrifuge tubes after centrifugation to equilibrium can not be used because some material (RNA-Cs?) sediments to the bottom. A new fractionation device was developed which collects fractions from the top of centrifuge tubes. It consists of a plastic float through which a suction tube leads into the fluid underneath. While the underlying principle is simple enough, the development of a working device took some effort and was accomplished with the help of personnel of the Instrumentation Division, WRAIR. Thirdly, among the few methods for the determination of conformational changes produced in supercoiled DNA by intercalants (sedimentation analysis, viscometry, measurement of circular dichroism), viscometry is the method of choice for theoretical and practical reasons. This required the development of a viscometer with hitherto not attained precision in the establishment and maintenance of constant temperature and in the timing of the passage of a column of liquid between the two markers of the viscometer capillary. Through the introduction of a Neslab water bath and a Wescan temperature control unit, the sample temperature can now be regulated and maintained to within 0.005°C.

Viscosities are derived from determinations of flow times of DNA test solutions in a Cannon semimicro dilution viscometer model K 100. An accuracy of flow times of  $\pm 0.05$  seconds is obtained when their determination is carried out with a Heath-Schlumberger electronic timer model SM-102A, coupled to the Wescan optical monitoring system of our viscometer by an electronic interface which was designed and built at WRAIR. The detectors of the optical system are two photosensitive resistors (Clairex CL 904L) which monitor the start and stop of the timing operation, respectively; as common light source of the system serves an incandescent lamp (Chicago Miniature Lamp-works #2307) which is placed relative to the detectors in such a manner that light reaching these detectors is reflected by air or by the test solutions inside the capillary of the viscometer. A change in refractive index inside this capillary during flow of our test solutions past the detectors alters the amount of light reaching these photosensitive resistors; the resulting change in voltage drop across each photoresistor is amplified by an operational amplifier module (Teledyne Philbrick 1026), subsequently limited to  $\pm 5$  volts by a Zener diode and finally shaped by an integrated circuit analog

voltage comparator (Signetics 526) to the appropriate trigger requirements (TTL level) of, respectively, the start or stop signal of the electronic timer.

After technical problems concerning binding studies of eliminating drugs to R-factor DNAs have been solved, work is now underway to study the reactive behavior of our most active R-factor eliminators toward R-factor DNA in vitro.

Studies on the methyl green-DNA complex and its dissociation by drugs.  
We reported last year the discovery of a linear correlation between the ability of compounds to displace methyl green from its complex with DNA and their ability to eliminate the kanamycin resistance determinant from R-factor R1, carried by Salmonella typhimurium. This suggests that endpoints of methyl green displacement may be useful biophysical indicators, predicting anti R-factor potency. In order to place this idea on a solid scientific basis, the nature of the methyl green-DNA complex and of its dissociation by DNA-complexing drugs was studied in detail with the following results. Methyl green binds stably to duplex DNA and to poly d(A-T) but to a lesser extent to single-stranded  $\phi$ X 174 DNA, transfer RNAs and poly dG-dC. RNAs and poly dG-dC exist preferentially in the A-conformation while duplex DNA and poly d(A-T) exist in the B-conformation. Exposing the methyl-green-DNA complex to graded concentrations of ethyl alcohol liberated part of the dye slowly by a zero order reaction, owing to partial denaturation of DNA. At higher alcohol concentrations which cause the B  $\rightarrow$  A transition of DNA conformation, the bulk of the dye was released. Evidently, methyl green binds to DNA and related polymers preferentially when they assume the B-conformation. The viscosity of the methyl green-DNA complex was significantly lower than that of uncomplexed DNA; this argues against the possibility of intercalation binding which appears also to be ruled out by the fact that the dye molecule is not planar but that its three phenyl residues are spatially arranged in a propeller-like conformation. The displacement of methyl green from its complex with DNA follows a bimodal pattern in which about 90 per cent of the dye are displaced by a process whose endpoint is strongly dependent upon the first order rate constant of the displacement reaction. This probably represents cationic competition for DNA phosphates. Displacements of >90 per cent of the dye shows little dependence of endpoints on reaction rates and is caused by strong intercalating drugs such as propidium, tilorone, quinacrine, ethidium, nitroacridines, proflavine and miracil D. The conformational changes (unwinding of helical turns and lengthening of the intervals between base pairs) which result from intercalation binding evidently cause the dissociation of methyl green from the double helix. Since R-factor DNA is closed circular supercoiled in nature, it is important that the endpoints of methyl green displacements were found by us also to be proportional to those concentrations of intercalants which precisely abolish superhelical

turns in closed circular DNA. Quantitative methyl green displacement analysis is not only a general test for the binding of drugs and other biologically active compounds to DNA but also forecasts biological activities of substances whose bioreceptor is on DNA. Our first communication, in 1971, has indeed been followed by numerous publications in which authors have used methyl green displacement analysis in the evaluation of DNA-binding drugs.

Distamycin A. This antibiotic continues to receive scientific attention in numerous laboratories because of its unique DNA-binding characteristics. It is of special interest to us because it inhibits the transfer of R-factors among Gram-negative bacteria. We have continued interaction studies of distamycin A with DNA-like polymers after reporting last year on the binding of the antibiotic to poly dA, poly dT, poly dG and poly dC as well as to single-stranded  $\phi$ X 174 DNA. The homopolymers were used as templates for the RNA polymerase reaction, that is for the transcription of RNA in vitro. Distamycin inhibited transcription from poly dA and poly dT but not from poly dC; poly dG was confirmed to have no template activity of its own. These biochemical studies in conjunction with biophysical results reported last year suggests that distamycin A exhibits base specificity for adenine and thymine, thereby explaining the A-T specificity of the antibiotic's binding to DNA species with various A-T/G-C ratios.

Mode of action of L-cycloserine. This project had a long history. In 1959, a paper from this laboratory reported the first systematic studies on the mode of action of the antibiotic, D-cycloserine, which is a structural analog and antimetabolite of D-alanine and inhibits two consecutive steps, involving D-alanine, in the biosynthesis of peptidoglycan which is the building block of bacterial cell walls. A small quantity of synthetic enantiomeric L-cycloserine was available, barely enough to show that this compound was a growth inhibitor of E. coli in a glucose-salts medium and that the mode of growth inhibition did not involve inhibition of the biosynthesis of the cell-wall polymer. The pair of enantiomers, hence, presented the unique problem of both being antibacterial but evidently on the basis of different modes and mechanisms of action. During the following years, Russian scientists under Braunstein found that cycloserine is an effective inhibitor of the transaminase reaction. A plausible hypothesis, explaining the antibacterial action of L-cycloserine, was therefore to assume that the compound was an analog and antagonist of L-alanine and interfered either with the formation of alanine (through transamination) or with its utilization, primarily in protein biosynthesis.

When L-cycloserine became commercially available, a study of its mode of antibacterial action was begun which is now complete with the following results. L-cycloserine inhibits the growth of E. coli

in mineral media with added sources of carbon, but not in broth. Growth inhibition can be reversed by supplying graded quantities of broth or of amino acids, especially L-alanine to the mineral medium. Evidently, L-cycloserine does not inhibit the utilization of amino acids when they are present in the growth medium. In a mineral-pyruvic acid medium, L-alanine, D-alanine, L- $\alpha$ -aminobutyric acid and L-isoleucine reversed in decreasing order of potency, the growth inhibitory effect of L-cycloserine. This suggested that the formation of L-alanine from pyruvate and amino acids through a transaminase step was inhibited by L-cycloserine. Since L- $\alpha$ -aminobutyric acid is not a constituent of proteins but a donor of the amino group in transaminations, reversal of growth inhibition by this compound was particularly suggestive of inhibition of an alanine transaminase. This was verified by studies demonstrating the inhibition of the  $\alpha$ -aminobutyrate-pyruvate transamination reaction in *E. coli* W by L-cycloserine. The inhibition is not strictly competitive but is of a mixed type because L-cycloserine interacts with pyridoxal phosphate which is a coenzyme in transamination. The mode of bacterial growth inhibition by L-cycloserine is, hence, L-alanine deficiency induced by a blockade of transaminations to pyruvate; bacteria, so inhibited, resemble phenocopies of starving alanine auxotrophs.

Computer modeling of chloramphenicol bound to ribosome. In 1956, Hahn and his associates<sup>5</sup> published a fundamental paper on structure-activity relationships in the chloramphenicol series. This has remained one of the basic reference articles on the subject. In the intervening years, many more derivatives of the antibiotic have been synthesized and their antibacterial effects quantitated. Additionally, the mode of action of the drug has been pinpointed to an inhibition of the enzyme, peptidyl transferase, which is an integral part of the larger ribosomal subunit and mediates the actual formation of peptide bonds in protein biosynthesis. Structure-activity relationships for compounds of the chloramphenicol series were, hence, re-analyzed in detail, too great to be reported here, with a view to using interactive computer graphics to model an archetypical chloramphenicol bound to its ribosomal receptor site. This was then done in collaboration with Dr. Peter Gund of the Computer Graphics Laboratory of Princeton University.

The antibacterial effect of chloramphenicol may only be fully understood in terms of the three-dimensional structures of drug and receptor site, and in terms of the energetics of their interaction. Although we are still a long way from knowing the detailed molecular structure of the ribosome, nevertheless, if we can determine the conformation of agents which complex to the ribosome by complementarity, we may infer major features of the topography of the active site. In an effort to derive a workable model which could serve as the basis for further work in designing improved antibiotic agents, we have attempted to determine the likely conformations of chloramphenicol, both as

free drug and when bound to the transpeptidase active site.

In principle, chloramphenicol has eight rotatable single bonds (Fig. 1). The amide bond ( $\phi_2$ ) is usually fixed as trans-planar, while rotation of hydroxy hydrogens ( $\phi_7$  and  $\phi_8$ ) should be facile. Rotation about the acetamide ( $\phi_1$ ) and aryl ( $\phi_5$ ) bonds should also be relatively easy. Of the remaining three axes, the C-C bonds ( $\phi_4$  and  $\phi_6$ ) have been assigned a rotation barrier of 2.7 kcal/mole, while the C-N bond ( $\phi_3$ ) barrier is only 0.5 kcal.

Despite the many degrees of conformational freedom, chloramphenicol was found to exist primarily in one conformation in the crystal (Fig. 2); the proximity of the alcohol groups suggested that an intramolecular hydrogen bond stabilized the structure. Bustard, Egan and Perun<sup>6</sup> have recently reached a different conclusion. They proposed from potential energy calculations that a similar, but non-hydrogen bonded, conformation prevailed (Fig. 3); a second conformer was less than 1 kcal higher in energy, and many others were energetically accessible. Furthermore, they demonstrated the absence of an intramolecular hydrogen bond by high dilution IR spectroscopy, and by the observation that the NMR of chloramphenicol phenylboronate (which contains a 6-membered ring) exhibited substantially different proton couplings. Consideration of solvent polarity effects suggested that the non-hydrogen bonded conformation was even more highly favored. One may speculate that, while solvation stabilizes an extended conformation, crystal forces favor the more compact, hydrogen bonded conformation.

Bustard's conformational calculations were based on nonbonded and torsional potential energy functions, and did not explicitly consider energetic effects of hydrogen bonding or partial charge interactions. Furthermore, no full geometry search was attempted, so that those results must be considered as suggestive rather than rigorous. Hltje and Kier<sup>7</sup> have recently investigated chloramphenicol conformations using quantum mechanical methods; two isoenergetic conformations -- one essentially identical to Bustard's first conformer, and one corresponding to his second conformer -- were calculated to be equally favored. Despite this essential confirmation of Bustard's results, extended Hckel calculations are known to be somewhat unreliable in determining preferred conformations and a definitive study remains to be done.

There thus appears to be no reason to expect that the conformation which chloramphenicol assumes in the crystal state is the same as obtains on the active site. Similarly, Cheney<sup>8</sup> has suggested that peptidyl tRNA exists in a bound conformation which is different from its form in solution.

One approach to determining the conformation of bound chloramphenicol was to compare the structure of various conformers with that of the natural substrate, or with that of antibiotics which are chloramphenicol antagonists, looking for similarities in the three-dimensional structure. Unfortunately, the structure of peptidyl-tRNA on the active site is unknown. However, Shipman, Christoffersen and Cheney<sup>9</sup> have modeled lincomycin free base by an ab initio molecular fragment technique, and obtained a structure which they believe corresponds to the biologically active conformation; Cheney<sup>8</sup> has used this structure and model calculations to propose a conformation for the natural peptidyl substrate when bound to the enzyme surface. Despite the rather tentative string of assumptions on which this line of reasoning hangs, the resulting model is fairly appealing.

We therefore searched for conformations of chloramphenicol which could be superimposed (utilizing interactive computer graphics) with the three-dimensional structure of lincomycin and/or the substrate model for peptide bond formation proposed by Cheney. We found several well-matching conformations, of which the two most likely are shown in Figs. 4 and 5. The first model is similar to one proposed by Harris and Symons<sup>10</sup> on the basis of examining Dreiding models of various transpeptidase substrates, while the second model is essentially the same as one proposed by Cheney by comparison of chloramphenicol crystal structure with his models of lincomycin and bound peptidyl-tRNA.

The examination of the relationship between structure and activity among chloramphenicol derivatives encourages us to propose a prototypical structure of compounds which interact with peptidyl transferase in the manner in which chloramphenicol does (Fig. 6). The propane chain with its amide group, -NH-CO-, binds to the peptidyl recognition site of peptidyl transferase. An acyl (or aminoacyl) residue is necessary for activity but its steric and electronic requirements for inhibition of peptidyl transferase are not very stringent. Of the two propane chain functional groups, one hydroxyl is accepted by the enzyme at the adenylyl ester oxygen site. Depending on which alcohol is thus bound, the nitrophenyl group binds hydrophobically to either the peptidyl side chain accommodating groove, or to the transpeptidase active site. We hope that further experimentation will offer greater insight into the mechanism of binding of this old and useful drug whose deceptively simple structure doubtless still holds a few surprises.

#### Conclusions.

The R-factor, R1, confers upon Salmonella typhimurium "intrinsic" resistance to ampicillin; bacteria recover and grow before significant hydrolysis of ampicillin by  $\beta$ -lactamase is evident. Studies have been

initiated on the elimination of an R-factor which has been transferred from Pseudomonas into E. coli; this permits separation of R-factor and chromosomal DNAs by intrinsic specific gravity differences. Special instrumentation has been developed for the viscometric study of the interaction of R-factor eliminating compounds with R-factor DNAs in vitro. Biochemical studies on the in vitro replication of R-factor DNA have resulted in the separation of three different polymerizing enzymes which are under study. The methyl green-DNA complex and its dissociation by DNA-complexing drugs have been studied in detail since this dissociation forecasts the R-factor eliminating potency of drugs. The antibiotic distamycin A which inhibits transmission of R-factors, binds to poly dA and poly dT and inhibits the template function of these polymers in the RNA polymerase reaction in vitro. Synthetic L-cycloserine inhibits bacterial growth by preventing the formation of alanine from pyruvate by transamination. A computer-generated model of chloramphenicol bound to its ribosomal receptor, explains those structural features of the antibiotic which are essential for antimicrobial action.

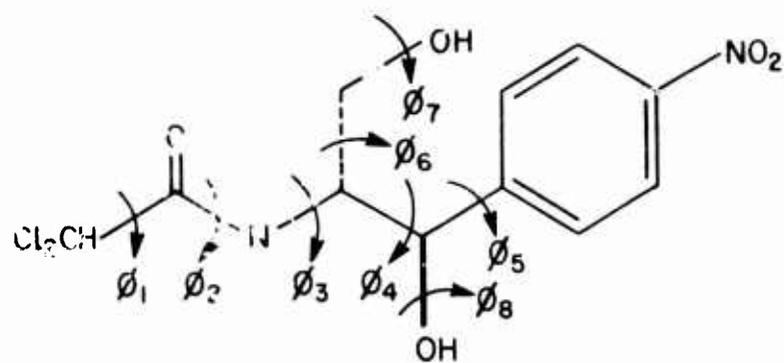


Fig. 1. Rotatable bonds in chloramphenicol.

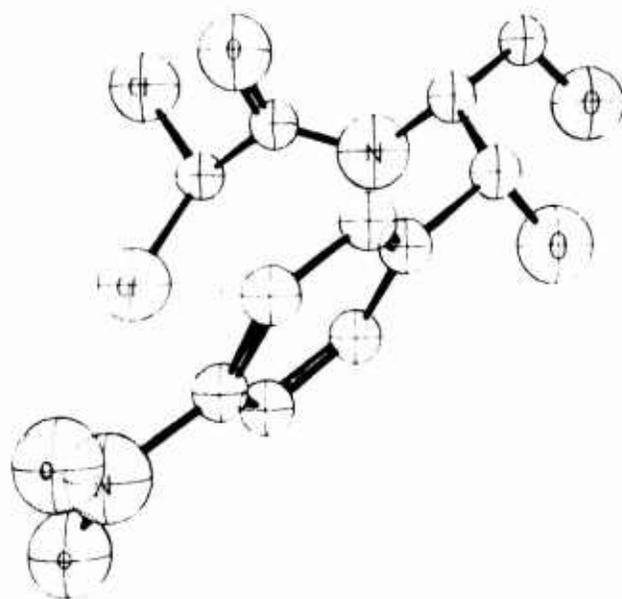


Fig. 2. Chloramphenicol, crystalline conformation.

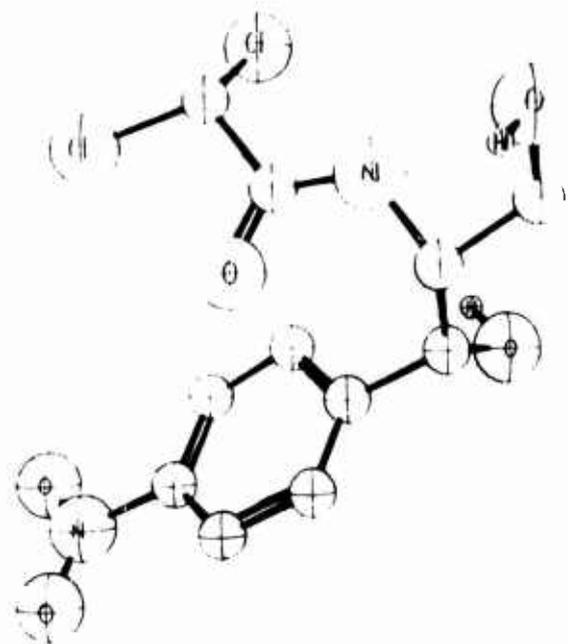


Fig. 3. Chloramphenicol, calculated (6) ground state conformation.

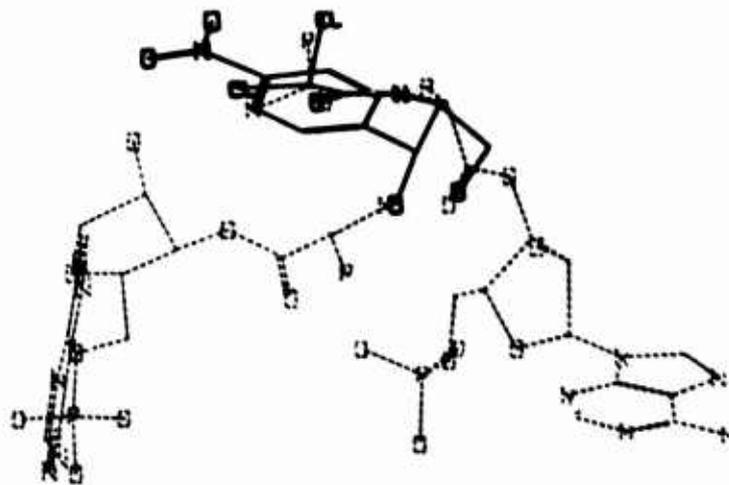


Fig. 4. Superposition of chloramphenicol (solid lines) and bound transpeptidase substrates.

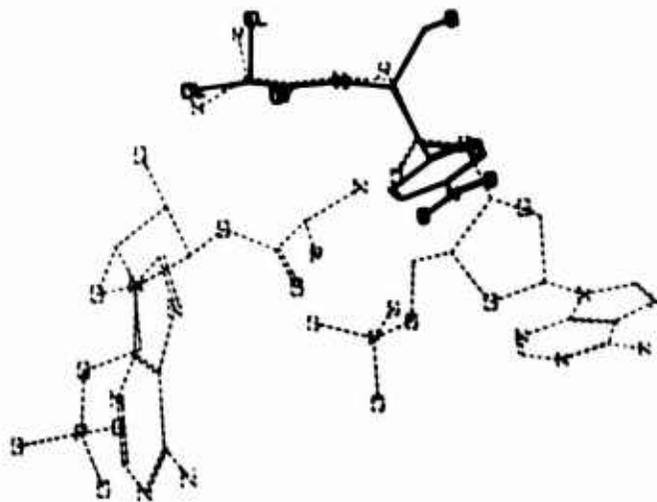


Fig. 5. Another superposition on which Fig. 6 is based.

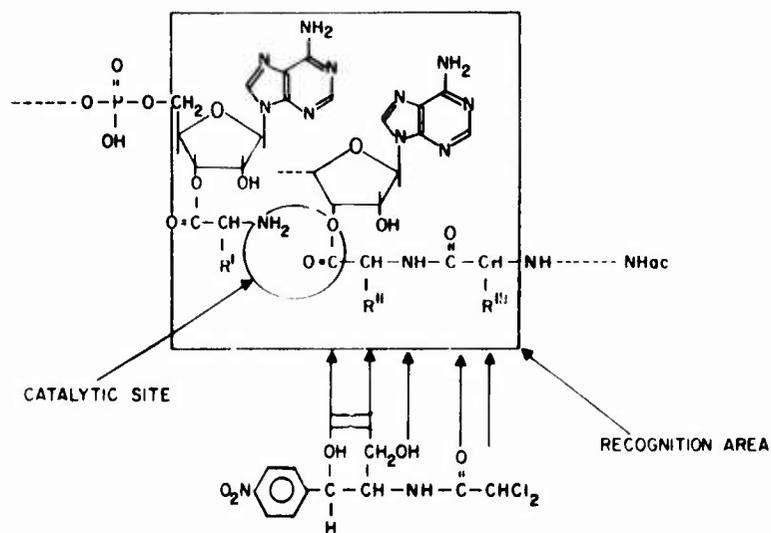


Fig. 6. Correspondence of chloramphenicol and peptidyl transferase structural features.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 074 Molecular basis of biological regulation and chemotherapeutic drug pharmacology

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1. Krey, A.K., and Hahn, F.E.: Base-specific interaction of distamycin A with polydeoxynucleotides. 9th FEBS Meeting, Budapest, 170, 1974.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA OC 6434	75 07 01	DD-DR&E(A)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8. C/SPN INSTR <sup>6</sup>	9a. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
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10. NO./CODES <sup>7</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3A161102B71P	01	075			
b. CONTRIBUTING							
c. XCODE/NUMBER/UNIT	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>8</sup>							
(U) Metabolic Problems and Biochemical Variations Associated with Disease and Injury							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>9</sup>							
002300 Biochemistry 003500 Clinical Medicine 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: <sup>10</sup>				FISCAL YEAR		CURRENT	
c. TYPE:				75		6	
d. KIND OF AWARD:				76		286	
e. AMOUNT:				6		286	
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				NAME: Mahan, CPT D.E.			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>16</sup>							
(U) Protein Synthesis; (U) Receptor Sites; (U) Macromolecules and Disease; (U) Antibody Synthesis; (U) Biochemistry of Parasites.							
23. TECHNICAL OBJECTIVE, <sup>17</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The technical objective of this work unit is to define the biochemical responses of the host to injury and to diseases of military importance in order to develop new approaches to the prevention and the early diagnosis of disease in military personnel.</p> <p>24. (U) Isolated and purified macromolecules from diseased tissues are characterized and compared with macromolecules derived from normal tissues in animal models. Macromolecular interactions between host and infecting agents are investigated. Binding sites for metabolic inhibitors and other drugs will be characterized and the nature of the agonist - antagonist binding will be characterized in order to establish the nature of drug tolerance and drug resistance in animal models. The biochemistry of the immunological responses and their modulation by drugs and other agents will be studied in order to evaluate their roles in the immunological responses. Antibodies to abnormal macromolecules will be prepared for use in diagnostic tests for disease.</p> <p>25. (U) 74 07 - 75 06 Studies which rule out binding of diphenylhydantoin by nucleic acids have been completed. Shigella flexnis toxin has been partially purified and characterized for use in immunological and biochemical studies. Macromolecular characterization of enterobacter pathogens has shown these are an extremely diverse genetic group and even within the Enterobacter agglomerans there is great diversity which emphasizes the diagnostic complexity for this group. Work has progressed significantly in the standardization of protein synthesis test systems for exploitation. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.</p>							

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Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 075 Metabolic problems and biochemical variations associated with disease and injury

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The objective of this work unit is to apply knowledge gained through basic and molecular research to existing military medical problems. Studies are conducted on disease-induced variations in cellular processes to define biochemical genetics and cellular biology by using physico-chemical and structural characteristics of macromolecules of bacteria, viruses and mammalian cells and their subcellular elements. These studies have been conducted:

1. The mechanism of protein synthesis in brain, liver and intestinal cells.
2. In vitro isolation and characterization of structural and functional genes.
3. Role of adenylyl cyclase and cyclic AMP in mixed lymphocyte cultures.
4. DNA relatedness among enterobacteria.
5. The binding of  $^{14}\text{C}$ -5,5-diphenylhydantoin to rat brain in vitro and in vivo, and its probable role in brain protein synthesis.
6. Isolation, purification and mode of action of S. flexneri toxin.
7. Isolation, purification and characterization of prostatic acid phosphatase to be used for production of antibodies for use in the early detection of prostatic carcinoma.

### Mechanism of cellular protein synthesis.

Cell-free protein synthesis systems using poly uridine as a messenger were developed from rat brain, liver and intestinal cells to be used to study the mode of action of dilantin, cytosine arabinoside and *S. flexneri* toxin. The system will also be used to study the translation of natural messenger RNA.

### Nucleotide sequence relatedness among Enterobacteriaceae.

Enterobacteriaceae contains pathogens that cause a variety of diseases including: enteric fever, diarrhea, urinary tract infection, food poisoning, and bacteremia. In addition, Enterobacteriaceae contains phytopathogenic bacteria. Our studies are designed to determine DNA relatedness in all organisms in this family of bacteria. The data obtained are used for the following purposes:

1. To develop a molecular (genetic) definition of a bacterial species.
2. To assess the lines of evolutionary divergence in pathogenic bacteria.
3. To develop a classification based on genotypic relatedness, instead of only a few phenotypic characteristics. The average bacterium contains sufficient DNA to specify approximately 3,000 average size genes, yet most organisms are classified on the basis of only 10-25 characteristics.
4. To identify atypical clinical isolates for purposes of treatment and epidemiology.
5. To accurately classify newly described organisms.

Data obtained previously indicated that enteric bacteria are all genetically related. In the majority of cases there is a core of about 20-25% relatedness among these organisms. The main exceptions are found in the genera Proteus and Providencia. Most of these organisms (proteaeae and providencia) exhibit about 10-15% relatedness to members of all other genera.

During the past year we have concentrated on assessing relatedness within specific genera of medical importance, and clarifying the status of recently described organisms, as well as atypical organisms.

Polynucleotide sequence relatedness among strains of Enterobacter agglomerans.

Until recently E. agglomerans strains have been classified with the genus Erwinia - as plant pathogens. These organisms have been isolated with increasing frequency from human infection. They were one of the two causative organisms in a serum bottle epidemic of bacteremial infection four years ago. These organisms have been isolated from human infection, soil and plants. Many are classified in Bergey's Manual as Erwinia herbicola, Er. stewartii, and Er. uredovora. Other synonyms for E. agglomerans include Er. ananas, Er. maydis, Er. mangiferae, Er. milletiae, Er. lathyri, and Xanthomonas trifolii.

DNA reassociation followed by separation of single- and double-stranded DNA on hydroxyapatite was used to assess relatedness within and between biogroups of E. agglomerans and to determine the relatedness of these organisms to other enterobacteria. Relatedness is defined as percent reassociation of heterologous DNA relative to percent reassociation of homologous DNA. More than 100 strains were studied, including at least four from each biogroup. (Biogroups were determined by Ewing and Fife; E. agglomerans is one species that includes seven anaerogenic biogroups and four aerogenic biogroups.)

A summary of the data follows:

1. In no case do all strains of a biogroup form one DNA relatedness group.
2. In several cases strains from different biogroups are in the same DNA relatedness group (70% or greater relatedness).
3. Anaerogenic strains are not highly related to aerogenic strains.
4. Strains from all biogroups are significantly (greater than 20%) interrelated.
5. Strains from all biogroups are significantly (greater than 20%) related to other enterobacteria.
6. At least 20 distinct DNA relatedness groups are in E. agglomerans, an extremely diverse group of organisms. Neither the three-species scheme (in Bergey's Manual) nor the one-species, 11-biogroup scheme correlates well with genetic relatedness. Additional biochemical data may help resolve this problem.

### Polynucleotide sequence relatedness in Yersinia enterocolytica.

The genus Yersinia has recently been included in the family Enterobacteriaceae. The genus contains three species: Y. enterocolytica, Y. pseudotuberculosis, and Y. pestis. Y. pestis is the causative organism of plague in man, rats, and other rodents. Although human plague is much less widely distributed than plague-infected rats it is of medical concern. Y. pseudotuberculosis is generally found in animals with pseudotuberculosis lesions, generally in the mesenteric glands. It has also been found in man. Y. enterocolytica has been isolated from the feces and lymph nodes of both sick and healthy animals and man (septicemia). Since 1955, the species has been isolated with increasing frequency from man and animals. It has also been isolated from material likely to be contaminated by feces, as milk and ice cream.

A collaborative project was carried out with Dr. D. J. Brenner, Center for Disease Control, Atlanta, Ga.

A summary of data follows:

DNA hybridization was used to determine relatedness among strains of Y. enterocolytica, between species of Yersinia, and Y. enterocolytica to other Enterobacteriaceae. Hybridization reactions were carried out at optimal (60C) and stringent (75C) incubation temperatures to assess distantly and closely related nucleotide sequences. Thermal elution chromatography was used to approximate the degree of unpaired bases within the related sequences. Twenty-eight strains of Y. enterocolytica were 85% or more related. All but one of 24 strains of Y. pseudotuberculosis, representing 6 different biogroups, were 45-62% related to Y. enterocolytica. At 75C relatedness decreases from 30% to almost 5-fold less than that seen at 60C. One strain of Y. pestis exhibited 45% relatedness to Y. enterocolytica. There are 0-2.5% unpaired bases in related sequences of Y. enterocolytica. This value increases to 5.5-15.0% in related sequences between Y. enterocolytica and other Yersinia species. The so-called Red Mouth bacteria show greater relatedness (30%) to Y. enterocolytica than to any other enterobacterium. Relatedness of Y. enterocolytica to most enterobacteria is 15-25%. E. tarda and P. mirabilis are 10% related to Y. enterocolytica. Fifteen strains of Pasteurella multocida showed 6-13% relatedness to Y. enterocolytica. These data are consistent with the recent addition of Yersinia to the Enterobacteriaceae.

Dr. B.D.W. Jarvis from Massey University, Palmerton North, New Zealand, collaborated for five weeks in this laboratory using DNA relatedness to resolve taxonomic questions with Rhizobium.

Studies on the purification and characterization of Shigella Dysenteriae 1 enterotoxin (in collaboration with Drs. S. Formal and P. Genski, DCDGI, WRAIR).

These studies are designed to isolate and characterize the S. dysenteriae toxin so that a stable toxoid can be obtained. A potent neurotoxin is elaborated into the culture medium upon autolysis of the bacteria. Both neurotoxic and enterotoxic activity can be recovered from the culture broth. Partial purification indicated that the substance is proteinaceous, heat labile, and has a molecular weight of approximately 55,000 to 60,000 by gel filtration. Attempts at toxoid production have been unsuccessful; the toxoid reverts to toxin.

Cell-free extracts of strain 3818, S. dysenteriae 1 were prepared by the method of Van Heyingen in the Department of Applied Immunology, WRAIR. The extracts were assayed for activity by means of HeLa cell cytotoxicity. Throughout the purification, assays were performed to determine specific activities. The purification procedure consists of the following steps: (1) Removal of nucleic acids from the cell extracts on Diethylaminoethyl Sephadex columns (DEAE Sephadex); (2) Ammonium sulfate precipitation of active fractions; (3) Molecular sieving on Sephadex G-200 or application to DEAE Sephadex and elution with a salt gradient, or a combination of both; (4) Polyacrylamide gel electrophoresis is performed to determine sample heterogeneity on an analytical scale. (Preparative electrophoresis of extracts are also being performed.) Molecular weight is determined by sodium dodecyl sulfate gel electrophoresis.

The effects of the enriched toxin preparation on protein synthesis were studied in a cell-free system from brain, liver, and intestinal cells using poly uridine as a messenger to direct the synthesis of poly-phenylalanine. Following is a summary of the results to date:

1. Crude cell extracts show cytotoxicity on HeLa cells at  $10^5$  dilution. The partially purified preparation reconstituted to 1 mg protein per ml has the same cytotoxicity at  $10^{11}$  dilution. This enriched fraction is not homogeneous as judged by electrophoresis and other physico-chemical methods.

2. Preliminary results show that the enriched fractions appear to inhibit protein synthesis at the tRNA aminoacylation steps in cell-free rat liver, rat brain, guinea pig intestine and E. coli preparations

Purification of prostatic acid phosphatase to develop a radioimmunoassay.

Acid phosphatase exists as several tissue specific isoenzymes, and prostatic acid phosphatase (PAP) is a reliable marker for both normal and transformed prostatic tissue. Elevated serum levels of prostatic

acid phosphatase have been used to diagnose prostatic carcinoma. However, in only 40% of the patients with diagnosed prostatic carcinoma is the blood PAP level elevated. Because this disease spreads first to the hard tissues during metastases, bone marrow aspirates and fluorine ( $^{18}\text{F}$ ) bone scans have been helpful tools in the diagnosis and staging of the disease. The utility of these two techniques is limited, however, as they can only detect the presence of the cancerous tissue in the latter stage of the disease.

The purpose of this study is to apply an immunological approach to improve the sensitivity in the detection of the enzyme in the aspirates of bone marrow allowing for earlier detection of prostatic carcinoma metastases. An antibody to PAP would be useful in two ways. First, the labeled antibody would provide a radioimmunoassay (RIA) which would be more sensitive and specific as a method for the detection of PAP in bone marrow aspirates than the activity of the enzyme itself. Secondly, through the use of fluorescent antibody techniques this antibody would also provide a means of visualizing cells of prostatic origin on slides of bone marrow aspirates.

This project is divided into three parts. Phase I consists of the purification of the antigen using seminal fluid as a source of PAP. Using purified PAP as an antigen, Phase II requires the production of antibodies to the enzyme in rabbits. Phase III consists of the development of the techniques for the RIA and the fluorescence staining procedures.

Relatively large amounts of PAP source material are required for obtaining sufficient quantities of purified enzyme. Human seminal fluid (100-200 ml) was centrifuged to remove insoluble material. The sample was dialyzed and applied to (10 x 100 cm) Sephadex G-200 columns. The specific activity of enzyme increased from 15 to 150 units/mg. The enriched PAP fractions were concentrated and equilibrated with starting buffer for DEAE-Sephadex column chromatography. The sample was applied and the column was developed with a salt gradient. Of the six protein peaks that were obtained, only one contained PAP. The specific activity of this fraction was approximately 350 units/mg protein. The DEAE-Sephadex enriched PAP fractions were applied to CM-cellulose at lower pH. An homogeneous protein-enzyme activity coincident peak was obtained with an essentially constant specific activity of 400-450 units/mg protein. This fraction appears to be electrophoretically homogeneous. The physico-chemical characterization of this enzyme is being carried out. Sufficient quantities of purified PAP have been obtained to proceed to the production of antibodies.

Production of antibodies against human prostatic acid phosphatase is in progress. Serum containing anti-PAP of sufficient titer has been obtained from rabbits by a series of weekly injections of 500 units of antigen in complete Freund's adjuvant. Antigen-antibody complexes have been demonstrated on agarose gel plates and shown to retain enzyme activity.

Studies on the presumed role of Dilantin<sup>TM</sup> in brain protein synthesis.

We reported last year that 5,5-diphenylhydantoin shows little or no binding to nucleic acids and cell fractions. These studies were done in vivo. The present study is extended to examine the possible involvement of DPH in brain protein synthesis using cell-free systems. This report deals with the details of previous findings as well as preliminary results of cell-free protein synthesis studies.

DPH in brains appears to be localized in grey matter in preference to white matter, and in neuronally enriched fractions rather than glial fractions. The drug localizes to myelin poorly, despite its striking lipid solubility. The drug is recovered after in vivo injections primarily in soluble fractions. Some DPH is isolated with particulate fractions and is associated primarily with the neuronal fraction and the microsomes. The specific activity of the neuronal fractions decreases with time from 2 to 12 hours post-injection. The microsome fraction shows increasing specific activity from 2 to 12 hours post-injection. This latter finding has not been substantiated by all investigators. <sup>14</sup>C-DPH associates in vitro to brain subcellular fractions to a much lesser degree than in vivo. Microsomes, which accumulate <sup>14</sup>C-DPH in vivo, do not do so when they are isolated from glial fractions. These studies would seem to indicate that DPH localizes to the parakaryon or nerve cell body, and subcellularly to its nucleus and perhaps to microsomes. DPH exists primarily in a free form in the soluble fractions of brain rather than localizing to any particulate fraction.

These studies do not rule out the possibility of DPH binding in vivo with nucleic acid, as localization to the nuclear fraction and microsomes might be related to the DNA or RNA content of these fractions. DPH has been reported to alter brain protein synthesis, although the mechanism of this inhibition of protein synthesis has not been carefully evaluated. Our results indicate that there is little or no binding of DPH to nucleic acids in vitro utilizing the techniques of UV spectral analysis, or gel chromatography. Also, DPH does not appear to interfere with the thermal transition of DNA or the reassociation of DNA. The results from these studies indicate that a mode of action of DPH in vivo related to this interaction with nucleic acid is unlikely.

Results of attempts to define the nature of the association of <sup>14</sup>C-DPH with brain tissue have been inconsistent. Woodbury and Kemp demonstrated binding of DPH to brain subcellular fractions released only by alkaline hydrolysis but not by ethyl acetate extraction. They also demonstrated binding of DPH to nucleic acid in vitro as evidenced by precipitation of Torula RNA in the presence of DPH and hypochromicity of UV spectra. We found essentially little or no in vitro binding to nucleic acids by our methods. It should be pointed out that the sources and varieties of nucleic acids employed in these studies include low molecular RNA such as tRNA from yeast, E. coli and mammalian

sources, high molecular weight RNA such as ribosomal RNA from the same sources and DNA from bacterial as well as mammalian sources. Other investigators using the techniques of equilibrium dialysis, differential ultracentrifugation, ultracentrifugation, repelleting and washing, and others, have not found any binding of  $^{14}\text{C}$ -DPH to brain tissue which is not reversible, nonspecific, and independent of ionic strength and osmolarity.

The results of our in vivo experiments indicate that the radioactivity from injection of  $^{14}\text{C}$ -DPH does accumulate in specific brain subfractions, but the association of radioactivity with the fractions is rapidly reversible with no evidence of bound drug when the fractions are chromatographed over Sephadex G-25 columns. On a column pre-equilibrated with  $^{14}\text{C}$ -DPH, no change in the elution pattern was noted with unlabeled fractions or those previously labeled in vivo. This indicates essentially no or very weak binding of  $^{14}\text{C}$ -DPH to the fraction studied. Increasing subcellular fraction concentrations as measured by protein contents did not result in increased DPH binding or association in our studies. Pretreatment of animals with DPH prior to  $^{14}\text{C}$ -DPH injections increased the specific activity of the brain subcellular fractions, but does not increase binding by the techniques employed in this study.

If the localization of the drug in the subcellular fractions was related to a drug interaction with a possible receptor, then the excess of DPH present in the pretreated animals should interfere with the  $^{14}\text{C}$ -DPH binding. If the  $^{14}\text{C}$ -DPH localization is related to the half-life of the free drug in the soluble fractions of brain, then the subcellular fractions might be expected to demonstrate an increase in  $^{14}\text{C}$ -DPH after DPH treatment, which was indeed the case. Also, the fact that the pattern of subcellular distribution did not change supports this conclusion.

The possible role of radioactive metabolites of  $^{14}\text{C}$ -DPH in our in vivo studies might be considered. There is little doubt that at 2 hours post-injection the formation of  $^{14}\text{C}$ -DPH metabolites represents insignificant amounts of the total  $^{14}\text{C}$  material in brain. At 12 hours post-injection this relationship probably remains similar, as the ionized radioactive metabolites would have to actively enter brain from blood. However, no studies on the distribution of metabolites of  $^{14}\text{C}$ -DPH in brain at 12 hours are presently available, so some caution is necessary in the interpretation of the 12 hour in vivo results until further metabolism studies are performed.

It can be concluded that there is little or no binding of DPH to nucleic acids in vitro as can be judged by the lack of hypo- or hyperchromicity or UV spectral shifts. This is in contrast to the reported

observations. In addition to the experiments described in our results, we varied the concentration of nucleic acid while keeping DPH levels constant, or varied DPH levels while keeping the nucleic acid level constant. The results were negative.

DPH has been reported to stimulate brain aldehyde reductase and to modify norepinephrine uptake by brain synaptosomes. The absence of strong binding of DPH to brain tissue may suggest an involvement of the drug with a small molecule or ion at the synaptic level, or an interference in the normal transport of such molecules or ions between the cell body and the synapse. An effect of DPH on cAMP, calcium-binding protein, or microtubular protein may be important aspects of future research.

These studies were extended to elucidate the possible involvement of DPH in brain protein synthesis. A cell-free protein synthesis preparation from rat brain was used. The following is a summary of the results.

1. At  $1 \times 10^{-5}$  M or  $2 \times 10^{-5}$  M concentration, DPH has no effect on amino acylation of mammalian tRNA. Limiting amounts of synthetase and varying amounts of tRNA and 15  $^{14}\text{C}$ -labeled amino acid mixtures were used. DPH did not inhibit this reaction.

2. DPH did not affect the poly uridine-dependent, polyphenylalanine synthetic system.

3. Pretreatment with DPH in vivo (two weeks daily injection of 80 mg/kg) produced no inhibition of protein synthesis.

4. Using polysomes and natural messenger RNA, no effect of DPH on brain protein synthesis was observed. It is thus concluded that DPH has no effect on the translation phase of protein synthesis in brain.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 075 Metabolic problems and biochemical variations associated with disease and injury

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA OB 6448	75 07 01	DD-DR&E(AR)436	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY CTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8. DRGPN HISTORY <sup>f</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	
74 07 01	D. Change	U	U	NA	NL	<input type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>g</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		61102A	3A161102B71P	01	076		
b. CONTRIBUTING							
c. NONCONFORMING		CARDS 114F					
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Basic Pharmacological Studies							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>i</sup>							
012600 Pharmacology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
68 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PREL. ESTIM.		b. FUNDS (in thousands)	
b. NUMBER <sup>j</sup> NA				FISCAL YEAR		75 1.0 80	
c. TYPE:				CURRENT YEAR		76 2.0 88	
d. KIND OF AWARD:				e. AMOUNT:		f. CUM. AMT.	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Einheber, Dr. A.			
				NAME: Miner, LTC, L. C. DA			
22. KEYWORDS (Precede each with Security Classification Code)							
(U) Pharmacology; (U) Medicinals; (U) Drugs; (U) Shock; (U) Toxicity							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Research is directed toward investigating special areas of the pharmacology of potential drugs of military importance, their interactions, their mechanisms of action, and the development, characterization and improvement of animal models for defining specific applicable parameters.							
24. (U) Drugs are tested in animal models specifically designed to pinpoint mechanisms of pharmacological effects, effects on physiological responses, and effects on protozoan systems. In vitro models are being used to correlate bioavailability with pharmacological efficacy.							
25. (U) 74 07 - 75 06 Isolated uterine strips are being used to demonstrate the complexity of the apparent adrenergic blocking effect of WR 181,023. WR 149,024 protected all dogs tested from a lethal dose of E. coli endotoxin; this correlated well with ameliorated histamine release and prevention of progressive hemoconcentration. No consistent relationship was seen between the antimalarial activity of several compounds and the respective degrees of chloroquine-induced pigment clumping inhibition or reversal they produced in P. berghei. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 076 Basic pharmacological studies

Investigators.

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1. Description.

The basic research efforts of the department are directed towards several major areas. They are: the pharmacology of promising medicinal agents and of certain toxic substances; drug interactions with, and the nature of, adrenergic receptors; and the development of new or modification of existing techniques to characterize drug effects.

Appropriate pharmacological, physiological, electrophysiological and biochemical studies are conducted both in vivo and in vitro. Many of these studies emphasize interactions of potential drugs with standard pharmacological agents. An important feature is ready access to the vast inventory of serially related and diverse chemicals which can be used in detailed studies of the nature of drug interactions with biological systems.

2. Effects of WR 181,023 on the isolated rat uterus.

a. Background:

While studying the cardiovascular effects of some 8-aminoquinolines, it was observed that 4-methylprimaquine (WR 181,023) abolished the blood pressure response to isoproterenol. This observation suggested that WR 181,023 might possess beta-adrenergic blocking properties. In order to explore this interesting possibility, studies have been initiated on the isolated rat uterus employing the in vitro technique reported by Levy and Tozzi (1963).

b. Methods:

The tissue was obtained from albino female rats which weighed between 175 and 300 g. Each rat was brought into estrus by injecting 0.15 mg/kg of diethylstilbestrol subcutaneously, 24 hr prior to testing. The animal was killed by a blow to the head and

the abdomen opened. The two horns of the uterus were dissected out and transferred to a dish containing fresh Locke's solution (NaCl 0.9%, KCl 0.042%, CaCl<sub>2</sub> 0.024%, glucose 0.1% and NaHCO<sub>3</sub> 0.05%). Then each uterine segment was freed from fat and cut open longitudinally to form sheets of tissue. These were suspended at 5 g tension in a 10 ml constant temperature bath (37°C) aerated with oxygen (95%) and carbon dioxide (5%). Spontaneous isotonic contractions were recorded on a Hewlett-Packard model 7868A recorder by means of a force displacement transducer.

When the contractions became regular, 0.01 µg/ml epinephrine was added to the bath and allowed to act for 3 min. Recovery of the spontaneous contractions was monitored after washing the bath 3 times with fresh Locke's solution. WR 181,023 was then added to the bath to provide a drug concentration in the bath of 10<sup>-5</sup>M. Five min later epinephrine, 0.01 µg/ml, was added to the bath solution.

c. Results:

Figure 1 shows the record from a typical experiment illustrating the effect of 10<sup>-5</sup> M 4-methylprimaquine in modifying the response to a test dose of epinephrine (0.01 µg/ml of bath solution). The presence of the drug in the bath did not abolish the inhibition produced by the dose of epinephrine in the spontaneously contracting rat uterus. However, the drug itself appeared to alter the rhythmicity of the contractions. This phenomenon, as well as interactions with smaller epinephrine doses, is under investigation.

3. Modification of experimental endotoxin shock with WR 149,024.

a. Background:

Despite advances in antibiotic therapy, mortality from endotoxin or gram negative shock remains high. Experimental evidence indicates that endotoxin may initiate prolonged and deleterious vasoconstriction either by excessive sympathetic activity or by direct vasoconstrictive action (Spink *et al.*, 1966, Cho, 1972). This has led to a growing interest in the treatment of endotoxin shock with vasodilating agents (Vick *et al.*, 1965, Siegel and Fabian, 1967). There is also substantial evidence for depression of cardiac function at sometime during the course of experimental endotoxin shock (Wangensteen *et al.*, 1971, Hinshaw *et al.*, 1972). As a consequence of these studies, the use of positive inotropic agents has been advocated (Hemreck and Thal, 1968).

Recently, in our laboratories, an agent has been found which causes both vasodilation and increased cardiac output (Caldwell et al., 1972). WR 149,024 (1,18-diamino 7,12 diaza 9,10 dithiaoctadecane tetrahydrochloride) rapidly produced vascular alpha adrenergic blockade but differed from other alpha adrenergic blocking agents by not blocking inhibition of intestinal motility caused by alpha adrenergic agonists.

This study was undertaken 1) to assess the ability of WR 149,024 to reduce the mortality in dogs given E. coli endotoxin and 2) to evaluate the effect of this compound on a variety of cardiovascular parameters normally affected by endotoxin. In addition, plasma levels of 2 humoral agents were followed in control and drug treated dogs: histamine, a substance whose early release and rise in plasma is implicated in the pathogenesis of endotoxin shock; and lysosomal protease activity, known to increase in animals subjected to hypoperfusion or shock states.

b. Methods:

Adult beagle or mongrel dogs weighing between 9 and 14 kg were anesthetized with intravenous sodium pentobarbital, 30 mg/kg, in all experiments. Twenty beagles were intubated with an uncapped endotracheal tube. A polyethylene catheter was placed in the abdominal aorta via the iliac artery for the measurement of arterial blood pressure and the rapid sampling of arterial blood. A femoral vein was cannulated for introduction of WR 149,024, 10 mg/kg (10 dogs) or an equal volume of saline (10 dogs) followed in 60 min by 1 mg/kg of E. coli endotoxin. The animals were kept on a heated pad (37°C) for 6 hr while blood pressure was observed and blood samples were taken. After this time, the catheters and endotracheal tube were removed, vessels ligated and wounds sutured. The dogs were then returned to their cages for observation. Survivors are defined as those dogs which lived for more than 72 hr following endotoxin.

Blood samples for histamine determination (2 ml) were taken at 0, 15, 30, 45 and 60 sec and at 5, 15, 30, 45 and 60 min after endotoxin injection. In those dogs that received WR 149,024 (60 min prior to endotoxin) additional histamine samples were obtained 0, 15, 30, 45 and 60 sec and at 5 min after WR 149,024. Plasma histamine determinations were made on platelet-free plasma after extraction into an ethanol-chloroform (30/20) mixture followed by a single alkaline wash. The samples were read on a fluorometer using the methods of Shore et al., (1959). In addition, central venous blood hematocrits were determined by the microcapillary method every 30 min following endotoxin.

An additional 10 mongrel dogs were used in cardiovascular studies. These dogs were opened at the chest by a sternum-splitting procedure and respired by positive-pressure ventilation. Ascending aortic blood flow was measured by an electromagnetic flow probe and meter. Catheters were placed in the abdominal aorta via the femoral artery for the measurement of arterial blood pressure, in the pulmonary artery for pulmonary arterial pressure, and in the left atria for left atrial pressure. EKG and heart rate were also monitored. All signals were displayed on a Grass polygraph. Pulmonary vascular resistance (PVR) was derived by utilizing the following relationship:

$$PVR = \frac{mPAP-LAP}{CO} \times 7,992 \frac{\text{dyne}\cdot\text{sec}}{\text{cm}^5}$$

where mPAP is mean pulmonary arterial pressure, LAP is left atrial pressure and CO is equated with ascending aortic blood flow.

WR 149,024 and/or endotoxin were administered along the same time course as described above.

In a group of 8 additional dogs the effects of WR 149,024 and endotoxin on plasma cathepsin-like activity and platelet number were determined. The dogs were anesthetized and prepared by inserting cannulae through the femoral artery and vein. Four dogs were pretreated with WR 149,024, 10 mg/kg, i.v., and 4 received saline 1 hr before the introduction of endotoxin, 1 mg/kg, i.v.

Plasma cathepsin-like activity is a measure of lysosomal disruption and plasma protease activity (Reich *et al.*, 1965). Arterial blood samples were obtained at 1 hr before endotoxin and just prior to WR 149,024 injection, at zero time just prior to endotoxin administration and at 1, 3 and 6 hr after endotoxin administration. These samples were then centrifuged at 2,000 g for 15 min. The plasma was quickly removed and refrigerated at 4°C. Plasma protease activity was determined by the method of Anson (1936). Enzyme activities are expressed as mEq of tyrosine produced per ml of plasma per hr at 37°C. Platelet number was assessed throughout the experimental period.

Statistical analyses were performed by Student's "t" test and Fisher's exact test for fit.

#### c. Results:

The fraction of animals surviving a potentially lethal dose of endotoxin was much greater after WR 149,024 pretreatment. In

the control group, only 1 of the 10 dogs receiving endotoxin survived 72 hr. The average time to death among the other control dogs was  $10.8 \pm 1.4$  hr. In contrast, all dogs in the WR 149,024 pretreated group survived the endotoxin insult for over 72 hr. In the control dogs, endotoxin produced an initial increase in plasma histamine within the first min with a secondary rise occurring between 5 and 30 min (Figure 2). However, following pretreatment of dogs with WR 149,024, endotoxin did not produce a rise in plasma histamine. As Figure 3 shows, WR 149,024 itself did produce a transient rise in plasma histamine levels with a return to low plasma values occurring within 5 min. In the only control dog which survived the endotoxin insult, the peak change in histamine concentration observed was 8.9  $\mu\text{g/L}$ .

Hematocrit values rose in both groups following endotoxin administration but the values for the WR 149,024 pretreated group returned to preendotoxin readings by 5 hr (Figure 4). Values for the control group remained elevated throughout the observation period of 6 hr. Plasma protein concentrations followed a similar rise in the control dogs from 4-6 hr after endotoxin suggesting a loss of fluid from the vasculature. Drug pretreated dogs did not show any change in plasma protein concentration.

Pretreatment of the dogs with WR 149,024 prevented the fall in blood pressure associated with endotoxin administration (Figure 5) even though WR 149,024 itself produces a transient hypotension.

Pulmonary vascular resistance (PVR) increased substantially 2 hr after endotoxin administration in the control group but no such increase occurred in the group pretreated with WR 149,024. In fact, there was a slight depression in that group as the experimental period progressed.

Endotoxin decreased ascending aortic blood flow drastically in the control group and this value remained low. WR 149,024, while producing a slight reduction initially, prevented the degree of depression seen with endotoxin alone. WR 149,024 also produced an initial bradycardia which rebounded within an hour. After the introduction of endotoxin, a generally slower heart rate was observed in the drug pretreated groups as compared with the control group.

An additional observation in all dogs receiving only endotoxin was a much more profound diarrhea with copious blood and mucous in the fecal matter.

Table 1 summarizes the results obtained from plasma cathepsin-like activity (PCLA) determinations. Control (zero time) protease activities (PCLA) in dogs which had been pretreated with WR 149,024 were similar to those which received only saline. Within an hr after endotoxin, however, differences were apparent between the 2 groups. Whereas the saline pretreated group displayed a marked continuous increase in plasma protease activity, those dogs pretreated with WR 149,024 showed no significant change throughout the 6 hr following endotoxin.

Analysis indicates that in control animals, endotoxin injection produced an abrupt reduction in platelet count. Platelet number, however, did show some recovery within a short period of time. WR 149,024 caused a slight reduction in platelet number but this pretreatment markedly ameliorated the subsequent effect of endotoxin on platelet number.

d. Discussion:

The dogs which received WR 149,024 prior to the injection of E. coli endotoxin had a significantly better survival record than the control dogs. Moreover, a number of other parameters measured during this study also revealed profound differences in the way in which WR 149,024 pretreated and control dogs reacted.

Plasma levels of histamine were shown to be elevated in very early stages of endotoxin shock but, in those dogs pretreated with WR 149,024, these levels remained in the normal range, even though WR 149,024 did cause an initial transient increase in plasma histamine. The blockade of the endotoxin-induced histamine release by WR 149,024 can not be attributed to a prior depletion of body histamine because the rise in plasma histamine caused by WR 149,024 was very transient and was much less than that produced in the control endotoxin dogs. It is possible that WR 149,024 may have in some way stabilized the mast cells or other histamine-containing cells such as basophils or platelets and made them resistant to lysis by endotoxin. In this regard, WR 149,024 has been shown by Kollmann, et al., (1971) to form mixed disulfides with a number of membrane proteins and this bridge formation could result in membrane stability.

In the WR 149,024 pretreated dogs, the absence of a blood pressure decrease following the administration of endotoxin was striking. This could have been related to the lack of a plasma histamine elevation in these pretreated animals or the absence of other vasodepressive substances such as bradykinin in their blood. The early reduction in aortic blood pressure and indeed the

reduction in cardiac output seen in dogs given endotoxin have been attributed by Kuida *et al.*, (1961) to hepatosplanchnic pooling of blood with resultant inadequate venous return. We have shown that WR 149,024 increased superior mesenteric blood flow in the dog. Moreover, a previous study from our laboratory has indicated that WR 149,024 is capable of increasing ascending aortic blood flow, decreasing total peripheral resistance and producing vascular alpha adrenergic blockage.

Pulmonary vascular resistance (PVR) rose markedly following endotoxin administration to control dogs. This phenomenon has been described by other investigators in the dog and the primate. Moreover, others have indicated that this pulmonary hypertension is at least partially due to aggregation of platelets and leukocytes in the pulmonary vasculature with a resultant mechanical blockade. However, they also implicate pulmonary vasopressor agents like catecholamines and serotonin which are released by platelet and leukocyte aggregation. Pretreatment of dogs with WR 149,024 blocked increases in pulmonary vascular resistance previously seen with endotoxin and it also prevented the loss of plasma platelets in our dogs. Sequestering of platelets in our control dogs' pulmonary vessels may have contributed to their pulmonary hypertension, but it should be noted that platelets disappeared from the control dogs' plasma long before (< 5 min post-endotoxin) the increase in PVR (> 1 hr post-endotoxin).

Aggregation of platelets and leukocytes in the control dogs, however, may have also released vasoactive agents such as histamine and serotonin that could account for the initial hypotension and the secondary increase in PVR. The observation that those dogs pretreated with WR 149,024 did not experience a significant reduction in circulating platelets following endotoxin administration or an elevation in plasma histamine is consistent with this suggestion. Several investigators (Hinshaw, *et al.*, 1957, Spink and Vick, 1961) have shown that plasma which contains platelets is necessary for the production of endotoxin shock.

Additional differences observed between the control and WR 149,024 pretreated dogs were in the hematocrit and plasma protein concentrations. The WR 149,024 group did not demonstrate the progressive hemoconcentration associated with the endotoxin control group. This hemoconcentration is considered a sign of poor prognosis and is probably due to a loss of water from the vasculature and may be a consequence of circulating histamine and kinins.

Endotoxin shock reduces the stability of lysosomes. Moreover, it is widely recognized that the cathepsins released from lysosomes during such periods of reduced blood flow and hypoxia can cause autolysis of the cells from which they originate. As these proteases leak from these cells into the systemic circulation, they produce deleterious effects through enzymatic digestion and may be responsible for the production of myocardial depressant factor by enzymatic cleavage of plasma proteins. Interestingly, WR 149,024 prevented the increase in plasma cathepsin-like activity observed in the endotoxin control dogs. Whether this lack of protease release was due to a direct action of WR 149,024 on the lysosomes or secondary to other beneficial cardiodynamic effects of WR 149,024 can not be determined from these experiments. If, indeed, WR 149,024 is an effective membrane stabilizer, other deleterious effects of endotoxin shock could also be avoided by maintenance of capillary endothelial membranes and prevention of platelet aggregation. An alternate possibility is that WR 149,024 inactivates circulating cathepsins or myocardial depressant factor by disulfide bridge formation.

#### 4. Inhibition and reversal of chloroquine-induced pigment clumping in Plasmodium berghei.

##### a. Background:

Clumping of cytoplasmic granules of hemozoin, a pigment produced from hemoglobin by intraerythrocytic malaria parasites, is the earliest morphological change detectable by light microscopy after exposure of these parasites to the antimalarial drug chloroquine (CQ) (Thurston, 1951). This CQ-induced pigment clumping (CIPC) can be inhibited by prior administration of various agents with known selective cytotoxicity as well as by other antimalarial drugs (Warhurst et al., 1971). While recent work on CIPC inhibition has emphasized in vitro techniques (Warhurst and Baggaley, 1972), we have explored the effect of oral administration of some candidate antimalarials, as well as that of a standard antimalarial drug, quinine, on CIPC (CQ given intraperitoneally) in Plasmodium berghei-infected mice. Since these candidate antimalarials are destined for oral dosing in humans, we sought to learn whether their bioavailability was demonstrable by a direct alteration of parasite morphology, viz., through their inhibition of CIPC after oral administration in an animal model.

In the course of studying the possible inhibitory effects on CIPC of the aforementioned antimalarials when given by gavage, we observed that one agent (WR 33,063) produced a response pattern suggestive of a novel effect. Following this lead, we found that

all of these test agents had the unanticipated capacity to restore pigment morphology, in a significant number of hemozoin-containing parasites, to a type present before CIPC, even if they were given after the clumping process due to CQ had been completed within virtually all pigmented parasites. To our knowledge, this phenomenon of CIPC reversal has not been reported elsewhere.

b. Methods:

Female CD-1 mice, Eperythrozoon-free, were used when 7 to 9 wk old. They were kept on hardwood bedding, allowed D & G mouse biscuits and water ad libitum at all times, and maintained under an alternating 12-hr dark-light (0730-1930 hr) cycle. Room temperature averaged between 20 to 25 °C during the course of the experiments. Experiments were begun between 0800 and 0900 hr. All inocula were freshly prepared. The injection volume of drugs or of their respective vehicles alone (controls) was 0.01 ml/g of the body weight. All doses, expressed as mg/kg of the body weight, are for the agent as the salt.

Infection was blood-induced and the parasite used was the drug-sensitive KBG 173 strain of Plasmodium berghei which was maintained by weekly mouse passage. Parasitized blood was collected and inocula were prepared (Einheber et al., 1970) from donor mice that had been inoculated one week previously with ca 100,000 parasitized erythrocytes.

In the experiments, all mice were inoculated (Day 0, between 0900-1200 hr) intraperitoneally with ca 10,000,000 parasitized erythrocytes. Approximately 72 hr later (Day 3), thin blood films were prepared from tail blood to determine percentage parasitemia and agents or their vehicles were administered and other procedures were performed as described below. Percentage parasitemia was determined from the Giemsa-stained thin blood films by examining 300 erythrocytes (including immature forms).

Potential CIPC-modifying antimalarials were prepared as suspensions (only quinine dissolved) in 0.9% saline containing 0.4% Tween 80 and 0.2% methyl cellulose (MCT) using a manual glass tissue homogenizer and were administered in single doses by gavage. Chloroquine phosphate USP (CQ) was dissolved in sterile water and was given once intraperitoneally, always at a dose of 40 mg/kg.

When testing for CIPC-inhibiting effects, the test agent or its vehicle (MCT) was given 30 min before CQ, which was given at "0" min. Unfixed unstained blood films (tail blood) were

taken at the time of each drug administration and 30, 80, 180, and 360 min after CQ or its vehicle (water) and were examined by phase-contrast microscopy. Thus, blood films from 3-day infected mice treated in 1 of 4 different ways were compared, viz., those given test agent + CQ; test agent + water; MCT + CQ; or MCT + water.

When testing for CIPC-reversing effects, CQ or its vehicle (water) was given at "0" min. while the test agent or its vehicle (MCT) was given 80 min after CQ. Unfixed unstained blood films were taken at the time of administration of each agent or its vehicle and at 110, 160, 260 and 440 min after CQ. Thus, blood films from 3-day infected mice treated in 1 of 4 different ways were compared, viz., those given CQ + test agent; water + test agent; CQ + MCT; or water + MCT.

Blood films were coded, read at random, and were not decoded until all readings were made. Fifty erythrocytes, each infected by a single pigment-containing parasite, were examined per blood film. The number of parasites with pigment types 1, 2, or 3, representing respectively more advanced stages in the process of CIPC as outlined by Warhurst and Robinson (1971) were determined and expressed as a percentage.

Percentage parasitemia was followed on Days 3, 6, 10, 14, 21 and 28, and cumulative mortality was noted regularly.

Antimalarials used were: quinine·2HCl·2H<sub>2</sub>O; WR 33,063 (6-Bromo- $\alpha$ -di-n-heptylaminoethyl-9-phenanthrenemethanol HCl); WR 171,669 (1-(1,3-Dichloro-6-trifluoromethyl-9-phenanthryl)-3-di-(n-butyl)amino-propanol·HCl); WR 30,090 (6,8-Dichloro-2-(3',4'-dichlorophenyl)- $\alpha$ -(Di-n-butylaminoethyl)-4-quinolinemethanol·HCl); WR 142,490 ( $\alpha$ -(2-Piperidyl)-2,8-bis(trifluoromethyl)-4-quinoline-methanol·HCl).

For electron microscopy, a separate series of experiments was performed on 3-day infected mice assigned to the various treatment groups as outlined above for the CIPC inhibition and reversal studies. However, in this series, blood samples were obtained only at one time interval. In the CIPC inhibition series, this was at 80 min after administration of CQ or water (i.e. 110 min after treatment with test agent or MCT), while in the CIPC reversal series, this was at 260 min after administration of CQ or water (i.e., 180 min after treatment with the test agent or MCT). At these times, 3 thin blood films (tail blood) were prepared from each mouse for light microscopy; 2 were unfixed and unstained, the other was fixed and stained with Giemsa. The mice

were then anesthetized with ether and bled from the cut groin vessels. Blood was collected in a heparinized syringe and fixed for 1 hr in phosphate buffered (0.05M, pH 7.4) 2% glutaraldehyde solution containing 4% sucrose. The mixture was then centrifuged at 200 g for 10 min, the supernatant discarded, and new fixative added. After fixation for several hr, the blood samples were postfixed in 1% osmium tetroxide, washed in 0.05M phosphate buffer, dehydrated in an ascending series of alcohol and in propylene oxide, and embedded in Epon 812.

The resulting blocks were cut on a Porter-Blum MT-2 ultramicrotome with a Dupont Diamond knife. Thin sections were mounted on 300 mesh copper grids and were stained with uranyl acetate and lead citrate. These sections were examined with a Siemens Elmiskop 101 electron microscope.

### c. Results:

In our 3-day infected mice, which displayed a parasitemia averaging about 15%, approximately 50% of the erythrocytic parasites possessed hemozoin. Among the latter, we could discern pigment of three morphologically different types, as described by Warhurst and Robinson (1971). When agent vehicles only were given to mice, almost 100% of the pigmented parasites possessed type 1 pigment (fine, well-dispersed granules), leaving but a very low percentage with the other types, 2 and 3, throughout the experiment (Figures 6a and 7a). Consistent with the findings of Warhurst and Robinson (1971), we found 30 min after CQ, a mixture among the pigmented parasites of type 2 (transitional forms consisting of less well-dispersed, coarser granules) and type 3 pigment (full pigment aggregation) (Figure 6b). Eighty minutes after CQ, essentially 100% of the pigmented parasites showed type 3 pigment. Inhibitory agent vehicle did not alter the course of clumping when given to mice before or after CQ; with this treatment, type 3 pigment, once formed, persisted throughout the experiment (Figs. 6b and 7b). All of the above control patterns have proven highly reproducible.

Similar to previous findings (Macomber *et al.*, 1964, Warhurst and Hockley, 1967, Macomber *et al.*, 1967 and Aikawa, 1972), electron microscopy on pigment-containing erythrocytic parasites from mice treated with CQ showed enlarged food vacuoles which contain malarial pigment particles, electron dense granules, vesicles and fibrillar material. Aggregates of crystalloid malarial pigment within these enlarged food vacuoles appear to account for the so-called type 3 pigment seen by light microscopy (Figure 9). This contrasts with the preponderant type 1 pigment seen in parasites

from concurrent control mice not given CQ. Electron microscopy on the type 1 pigment showed small food vacuoles each containing one crystalloid electron dense particle (Figure 8). The type 2 pigment is a mixture of both type 1 and type 3 particles.

None of the test agents produced pigment clumping when given alone. The inhibition of CIPC by prior administration of these agents (Fig. 6) confirms this previously noted property of each (Warhurst *et al.*, 1972), except for WR 171,669, whose effects are reported here for the first time. Comparisons against the expected patterns produced by concurrent controls, which received only agent vehicle followed by CQ, revealed that each agent (Figure 6c-i) had a direct effect on pigment morphology within an hour after oral administration (i.e. by 30 min after CQ). Electron-microscopic studies on these materials confirmed the findings.

WR 33,063, the only agent tested for its CIPC inhibitory properties at more than one dose level, caused an increased degree of CIPC inhibition with increased dosage by 80 min after CQ. At this time after each dose of WR 33,063, type 1 and type 2 pigment were present such that significantly less than 100% of the pigmented parasites displayed type 3 pigment. By periodic sampling of tail blood up to 6 hr after CQ, and thereby extending the observation time after CQ beyond the 80 min period of previous workers (Warhurst *et al.*, 1971), we found that after each dose of WR 33,063, especially 80 mg/kg and 160 mg/kg, the proportion of parasites with type 3 pigment had progressively declined from that seen at 80 min after CQ, concomitant with an increased proportion of those with types 1 and 2 (Figure 6 c-e).

This observation of an apparent breakdown of some of the already formed pigment clumps led us to administer each of these CIPC-inhibitory agents 80 min after CQ, at which time pigment clumping is essentially complete. Periodic examination of pigment morphology up to 6 hr after such post-treatment of mice with each test agent revealed the appearance of pigment types like those present before full CIPC sets in (Figure 7c-g). Evidence of CIPC reversal was seen as early as 30 min after each agent, except for WR 33,063; reversal first occurred between 30 and 80 min after its administration.

Electron microscopy on the CIPC-reversing effect of WR 30,090 (80 mg/kg p.o.) and WR 33,063 (640 mg/kg p.o.) further elucidated the light-microscopic observations. By 180 min after treatment with either of these agents, each given 80 min after CQ administration, clumped pigment particles produced by CQ (Figure 7)

are rare. Instead, individual pigment particles are scattered throughout the parasite cytoplasm and each is surrounded by a single membrane (Figure 10), as is preponderantly the case with pigment-containing parasites from mice not exposed to CQ (Figure 8). Occasionally, intermediate forms which consist of a few pigment particles surrounded by a membrane can be seen (Figure 11). These forms often show a small vesicular extension from the food vacuole containing an aggregate of pigment particles. A pigment particle is often present in the vesicular extension (Figure 12). This suggests that the process of CIPC reversal is effected by a process of pinching off of pigment particles from the food vacuole.

In suppressing parasitemia and delaying or preventing death from malaria, several agents (WR 33,063, 80 mg/kg; quinine, 200 mg/kg), when given alone, were equivalent to CQ, while others (WR 33,063, 160 mg/kg or 640 mg/kg; WR 171,669, 20 mg/kg; WR 30,090, 80 mg/kg; WR 142,490, 20 mg/kg) were more potent. We discerned no consistent relationship between the antimalarial activities of the various agents, in the doses given, and the respective degrees of either CIPC inhibition or reversal they produced. Nor did the combination of agent and CQ, regardless of their order of administration, potentiate antimalarial efficacy. If the agent proved more efficacious than CQ, their combined effect on the course of infection was comparable to that of the agent alone; if the agent and CQ had approximately equal antimalarial efficacy, the results were like those obtained when either was given alone.

The patterns of both CIPC inhibition (Figure 6) and reversal (Figure 7) produced by the various test agents differed. Some agents caused a potent reversal, featuring eventual reappearance of parasites with type 1 pigment (Figure 7c-e); reappearance of only type 2 pigment marked the reversal response to others (Figure 7f, g). Comparisons of the inhibition and reversal patterns yielded by the individual agents reveal that the more potent inhibitors also produced a more potent reversal. Both patterns after quinine are especially interesting as they show a wearing off of its CIPC-inhibiting (Figure 6h) and -reversing (Figure 7f) effects with time. Waning of reversal was evidenced by an increase in the proportion of parasites with type 3 pigment between 80 and 180 min after quinine (Figure 7f), whereas inhibition decreased to the extent that complete clumping occurred between 3 and 6 hr after CQ (Figure 6h).

#### d. Discussion

Although our purpose here is not to delineate the complex interplay of factors underlying the patterns of CIPC inhibition and

reversal we have observed, our results, especially those with quinine, merit some consideration in the light of recent information. Fitch (1972) has shown that phenanthrenemethanols and quinolinemethanols compete with CQ, presumably through structural similarities, for a binding site(s) within the infected erythrocyte. Warhurst et al., (1972) consider Fitch's binding site(s) to be closely related, if not identical, to the intracellular locus at which CQ induces clumping. Radioactive CQ studies suggest reversible CQ-binding to this site(s) (Macomber et al., 1966, Polet and Barr, 1969), a concept extended by Fitch (1972) to the binding of CQ-competitive agents, including quinine. If CQ were becoming selectively concentrated in the erythrocyte-parasite complex, as suggested by the work of Macomber et al., (1966) and demonstrated by Aikawa (1972) by using <sup>3</sup>H-chloroquine, while quinine, in the form (parent chemical, metabolite(s), or both) that competes with CQ, were being depleted therefrom, then such patterns of increasingly more potent CIPC effects as we saw with quinine would not be unexpected. Indeed, if studies involving exclusive administration of these agents are applicable, then these patterns appear to be consonant not only with quinine's "rapid absorption and excretion and extensive metabolism" (Thompson and Werbel, 1972) but also with CQ's attainment of a peak concentration in populations of infected erythrocytes as late as 4 hr after its administration to P. berghei-infected mice at the same dosage and by the same route we used (Macomber et al., 1966).

Warhurst and his associates have shown that clumping of malarial pigment after treatment with CQ in vitro (Warhurst and Baggaley, 1972) or in vivo (Warhurst et al., 1971) is a synthetic process which requires energy. In consequence, they have proposed and used (Homewood et al., 1972) the extent of clumping after CQ treatment of parasitized erythrocytes in vitro as a measure of the parasites' ability to produce energy. Because electron microscopy suggests that the reversal process of CIPC is accomplished by a pinching off of pigment-containing vesicles from the post-CQ enlarged food vacuole (Figure 12) and, in any event, shows that this process involves a reacquisition of membrane by the pigment particles, it may be that CIPC reversal, like CIPC itself, is also a synthetic process and requires energy production by parasites with clumped pigment. The fact that some parasites with clumped pigment exhibit reversal whereas others do not suggests that not all members of a morphologically similar population of CIPC parasites are competent to respond to the various reversing agents. This variable competence to respond may reflect different degrees of physiological impairment among the members of such a population.

Suggestive of such a progressive, CQ-induced impairment is the persisting but greatly reduced ability of a population of parasites with clumped pigment to respond with reversal to WR 30,090 when the interval between CQ and this agent was extended up to 240 min in a separate study (Palmer and Einheber, unpublished results).

Possible differences among the candidate antimalarials in such factors as binding site affinity (Fitch 1972), and host absorption, distribution, biotransformation, and excretion, singly or collectively, could contribute significantly to the apparent differences in the CIPC inhibitory and reversal patterns each produced. Lack of detailed information on these variables, however, precludes a meaningful interpretation of interagent differences in these patterns at present. Notwithstanding these uncertainties, comparisons of such patterns produced by different preparations (vehicles, formulations, salts) of a given agent possessing CIPC-inhibiting or -reversing properties should yield relatively prompt and valid information on improved bioavailability by the oral route. The dose response shown by WR 33,063 in CIPC inhibition at 80 min after CQ (Figure 6 c-e) suggests that different degrees of either CIPC inhibition or reversal by different forms of an orally administered drug should reflect lesser or greater absorption and accessibility to the parasite. In such a system, economy in the use of agents in short supply and rapidity of data acquisition, in comparison with more prolonged studies which follow agent effects on the course of malaria infection, should prove advantageous for preclinical evaluation of appropriate candidate antimalarials, many of which do show absorption problems and for which suitable blood analytical procedures are still lacking.

Table 1

Plasma Cathepsin-like Activity (mEq tyrosine/ml/hr)<sup>a</sup>

	Time (min)				
	-60 <sup>b</sup>	0 <sup>c</sup>	+60	+180	+360
Saline pretreatment	4.3±0.4	4.5±0.7	7.3±1.1	11.2±1.9	13.9±2.8
WR 149,024 pretreatment (10 mg/kg, i.v.)	4.9±0.6	4.3±0.7	5.1±0.9	5.8±0.7	5.6±1.2

<sup>a</sup> All values are means of five assays ± S.E.M.<sup>b</sup> WR 149,024 given immediately after this blood sample.<sup>c</sup> E. coli endotoxin given immediately after this blood sample.

The Effect of WR 181,023 on the Epinephrine Response in the Isolated Rat Uterus

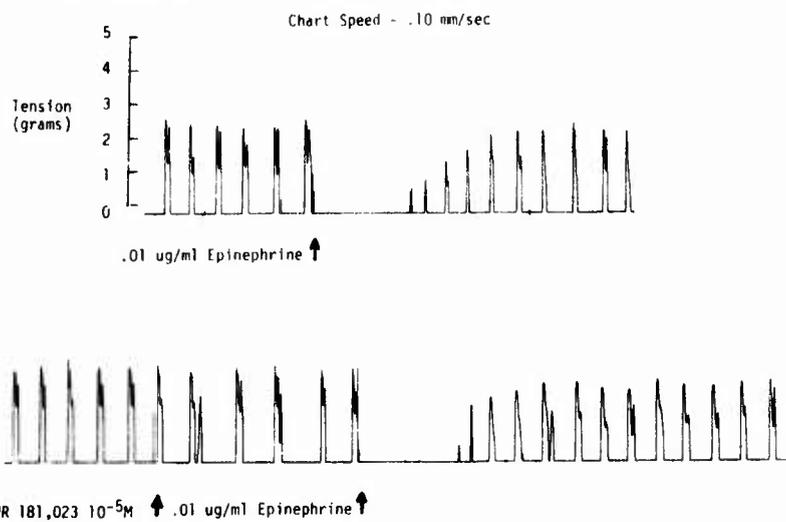


Figure 1

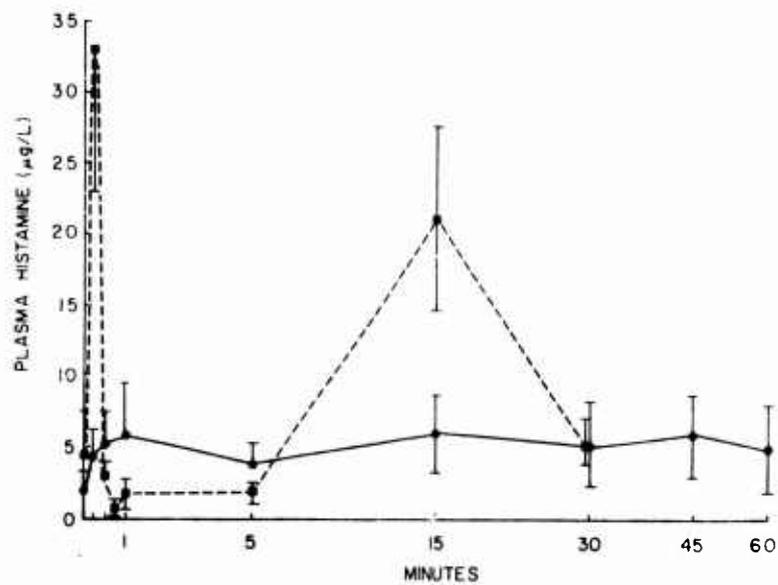


Figure 2. Plasma histamine concentrations following i.v. administration of *E. coli* endotoxin (1 mg/kg) in control dogs (■ --- ■, N = 10) and dogs pretreated with WR 149,024, 10 mg/kg (■ — ■, N = 9). Brackets indicate  $\pm$  S.E.M.

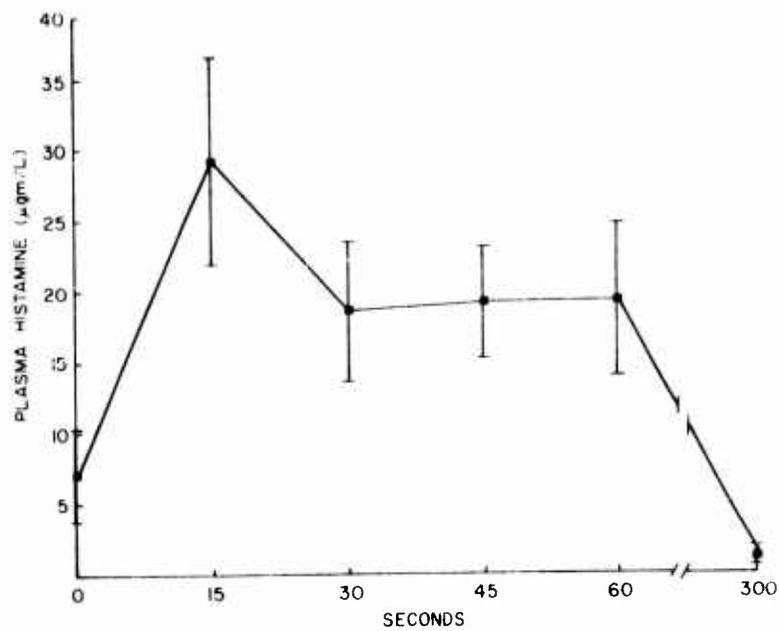


Figure 3. Plasma histamine concentrations ( $\mu\text{g}/\text{liter}$ ) immediately before and immediately following intravenous injection of WR 149,024. Each point represents the mean of 9 animals and brackets indicate  $\pm$  S.E.M.

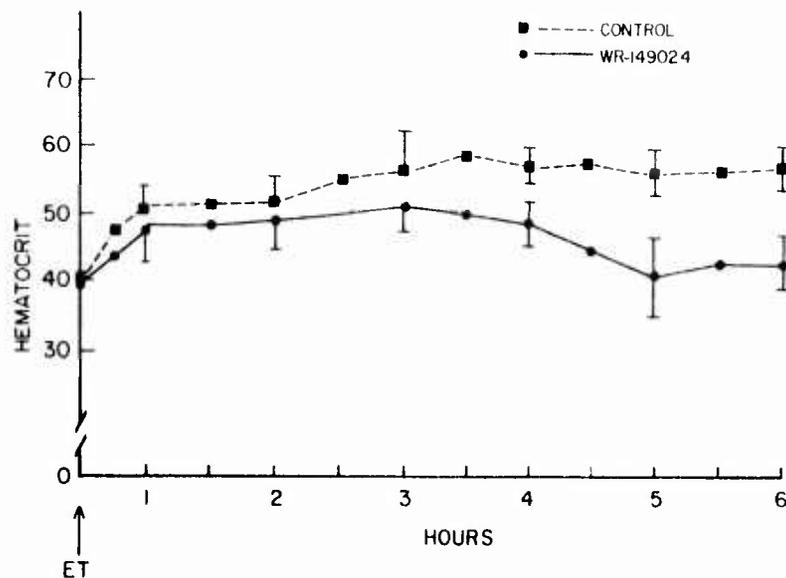


Figure 4. Hematocrit values following *E. coli* endotoxin administration (ET, 1 mg/kg) in control dogs (■---■, N = 10) and dogs pretreated intravenously with WR 149,024 (10 mg/kg) (■—■, n = 9). Brackets indicate  $\pm$  S.E.M.

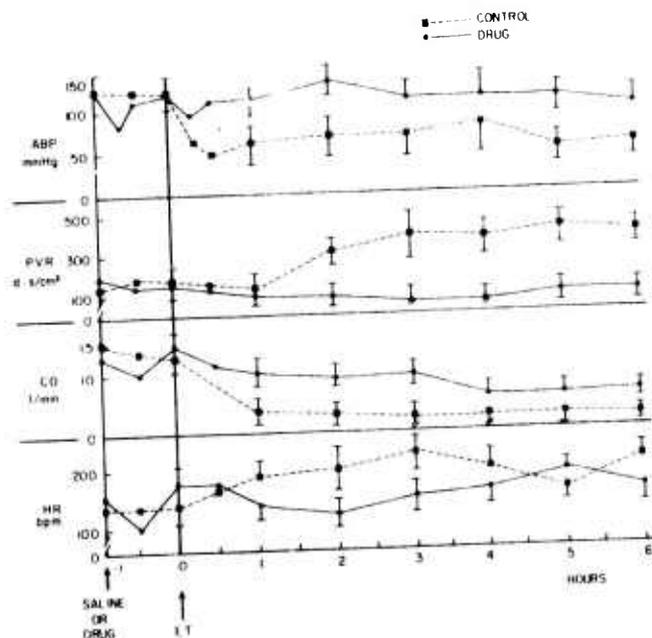


Figure 5. Cardiovascular parameters following intravenous WR 149,024, 10 mg/kg (●—●) or saline (■—■) and intravenous endotoxin, 1 mg/kg. ABP denotes mean arterial blood pressure, PVR indicates pulmonary vascular resistance, CO indicates cardiac output (actually ascending aortic blood flow), and HR denotes heart rate. Each point represents the mean of 5 values and brackets indicate  $\pm$  S.E.M.

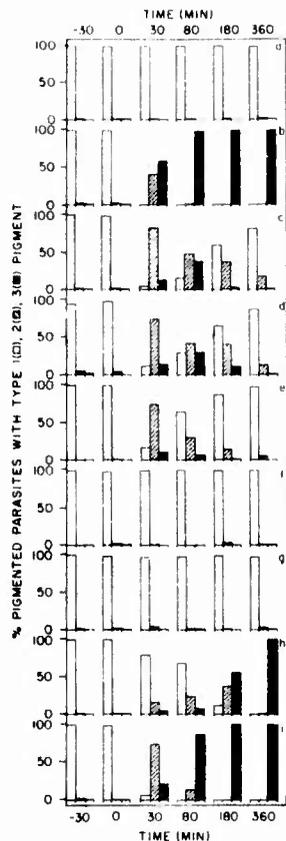


Figure 6. Inhibitory effect on chloroquine (CQ)-induced pigment clumping of oral administration before CQ of several antimalarial agents. The test agent or its vehicle (MCT) was given by gavage at -30 min and CQ or its vehicle (water) was given intraperitoneally at "0" min. The mean percentage of parasites with the various pigment types for each treatment group at each sampling interval is represented by bars above. The number of mice per group (n) is indicated below. a, control group receiving agent vehicle (MCT) and CQ vehicle (water), n=5. b, control group receiving MCT and CQ, n=8. c-i, results of administration before CQ of the various tests agents, as follows: c-e, WR 33,063 in doses of 80(c), 160(d), and 640(e) mg/kg, n=5, 3, 3 respectively. f, WR 171,669, 20 mg/kg, n=5. g, WR 30,090, 30 mg/kg, n=6. h, Quinine·HCl·2H<sub>2</sub>O, 200 mg/kg, n=6. i, WR 142,490, 20 mg/kg, n=6.

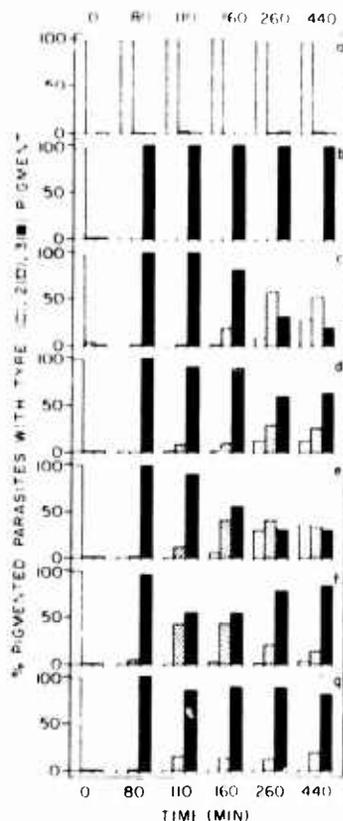


Figure 7. Reversing effect on chloroquine (CQ)-induced pigment clumping of oral administration after CQ of several antimalarial agents. CQ or its vehicle (water) was given intraperitoneally at "0" min while the test agent or its vehicle (MCT) was given by gavage at 80 min. The mean percentage of parasites with the various pigment types for each treatment group at each sampling interval is represented by the bars above. The number of mice per group (n) is indicated below. a, control group receiving water and MCT, n=5. b, control group receiving CQ and MCT, n=5. c-g, results of treatment with the various test agents after CQ, as follows: c, WR 33,063, 640 mg/kg, n=6. d, WR 171,669, 20 mg/kg, n=5. e, WR 30,090, 80 mg/kg, n=15. f, Quinine HCl·2H<sub>2</sub>O, 200 mg/kg, n=4. g, WR 142,490, 20 mg/kg, n=5.

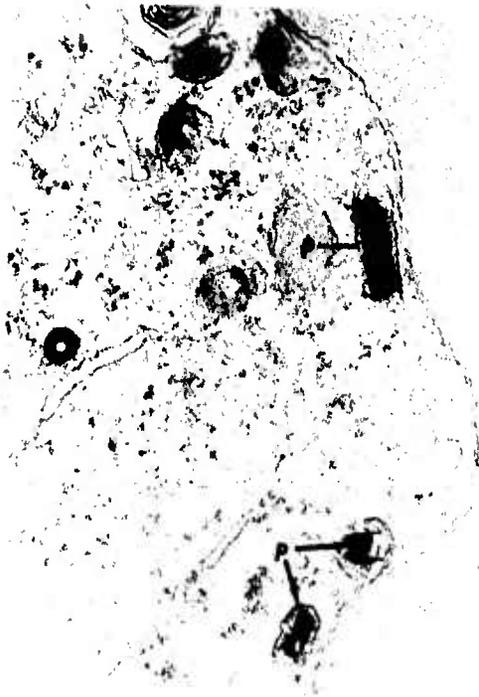


Figure 8. Electron micrograph of food vacuoles of intraerythrocytic *P. berghei* from a 3-day infected mouse given control treatment. Individual pigment particles (P) are contained in small food vacuoles that are well dispersed in the parasite cytoplasm. This appears to correspond to the type 1 pigment as seen by light microscopy. x51,000.



Figure 9. Electron micrograph of a food vacuole (F) of intraerythrocytic *P. berghei* from a 3-day infected mouse treated with chloroquine 80 min previously. A large food vacuole contains aggregates of crystalloid pigment particles (arrow). This appears to correspond to the type 3 pigment as seen by light microscopy. x60,000.

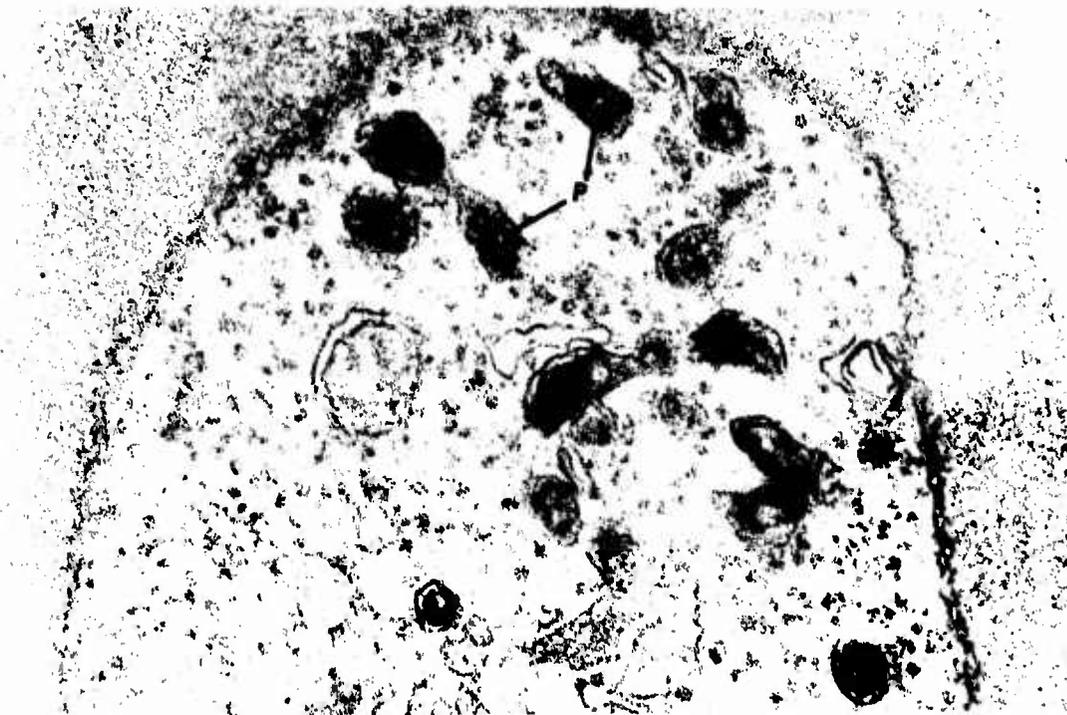


Figure 10. Electron micrograph showing the CIPC-reversing effect of WR 33,063. Three-day infected mice were given chloroquine followed in 80 min by WR 33,063, and blood was sampled 180 min thereafter. Each pigment particle (P) is surrounded by a single membrane and is scattered in the cytoplasm. x60,000.



Figure 11. Electron micrograph showing the CIPC-reversing effect of WR 30,090. Three-day infected mice were given chloroquine followed in 80 min by WR 30,090, and blood was sampled 180 min thereafter. A few pigment particles (P) are present in a food vacuole (F). x54,000.



Figure 12. Electron micrograph showing the CIPC-reversing effect of WR 33,063. Small vesicles containing pigment particles (P) extend (arrow) from a food vacuole (F). x66,000.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 076 Basic pharmacological studies

Literature Cited.

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PROJECT 3A161102B71Q  
COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00  
Communicable Diseases and Immunology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA OA 6440	75 07 01	DD-DR&B(AR)636	
3. DATE PREV SUPPLY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8. ORG'N INST'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
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11. NO./CODES <sup>6</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		61102A		3A161102B71Q		00	
b. CONTRIBUTING						165	
c. JERARCHICOMEN		CARDS 114F					
11. TITLE (Precede with Security Classification Code) <sup>7</sup>							
(U) Parasitic Diseases of Military Importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>8</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER <sup>9</sup>				FISCAL YEAR		75	
c. TYPE:				CURRENCY		6	
d. KIND OF AWARD:				76		6	
10. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME <sup>10</sup> : Walter Reed Army Institute of Research				NAME <sup>10</sup> : Walter Reed Army Institute of Research			
ADDRESS <sup>10</sup> : Washington, DC 20012				Div of CD&I			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DOD # if U.S. A school institution)			
NAME: Buescher, COL E. L.				NAME <sup>11</sup> : Burke, COL J. C.			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Moon, A. F. DA			
				NAME:			
22. KEYWORDS (Precede each with Security Classification Code) (U) Parasite; (U) Schistosomiasis; (U) Pathology; (U) Primate; (U) Chemotherapy; (U) Immunology; (U) Trypanosomiasis; (U) Filariasis							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23(U) The purpose of this research is to study various physiological, immunological and ecological aspects of parasitic diseases of military importance toward the goal of gaining a better understanding of natural susceptibility, acquired resistance and effectiveness of therapeutic agents for the prevention, suppression and treatment of these infections.							
24(U) Through careful perusal of pertinent literature and discussion with other scientists, both classical and new methods are used to set up controlled experiments.							
25(U) 74 07 - 75 06. Trypanosoma rhodesiense infections in rhesus monkeys led to development of antibodies to nucleic acids. The most dramatic response was to single-stranded DNA; lesser but significant responses were directed to native DNA and double-stranded RNA. Maximum responses occurred after 4 weeks. The antibodies to ssDNA were largely in the 19S fraction early in the course of infection, later they were predominantly in the 7S fraction. Antibodies to dsDNA were present almost entirely in the 7S fraction. Trypanosoma gambiense infections in humans were associated with the development of antibodies to ssDNA but no dsDNA or dsRNA. These studies demonstrate that antinucleic acid antibodies develop during the course of African trypanosomiasis. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 74 - 30 Jun 75.							

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DD FORM 1498  
1 MAR 66

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 165 Parasitic diseases of military importance

Investigators.

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1. Nucleic acid antibodies in African trypanosomiasis.

Antinuclear antibodies including antibodies to single-stranded DNA (ssDNA) occur in a number of pathological states. However, high levels of antibodies to native DNA (nDNA) and double-stranded RNA (dsRNA) have been found primarily in systemic lupus erythematosus (SLE) and in New Zealand mice. Antibodies to ssDNA and dsRNA, but not nDNA, can be induced by immunization. Recently protozoan infections have been associated with the formation of antinuclear antibodies. Rats infected with Plasmodium berghei developed agglutinating antibodies to RNA and DNA. In addition, fluorescent antinuclear antibody titers in Africans showed a positive correlation with the titers of malarial antibodies.

Development of a proliferative glomerulonephritis in rhesus monkeys infected with Trypanosoma rhodesiense has recently been reported. A follow-up study indicated the presence of glomerular immune deposits including complement components (C<sub>3</sub> and C<sub>4</sub>), IgM and properdin. The finding of glomerulonephritis coupled with the previous observations of antinuclear antibodies in protozoan disease prompted the present investigation of antibodies to specific nucleic acids during the course of African trypanosomiasis in the rhesus monkey. In brief, 62% of the monkeys showed antibody to ssDNA sometime during the course of infection; 31%, antibodies to nDNA; and 25%, to dsRNA. In addition several humans infected with Trypanosoma gambiense had antibodies to nucleic acids.

Preparation of Sera and Immunoglobulin Fractions

Sixteen rhesus monkeys (*Macaca mulatta*) were infected and bled as reported previously. Sera were stored at -70°C until studied. Each animal received 5,000 Trypanosoma rhodesiense organisms (EATRO strain #1886) intravenously and serial bleedings were obtained until death or necropsy. Human sera were obtained in Zaire from African patients and asymptomatic volunteers. These sera were stored and transported in liquid nitrogen. The diagnosis of trypanosomiasis (Trypanosoma gambiense) was confirmed by microscopic observations of motile organisms from lymph node aspirates, peripheral blood or cerebrospinal fluid.

Malaria was endemic in the region in which these sera were obtained. Serum fractionation was accomplished by Sephadex G200 gel filtration using known reference standards. The 19S and 7S peaks were eluted with a buffer composed of 0.1 M Tris HCl, pH 7.5, and 0.15 M NaCl. Each peak was concentrated to the original serum volume with an Amicon system using an XM50 membrane.

Assay for antibodies to nucleic acids. Antibodies to dsRNA, ssDNA and nDNA were measured by a modified Farr ammonium sulfate micro-precipitation assay described previously. The dsRNA used as ligand was synthetic  $^{14}\text{C}$ -polyriboinosinic polyribocytidylic acid (4,000 dpm/ $\mu\text{g}$ ) purchased from Miles Laboratory, Elkhart, Indiana.  $^{14}\text{C}$ -nDNA (10,000 dpm/ $\mu\text{g}$ ) prepared from human KB cells was used unaltered or heated to  $100^\circ\text{C}$  for ten minutes and plunged into an ice bath with constant stirring to obtain heat denatured  $^{14}\text{C}$ -ssDNA. For ssDNA sera were heated to  $56^\circ\text{C}$  for thirty minutes prior to assay to prevent nonspecific binding of this  $^{14}\text{C}$ -nucleic acid by complement components and other heat labile proteins. The reaction mixture consisted of a total of 100  $\mu\text{l}$  in borate buffer, pH 8.0. The standard assay for antibodies to dsRNA employed 25  $\mu\text{l}$  of serum and 80 nanograms (ng) of ligand. A total of 10  $\mu\text{l}$  of serum and 50 ng of ligand was used in the standard assays for nDNA and ssDNA. The mixtures were incubated for 30 minutes at  $37^\circ\text{C}$  and overnight at  $4^\circ\text{C}$ , followed by precipitation with an equal volume of 70% ammonium sulfate (final concentration 35%). After 60 minutes at  $0^\circ\text{C}$  the precipitate and supernate were separated by centrifugation at 1000 g for 20 minutes; 100  $\mu\text{l}$  of supernatant were removed, placed in Bray's solution and the radioactivity measured in a Nuclear Chicago liquid scintillation counter with correction for quenching and background. From this determination the per cent  $^{14}\text{C}$ -ligand bound by the serum was calculated. In this assay, the  $^{14}\text{C}$ -ligand bound to antibodies was precipitated, whereas the unbound ligand remained in the supernate. The purity of the  $^{14}\text{C}$ -nDNA used in this assay was periodically tested using a rabbit antibody specific for ssDNA but not nDNA; this antibody precipitated  $<10\%$  of the radioactivity.

Selected sera and immunoglobulin fractions were serially diluted and assayed as above. The per cent binding was plotted against the log of the serum dilution. From such plots the serum volume required to bind 50% of each  $^{14}\text{C}$ -ligand was determined and called the antigen binding capacity (ABC). The ABC of each serum represents the quantity of ligand bound per unit volume of serum expressed herein as micrograms nucleic acid bound/ml serum.

Fluorescent Antibody Test. Antibodies to soluble trypanosomal antigens were measured using a modification of a standard method.

Statistical analysis. The Wilcoxon matched-pairs signed-rank test was used to compare results obtained with the rhesus monkeys. In analyzing the human data the Mann-Whitney U test was applied. For each statistical test the  $\gamma = 0.025$  level of significance (one-tailed test)

was chosen.

**Monkey sera.** All sera were tested for binding of three  $^{14}\text{C}$ -nucleic acids: heat denatured single-stranded DNA (ssDNA), native DNA (nDNA), and double-stranded RNA (dsRNA). In two separate experiments rhesus monkeys were bled prior to inoculation with *Trypanosoma rhodesiense*, then serially bled thereafter. All animals had patent infections by the eighth day after inoculation with subsequent parasitemias ranging from  $10^6$  to  $10^8$  organisms/ml blood.

Anti-nucleic acid antibodies generally rose between 20 and 30 days following inoculations, after which a plateau was reached. Control monkeys included in each experiment were given buffer alone without organisms. In the first experiment control monkeys showed no change in antibody to the nucleic acids. However, a rise in these antibodies was noted in the infected monkeys (Fig. 1A). When the experiment was repeated, essentially similar results were obtained (Fig. 1B). Again control monkeys showed no increase in antibody.

Most striking was the response to ssDNA. A significant rise was observed in both experiments ( $p < 0.005$  for experiment 1, Fig. 1A;  $p < 0.025$  for experiment 2, Fig. 1B; Wilcoxon test). After infection 10 of 16 animals showed a binding of  $>50\%$  as compared with pre-infection levels of 30% or less.

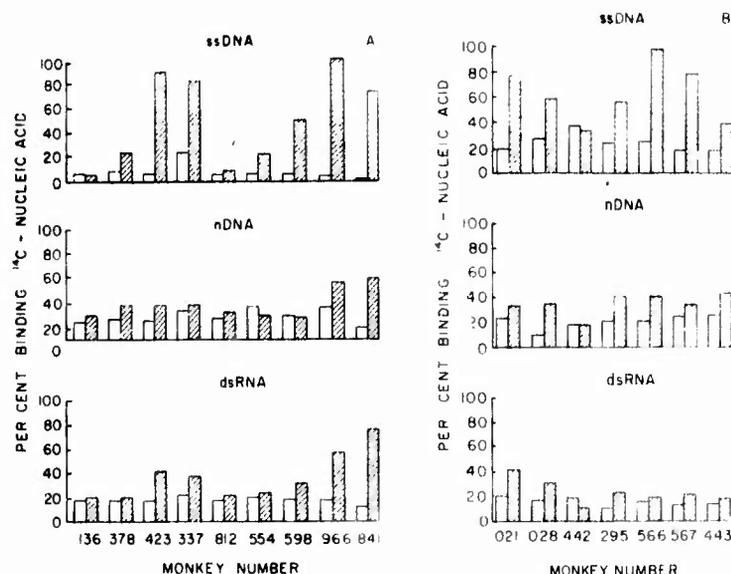


Figure 1A and B. Peak binding of nucleic acids by monkey sera. The open bars indicate pre-infection levels. The shaded bars indicate peak binding of a particular nucleic acid during the course of infection. The vertical axis represents percentage binding of each radiolabeled nucleic acid. Figures 1A and B represent the results of experiments one and two respectively. In experiment one there was a significant rise in antibodies to ssDNA, nDNA and dsRNA. In experiment two there was a significant increase in binding to ssDNA and nDNA but not to dsRNA.

The immune response to nDNA was less impressive but still significant ( $p = 0.025$ , both experiments, Wilcoxon test). Only 2 of 16 animals had a peak antibody response to nDNA greater than 50%; these two animals also showed the highest response to dsRNA. Three additional animals showed maximum response to dsRNA. Three additional animals showed maximum responses in the range of 40-50%. The antibody response to dsRNA was least impressive of the three ligands. The rise in antibodies to dsRNA was significant in the first experiment ( $p < 0.005$ , Fig. 1A; Wilcoxon test) but not in the second ( $p < 0.025$ , Fig. 1B; Wilcoxon test).

Time Course. The time course of the development of antibodies in a single monkey (No. 966) is shown in Figure 2. This animal showed a marked response to ssDNA and a moderate response to nDNA and dsRNA. Antibody levels were maximum by thirty days post-infection, after which they remained elevated for at least thirty days before declining slightly.

Quantitation of nucleic acid antibodies. Antigen binding capacities were determined for sera of two animals serially bled (Table 1). These animals were chosen because they developed an immune response to all three nucleic acid antigens. In animal 841 the initial response to ssDNA and dsRNA occurred after fifteen days, rose to a maximum within the next two weeks and declined thereafter. Quantitatively the maximum response to all three antigens was in the same range. In animal 966 the

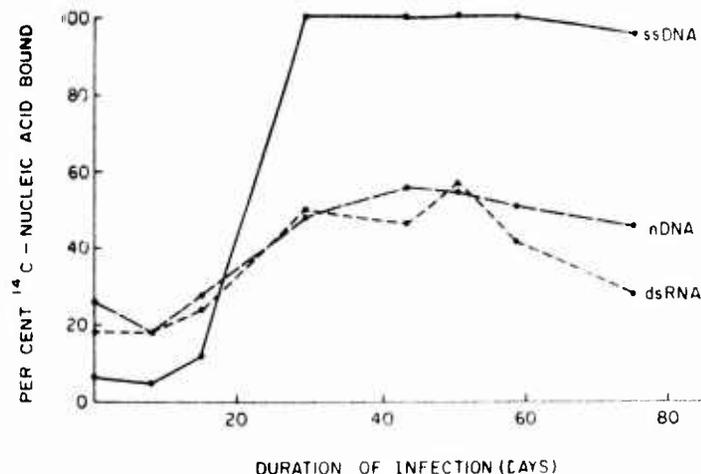


Figure 2. Time course of nucleic acid binding in one monkey. Three  $^{14}\text{C}$ -labeled nucleic acids were tested: ssDNA (●—●), nDNA (●---●), and dsRNA (●.....●). One month after infection, increased binding was observed with each nucleic acid.

initial immune response to all three ligands occurred by the fifteenth day after infection and rose to a maximum within one week. This animal differed from 841 in that its maximum response to ssDNA was four times higher than that of 841. For ssDNA there was a slight tendency in both monkeys for the immune response to peak, whereas with nDNA and dsRNA

the immune response tended to plateau.

Fractionation of antibodies to ssDNA and nDNA. Because of the marked increase in total IgM in these monkeys as well as in other animals infected with African trypanosomes, the type of antibodies to nucleic acids was of special interest. Antibodies to ssDNA were first chosen for further study because of the higher antibody levels to this ligand. Sera from animals 841, 566 and 966 were fractionated by Sephadex G200 chromatography. Since the quantities of sera available for fractionation were limited, pools of 2 consecutive bleedings were generally used. In one instance a single bleeding obtained at necropsy was fractionated. A pool from days 23 and 29 from monkey 841 had slightly more antibody to ssDNA in the 19S than in the 7S fraction (Table II). A later pool from days 43 and 50 obtained from monkey 566

TABLE I  
Quantitative determination of antibody response of two monkeys to native DNA (nDNA), heat-denatured DNA (ssDNA) and double-stranded RNA (dsRNA)

Monkey No.	Days Post-infection	ABC*		
		nDNA	ssDNA	dsRNA
		<i>µg/ml</i>		
841	0	<0.5	<0.5	<1.0
	8	<0.5	<0.5	<1.0
	15	<1.0	2.0	2.2
	23	7.5	7.7	8.8
	29	4.1	11	4.6
	36	3.2	6.5	3.6
966	0	<0.5	<0.5	<1.0
	8	<0.5	<0.5	<1.0
	15	4.3	10	6.2
	29	4.7	42	5.8
	43	5.0	44	5.0
	48	3.8	26	4.9

\* ABC = Antigen binding capacity expressed as µg antigen bound per ml of serum. These ABC's are accurate to two significant digits.

the greater amount of antibody to be in the 7S fraction. These results led us to study a single monkey (No. 966) at several times after infection. The first two pools available, one from days 23 and 29 and the other from days 43 and 50, had slightly more antibody in the 19S fraction (Table II). Antibodies in the final bleeding at necropsy were predominantly in the 7S fraction. The two fractionated pools from this monkey, which were tested against nDNA, had all the antibody in the 7S fraction (Table II).

Human sera. Availability of human sera from known infections with Trypanosoma gambiense permitted assessment of anti-nucleic acid anti-

bodies in naturally acquired trypanosomiasis. Antibodies to trypanosome antigens were detected by indirect immunofluorescence in all sera from patients with trypanosomiasis and in none of 11 control sera. There was a significant increase in antibodies to ssDNA ( $p < 0.0005$ , Fig. 3; Mann-Whitney U test). Six of 24 patients with trypanosomiasis had binding  $> 40\%$ ; all control sera had levels  $< 40\%$ . The distribution of antibodies to nDNA and to dsRNA were similar in the two groups; no significant difference between patients and controls was shown for either ligand.

TABLE II  
Relative proportions of antibodies to heat-denatured DNA (ssDNA) and native DNA (nDNA) in the 19S and 7S fractions of sera from monkeys infected with *Trypanosoma rhodesiense*

Monkey No.	Days Post-infection	% ABC to ssDNA <sup>a</sup>		% ABC to nDNA	
		19S <sup>b</sup>	7S <sup>c</sup>	19S	7S
841	Pool 23 + 29	54	46	ND <sup>d</sup>	ND
566	Pool 43 + 50	14	86	ND	ND
966	Pool 23 + 29	56	44	<15	95
	Pool 43 + 50	61	39	<15	96
	75	26	74	ND	ND

<sup>a</sup> For each pool the ABC for a given ligand in each fraction was calculated and the percentage of the total ABC for that ligand found in each fraction presented.

<sup>b</sup> Exclusion peak, Sephadex G-200 chromatography.

<sup>c</sup> 7S peak, Sephadex G-200 chromatography.

<sup>d</sup> Not determined.

Antibodies to nucleic acids developed in rhesus monkeys infected with *Trypanosoma rhodesiense*. Significant responses were detected to all three ligands tested. The most dramatic response was to ssDNA. Lower antibody responses were observed with nDNA and dsRNA. Maximum binding levels occurred at about 30 days after infection. This coincided with the onset of glomerulonephritis as determined by open renal biopsy.

Detailed quantitative studies of sera from two animals having an immune response to all three ligands showed an increase of at least 5-10 fold in antigen binding capacity. A single monkey (No. 966) had a 50-fold increase in antibodies to ssDNA. The quantities of antibody produced during trypanosomal infections are in the same range as is found in patients with SLE and in New Zealand mice.

A marked increase in serum IgM has been reported in African trypanosomiasis. These observations led us to carry out fractionations of sera to determine binding by 19S and 7S antibodies. Studies in three animals showed antibodies to ssDNA in both 19S and 7S fractions.

There tended to be a slight predominance of 19S antibodies early in the course of infection and 7S antibodies late in the course. In contrast, antibodies to nDNA were almost entirely in the 7S fraction. Previously anti-nucleic acid antibodies have been found in both 19S and 7S fractions.

The one other protozoan infection in which anti-nucleic acid antibodies have been reported is malaria. Rats infected with *Plasmodium berghei* developed agglutinins primarily to RNA with a lesser response to DNA; no information was given as to whether the DNA was native, denatured or a mixture of both. Malarial antibodies in healthy Africans showed a positive correlation with antinuclear factors detected by indirect immunofluorescence. These antibodies gave a speckled immunofluorescent pattern, apparently reacting with a nuclear glycoprotein rather than a nucleic acid.

Our studies with African patients infected with *Trypanosoma gambiense* showed a significant increase in binding to ssDNA but not to nDNA or dsRNA. Since all patients and controls lived in a region endemic for malaria, it seems unlikely that this infection would account for the increased levels of binding to ssDNA in trypanosomiasis.

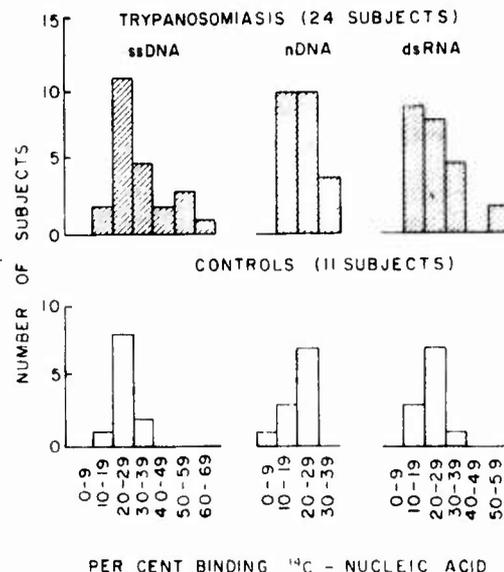


Figure 3. Binding of nucleic acids by human sera. The horizontal axis denotes percentage of radioactive nucleic acid bound in increments of 10%. The vertical axis indicates the number of subjects with binding in each increment. Increased binding of ssDNA was quite significant for patients infected with trypanosomiasis ( $p = 0.004$ , Mann-Whitney U test); there was no significant increase in binding of nDNA or dsRNA.

It should be emphasized that the source of the nucleic acid immunogens in African trypanosomiasis, in humans and in monkeys, remains undetermined. DNA could have come from damaged host cells or from trypanosomes which are notable for their kinetoplasts, an extranuclear DNA-containing organelle. With regard to the monkeys, infections with *Trypanosoma rhodesiense* could have led to activation of a latent viral infection resulting in production of anti-nucleic acid antibodies. This is supported by the intracytoplasmic viral-like inclusions that have been found in glomerular capillary endothelial cells from most monkeys infected with *Trypanosoma rhodesiense*. Similarly, interaction of two infective agents may have been responsible for the appearance of anti-nucleic acid antibodies in a patient with HAA-positive hepatitis and tuberculosis.

In patients with SLE and in New Zealand mice, autoimmune glomerulonephritis is associated with antibodies to nucleic acids. Similarly, monkeys infected with *Trypanosoma rhodesiense* developed glomerulonephritis and antibodies to nucleic acids. It is possible that in trypanosomal infections the antinuclear antibodies may be pathophysiologically related to the renal disease. To establish this relationship it will be necessary to demonstrate the concentration of antinuclear antibodies and their antigens in the kidneys of these monkeys.

## 2. Histochemical differentiation of the microfilariae of *Brugia pahangi* and sub-periodic *Brugia malayi*

*Brugia pahangi* and sub-periodic *B. malayi* co-exist in many areas of Malaysia where they are found in many of the same animal hosts. The latter, however, infects man as well as animals while the former is not thought to be a parasite of humans. These two species are difficult to differentiate morphologically, particularly in the microfilarial stage. Since the diagnosis of most human cases of filariasis is based on the detection of microfilariae in the peripheral blood, it is possible that a significant number of infections in Malaysia diagnosed as due to *B. malayi* may, in fact, have been due to *B. pahangi*. Experimental infection of *B. pahangi* in man has been reported. Also, clinical manifestations similar to tropical eosinophilia syndrome were reported in a human volunteer experimentally exposed to *B. pahangi*. Consequently, a relatively simple and reliable method of differentiating these two parasites would be advantageous in any field or laboratory study.

Chalifoux and Hunt introduced a histochemical method for demonstrating differences between the microfilariae of two common canine filarial worms, *Dirofilaria immitis* and *Dipetalonema reconditum*, based on the specific distribution of acid phosphatase activity in these organisms. Later, Chalifoux *et al.* using the same technique, were able to differentiate 11 types of circulating microfilariae isolated from seven species of New World monkeys.

The present study was designed to determine whether microfilariae of *B. malayi* and those of *B. pahangi* could be accurately and consistently

differentiated on the basis of the specific distribution of their acid phosphatase activity.

Thin blood smears were made from fresh syringe-drawn peripheral blood of patas monkeys (Erythrocebus patas) and gerbils (Meriones unguiculatus) experimentally infected with B. malayi and of cats and dogs with laboratory infections of B. pahangi. No anticoagulants were used. The smears were air dried, fixed on the same day in absolute acetone at 4°C, and stored in a refrigerator at 4°C. The activity of acid phosphatase of the microfilariae from each host animal was demonstrated according to the method of Barka and Anderson on freshly fixed slides and on slides that had been stored for 1, 2, 3, 4, 8, and 12 weeks. Naphthol AS-TR was used as the substrate and diazotized pararosaniline as the capturing agent. Slides were placed for one hour at 37°C in the incubating solution adjusted to pH 5.0. Three types of controls were used: 1) slides incubated without substrate, 2) those incubated in the presence of the specific inhibitor sodium fluoride (0.01M), and 3) slides incubated following exposure for five minutes to boiling distilled water. To establish the stability of the enzyme during storage, freshly prepared smears were processed together with slides which had been stored for increasingly longer periods of time.

A small number of slides also were processed according to the method of Gomori. The results were comparable to those obtained with the Barka and Anderson method and consequently the relatively more complicated Gomori technique was not used routinely.

The characteristic distribution of acid phosphatase activity as demonstrated by the Barka and Anderson method in B. malayi and B. pahangi microfilariae is shown in Figures 4 and 5, respectively. In B. malayi the excretory and the anal pores were the two most prominent sites of red azo dye precipitation, and each could be seen easily even at low magnification. Two other sites of somewhat lesser enzymatic activity were located one at the extreme anterior end and the other in an area equidistant from the anal pore and the tail tip. These locations corresponded to the position of the "roten Mundgebilde" or amphid and the "Schwanzgebilde" or phasmid organelles, respectively. The remainder of the body exhibited practically no enzymatic activity.

The microfilariae of B. pahangi, in contrast, showed heavy diffuse acid phosphatase activity along their entire length. The excretory and anal pores, however, were still recognizable because of their more intense staining.

The observed differences in acid phosphatase activity between B. malayi and B. pahangi appeared to be independent of the host source of either parasite.

These differential staining characteristics were closely dependent upon the pH. The intensity of the reaction was greatest at pH 5.0 but decreased significantly if this pH was raised or lowered more than 0.5



Fig. 4. Brugia malayi microfilaria. Sites which exhibit acid phosphatase activity are (from anterior to posterior) the amphid area, the excretory pore, the anal pore, and the phasmid area.

of a unit. Microfilarial staining was best defined when slides were processed immediately after fixation. Satisfactory differentiation could still be obtained on slides stored up to four weeks; however, progressively less enzymatic activity was noted as a function of time in storage. All control slides were negative for acid phosphatase activity.

Numerous methods have been described in attempts to differentiate microfilariae of sub-periodic B. malayi from those of B. pahangi. Schacher discussed his own attempts and those of numerous other workers to separate the two species by standard measurement techniques. Other investigators have described differences in the length of the "Innenkorper", variations in sheath-casting frequency, and morphologic differences in rectal protrusions. In addition, attempts have been made to separate the two filarial species by biologic means using mosquitoes

of the genus Armigeres which are very good vectors for B. pahangi but poor vectors for B. malayi. All these techniques, however, are time consuming, tedious, or not completely reliable for consistent differentiation. Finally, electron microscopic examination of the microfilarial stages of these two parasites has demonstrated no distinguishing characteristics.



Fig. 5. Brugia pahangi microfilaria. Acid phosphatase activity is heavy and diffuse along the entire body length. The excretory and anal pores are still recognizable.

The distribution of acid phosphatase activity in microfilariae offers an easily recognizable parameter for differentiating B. malayi from B. pahangi. Screening stained slides even at a low magnification becomes a relatively simple and fast operation which affords a high degree of confidence. The method can be taught easily to inexperienced technicians; all chemicals are inexpensive and commercially available. This histochemical method therefore represents a more advantageous and reliable technique than those previously described.

3. Effects of portocaval shunting on *Schistosoma japonicum* infection in chimpanzees.

Schistosomal nephropathy in man and in chimpanzees has been associated consistently with the presence of Symmers' pipe stem fibrosis of the liver, although some glomerular changes in mice, hamsters and monkeys have been observed in the absence of pipe stem hepatic fibrosis. Mild nephropathy in rabbits has been associated with portal fibrosis. Humans and chimpanzees with Symmers' fibrosis generally are more heavily infected than those without this lesion; therefore it has not been possible to differentiate between the effects of infection intensity and other possible etiologic links with Symmers' fibrosis. The present study was designed to dissociate intensity of infection and Symmers' fibrosis in *Schistosoma japonicum* infected chimpanzees by surgically creating end-to-side portocaval anastomoses on the eighth week of infection, so that most of the eggs were shunted from the mesenteric circulation into the lungs.

Eight young chimpanzees from West Africa were experimentally infected with the Japanese strain of *S. japonicum*. Prior to exposure routine physical examinations were performed, and stool and blood examinations were done to detect possible natural parasite infections. All animals appeared to be in good health.

Four animals (Nos. 204, 358, L364 and 367) had been utilized previously in *Plasmodium falciparum* experiments and had been cured of their malarial infections. One animal (No. 618) had a light microfilaremia. Stool examinations revealed the usual intestinal parasites (*Entamoeba coli*, *Balantidium coli*, *Strongyloides* sp. etc.). No schistosome eggs were seen.

*S. japonicum* cercariae were obtained from a pool of infected *Oncomelania hupensis nosophora* snails that had been exposed to miracidia approximately three months previously. Cercariae collections and percutaneous exposures of chimpanzees (Table III) were carried out as previously described. Beginning 33 days after exposure, feces from each chimpanzee were concentrated by the formalin-ether-buffered alcohol technique and the entire sediment was examined for schistosome eggs. After the onset of patency the procedure was repeated weekly until the termination of the experiment.

One week after eggs appeared in the feces, one uninfected and six infected chimpanzees (Table III) were subjected to an end-to-side portocaval shunt. The chimpanzees were sedated with intramuscular ketamine (10 mg per kg body weight), intubated and anesthetized with nitrous oxide and oxygen. The abdomen was opened by a midline incision and a Kocher maneuver performed in order to expose the portal vein and vena cava. The portal vein was divided and the proximal stump ligated. The distal portal vein was anastomosed to the vena cava in an end-to-side fashion utilizing 6-0 Epiflex running vascular sutures. The portal

TABLE III

Exposure of chimpanzees to cercariae of *S. japonicum* and the timing of subsequent surgical and necropsy procedures

Number	Chimpanzee		Total No. cercariae (35 per kg)	Shunt surgery (week post-exposure)	Duration of experiment (weeks)	
	Sex	Weight (kg)			Post-exposure	post-shunt
204	M	25.9	910	8	19	8
358	F	17.3	610	8	12	4
L364	M	17.7	630	8	38	30
367	M	15.6	580	8	38	30
618	M	13.0	715	8	15	7
761	M	12.8	410	8	38	30
759*	F	11.1	320	ND**	19	ND
758*	F	11.0	420	ND	19	ND
478	M	15.0	None	ND	38	ND
376	M	11.4	None	8	38	30

\*Chimpanzee #759 was treated on post-exposure week 11 with SQ 18,506; chimpanzee #758 was used as an infected, untreated control animal.

\*\*ND, not done.

TABLE IV

Egg excretion and worm recovery in chimpanzees infected with *S. japonicum*

Chimpanzee number	No. of cercariae	First eggs detected (weeks)	Egg excretion data			Worm recovery data		
			Peak egg count	Mean	Max.	Male	Total	Percent recovery
204 (S)**	910	7	18	60	118	6	12	1.3
358 (S)	610	7	7	2	3	ND	ND***	ND
1364 (S)	630	7	9	7	26	1	5	0.8
367 (S)	580	6	13	29	111	21	42	7.3
618 (S)	715	7	7	<1	1	0	0	0
761 (S)	410	7	10	23	136	7	14	3.4
759 (U)	320	7	7	1	6	0	0	0
758 (U)	420	7	12	20	62	59	79	18
478 (U)	None							uninfected, unshunted, control
376 (U)	None							uninfected, shuntea, control

\*NEIFPG, number of eggs per gram of feces

\*\*(S), shunted; (U), unshunted

\*\*\*ND, not done

vein was occluded for approximately 30 minutes for completion of the vascular anastomosis. No anticoagulants were utilized in the procedure. All of the animals tolerated the procedure well and were not routinely given prophylactic antibiotic therapy. They were allowed standard laboratory diet within 48 hours of the operation and in those cases where hepatic encephalopathy became clinically apparent, a low protein diet was substituted. All wounds healed satisfactorily.

Chimpanzee No. 358 died unexpectedly on week 12 after exposure. Chimpanzee No. 618 became extremely ill and was killed 15 weeks after exposure. Three animals were killed 19 weeks after exposure and the remaining 3 were killed 38 weeks after exposure. Before necropsy, cardiovascular function was evaluated with the animals under phencyclidine anesthesia. The left femoral artery was cannulated and a Swan Ganz thermodilution catheter was inserted into the right jugular vein and passed to the pulmonary artery, which was identified by the typical pressure contour. A systemic arterial catheter was placed in the femoral artery. The catheters were connected to calibrated pressure transducers for pressure measurement. Thermal dilution determinations of cardiac output were performed in triplicate using iced saline. Calculations were done by standard techniques supplied by the manufacturer. Subsequently, the glomerular filtration rate was determined using inulin. Following hydration with approximately 500 ml of intravenous 5% dextrose in water, the bladder was catheterized and 132 mg per kg body weight of inulin were injected intravenously. Half an hour later the bladder was flushed and urine was collected over two 20 minute periods and blood samples were taken after 10 minutes of each urine collection. Inulin concentrations in blood and urine were determined and the rates of inulin clearance in ml per kg body weight were calculated.

At necropsy, gross pathological manifestations were noted and adult worms were recovered by perfusion with 0.85% saline. The major organs were then dissected free, weighed and processed for future histopathological examination (10% buffered formalin fixation) and organ egg assay (unfixed, frozen). Organ egg loads were assayed after tissue digestion with 4% potassium hydroxide.

The percentages of cercariae recovered as adult worms were low and variable and ranged from 0 to 18.8%. All exposed animals passed eggs in their stools (Table IV). Data concerning exposure, portacaval shunting, and the numbers of worms and eggs found are detailed in Tables III to V. Higher percentages of eggs were found in the lungs and a lower percentage in the livers of shunted chimpanzees (Table V) than in unshunted chimpanzees studied previously. The lungs were perfused in three shunted chimpanzees. Only one male worm and no females were recovered (Table IV). The number of eggs per worm pair in the tissues was high in the shunted chimpanzees (Table V).

Two of the six *S. japonium* infected, shunted chimpanzees (Nos. 358 and 618) were experimental failures with low grade infections and early

TABLE V

Number and distribution of *S. japonicum* eggs in various organs

Chimpanzee number	Mean number of eggs per gram of tissue (X 1,000)						Eggs per worm pair (X 1,000)
	Liver	Small intestine	Large intestine	Lung	Other*		
204 (S)**	1.8 (47)***	0.3 (1.4)	0.7 (13)	3.0 (37)	0.3 (2.3)	ND	
358 (S)	0.1 (28)	<0.1 (1.0)	0.4 (58)	0.1 (12)	<0.1 (0.18)	ND	
1364 (S)	0.3 (9)	<0.1 (0.5)	2.2 (53)	3.6 (38)	ND	370	
367 (S)	3.4 (28)	<0.1 (0.4)	2.8 (23)	7.7 (47)	0.9 (2.0)	289	
618 (S)	<0.1 (64)	0 (0)	<0.1 (13)	<0.1 (20)	<0.1 (2.6)	ND	
761 (S)	1.3 (9)	0.6 (9.9)	2.8 (39)	8.7 (42)	ND	440	
759 (U)#	0.1 (12)	0.2 (17.4)	0.7 (62)	0.1 (2)	0.2 (6.9)	ND	
758 (U)	1.6 (50)	0.1 (2.3)	2.1 (47)	0.1 (0.5)	ND	109	
478 (U)							
376 (S)							

\*Other, the combined results of other organs examined for tissue egg deposition.

\*\* (S) shunted; (U) unshunted.

\*\*\*Numbers in ( ) indicate the percent distribution of eggs in the various organs.

#Chimpanzee #759 treated on post-exposure week 11 with SQ 18,506.

TABIE VI

Cardiovascular and renal function in S. japonicum infected chimpanzees

Chimpanzee number	Systemic arterial pressure (mm Hg)	Pulmonary arterial pressure (mm Hg)	Pulmonary systemic resistance*	Cardiac output (ml/min)	Cardiac index (ml/min/kg)	Renal inulin clearance (ml/min/kg)
204	190/140 (155)**	40/20 (30)	.19	2,540	98	2.1
1364	165/85 (115)	18/5 (10)	.10	1,280	72	1.9
367	145/95 (125)	25/7 (13)	.10	1,380	83	3.5
761	115/70 (85)	20/5 (12)	.14	800	68	2.3
759	160/105 (130)	40/15 (26)	.20	950	105	2.5
758	ND	ND	ND	ND	ND	3.4

\*Pulmonary systemic resistance =  $\frac{\text{Mean pulmonary arterial pressure}}{\text{Mean systemic arterial pressure} - \text{cardiac output}}$

the normal upper limit for the ratio of pulmonary to systemic resistance is <0.2.

\*\*Systolic/diastolic, the mean pressure is in parentheses.

demise. Schistosome lesions were limited to sporadic active granulomas of the gut, liver and lung. The surgical anastomoses were intact and patent. No pipe stem fibrosis or nephropathy were seen. Significant non-schistosomal lesions were present (see below), but the causes of death of these animals were not determined with certainty.

The portacaval anastomosis was patent and well-healed. The colon showed extensive, patchy active areas of inflammation and egg deposition (Fig. 6) with bilharziomata occurring near the cecum, rectosigmoid and surrounding involved lymph nodes. The liver showed moderate numbers of active and healing granulomas containing viable immature and mature, as well as degenerated, *S. japonicum* eggs. In addition, numerous viable eggs of *Capillaria* sp. were seen; these tended to be massed together in the midst of relatively milder and more densely fibrotic granulomatous reactions than those of *S. japonicum*. Only mild pipe stem liver fibrosis was noted on gross examination (Fig. 7). Microscopically the active portal lesions were milder than but comparable in degree of inflammatory activity to the 4 1/2 month infected control chimpanzee (No. 758) and to previously studied unshunted chimpanzees.

The lungs showed marked active granulomatous involvement. In addition, there were diffuse lymphoreticular hyperplasia of lymph nodes, focal hyaline droplet arteritis of the myocardium, esophagus (Fig. 8), liver and kidney, and Grade III active nephropathy of mixed granular and fibrillary type (Fig. 9). PAS staining hyaline granules also were seen occasionally in Kupffer cells of the liver (Fig. 10). These were smaller than but similar to those seen in the glomeruli and inflamed arteries. A sample of peripheral nerve showed focal lymphoid cell infiltration (Fig. 11).

All three portacaval shunts of these infected chimpanzees were patent and well healed. Active, as well as some involuting colonic patches of inflammation and egg deposition were seen in variable numbers and distribution. Animal No. 367 had a pararectal bilharzioma, and eggs were found in microscopic sections of the stomach and pancreas. None of the three animals had grossly evident pipe stem fibrosis of the liver. No active liver granulomas were seen in histological material, but there were degenerated and calcified eggs with involuting or healed granulomas in relatively small numbers. One animal (No. L364) had no significant portal fibrosis in microscopic sections; the other two animals (Nos. 761, 367) had mild increases of portal fibrous tissue of moderate density. This tissue lacked significant active inflammatory infiltration (Figs. 12, 13, 14). The focal occurrence of fibrous intimal narrowing, disruption or recanalization of middle sized portal veins represented healed schistosomal endophlebitis (Figs. 12, 13, 14). Hepatic artery branches were mildly or markedly dilated (No. L364) (Fig. 13) or thickened (Nos. 761, 367) (Fig. 12) with focal budding and branching (No. 367). These arterial changes were more pronounced in the shunted animals than in comparable unshunted controls.

g. 6. Chimpanzee No. 204, Sigmoid colon. Numerous maturing eggs of S. japonicum are approaching the mucosal surface in convoy-like fashion; there is active, patchy inflammation of the lamina propria and distortion of the mucosal pattern.

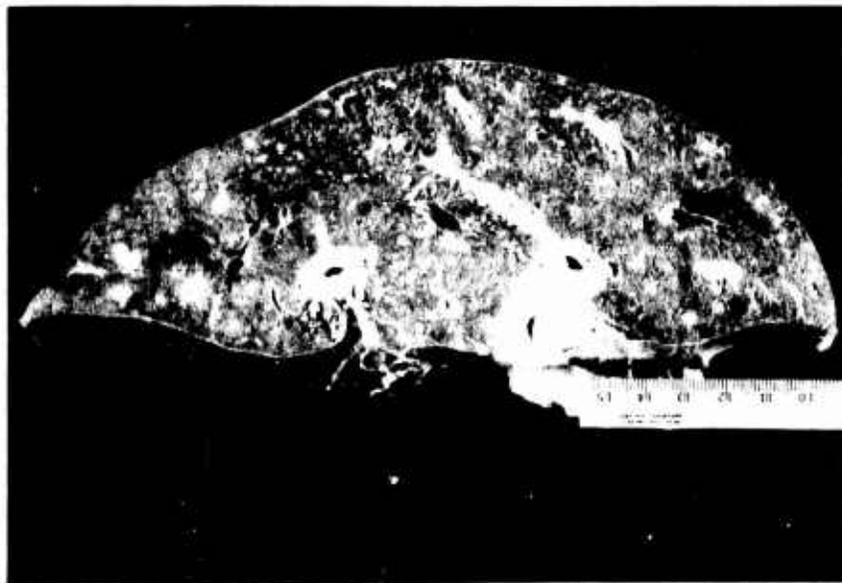
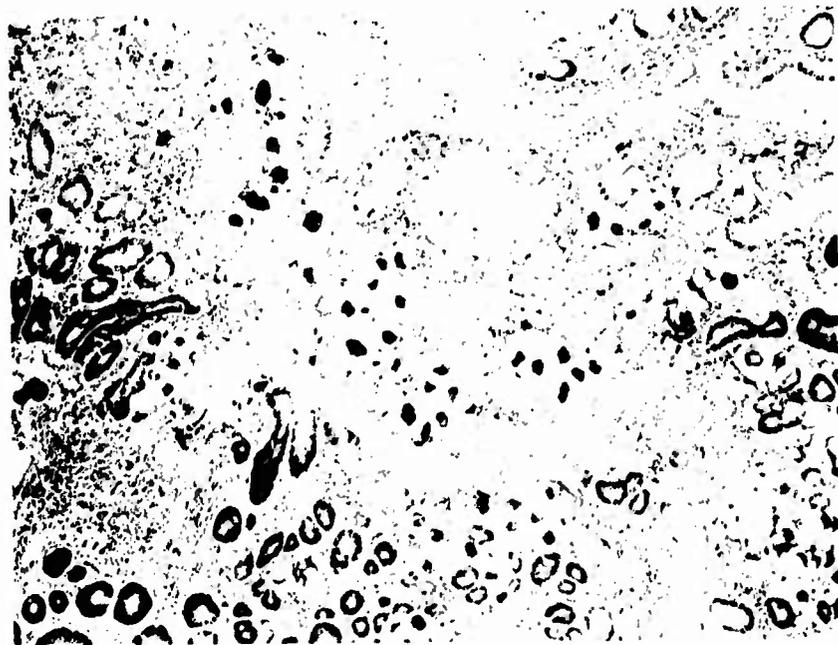


Fig. 7. Chimpanzee No. 204, Liver. The liver surface shows mild pipe stem fibrosis and focal, composite granulomas. The architecture is otherwise intact.

Fig. 8. Chimpanzee No. 204, Esophagus. Hyaline droplet arteritis (arrows) of a small artery is situated deep between smooth muscle bundles.

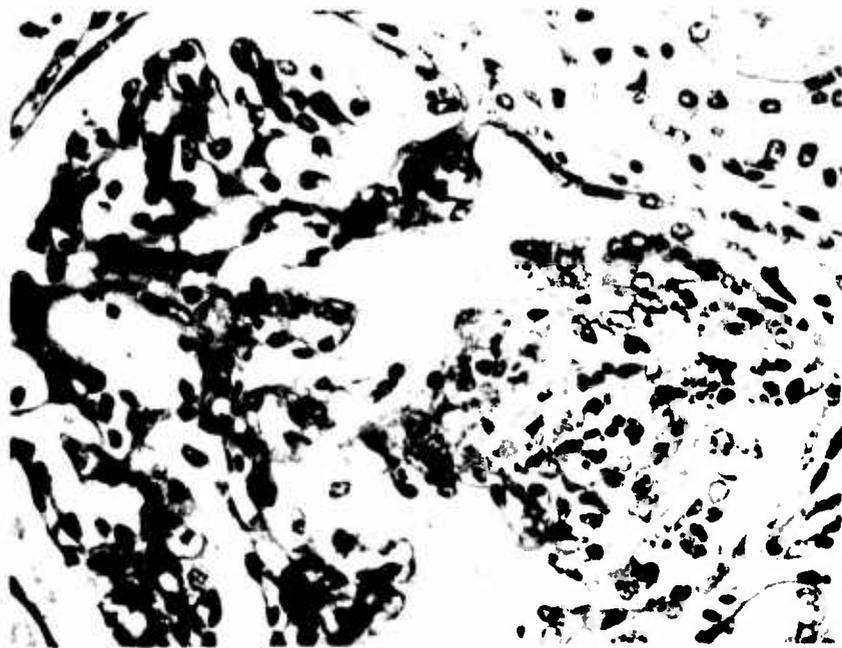
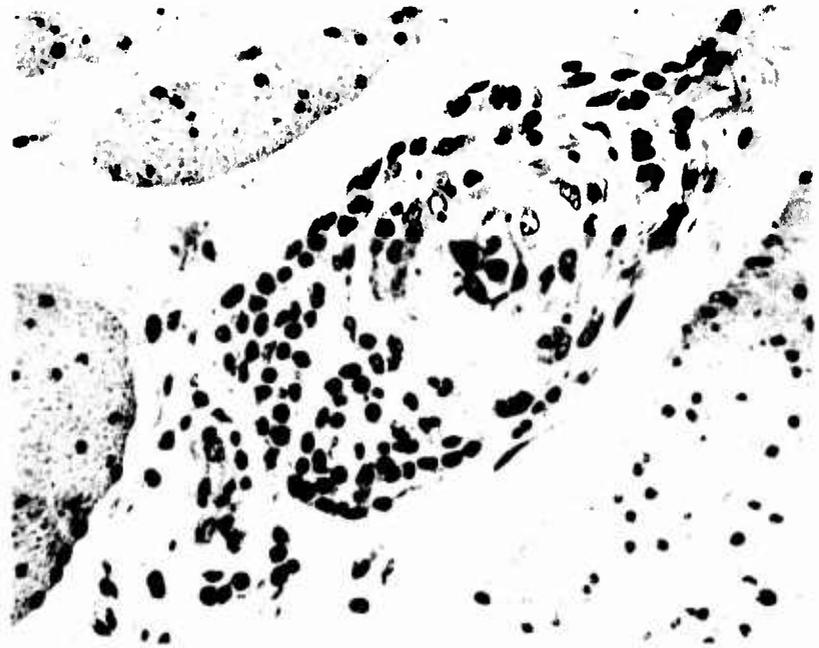


Fig. 9. Chimpanzee No. 204, Kidney. A section of glomerulus illustrates prominent focal fibrillary thickening and lobular foci of mesangial cell proliferation, the largest of which shows numerous hyaline granules.

The numbers of eggs and granulomas varied but were relatively abundant. Active granulomas with viable eggs were present in all three animals (Fig. 15), but significant pulmonary arteritis was not observed. Abdominal lymph node involvement at egg foci was marked. Animal No. 367 also showed mild focal thickening and inflammation of occasional systemic arterial branches without necrosis or hyaline droplets. Animal No. L364 exhibited nephropathy Grade I; no. 367 showed active nephropathy Grade II (Fig. 16); and No. 761 showed no significant renal lesions. Hyaline granules were not seen in the kidneys or the Kupffer cells of the liver. In summary, the schistosomal lesions of the liver in all three animals were inactive and contrasted with the marked inflammatory activity in the other infected organs. The least severe pathology was found in chimpanzee No. L364, the most severe in No. 367, while that in No. 761 was intermediate.

The infected, untreated, unshunted control animal (No. 758) showed the severe S. japonicum lesions usually present 4 1/2 months after exposure. These included pipe stem hepatic fibrosis, nephropathy and focal systemic arteritis. These lesions did not develop in the infected SQ-18,506 treated control (No.759).

In addition to hepatic capillariasis (No. 204), lung mites (Pneumonyssus sp.) were found in two animals (Nos. 618, 761); strongylids in two animals with severe (No. 761) or mild (No. L364) enteritis; a one foot long cestode (Bertiella studeri) in No. 761; and Oxyurus sp., Oesophagostomum sp. or unidentified intestinal helminths in several animals.

Animal No. 358 showed strikingly severe acute pulmonary congestion and edema; No. 618 had a colonic abscess and granulomas of the liver in which no organisms could be detected. Animal No. 367 also had several accessory spleens showing fibrosis of the pulp. A number of chimpanzees showed evidence of interstitial lymphoid cell aggregates of the renal cortex associated with atrophic glomeruli and tubules. This involvement appeared to be unrelated to the presence or absence of schistosomal nephropathy.

The ratios of pulmonary to systemic vascular resistance were within the expected normal range in all animals (Table VI). Inulin clearance was also in the normal range and was unrelated to the degree of nephropathy (Table VI).

Since the experimental protocol was successfully completed in only four of the shunted chimpanzees, conclusions must necessarily be limited.

The initial infection intensity was low and irregular; however, both parasitological and pathological findings suggest that there was a further decrease following the shunt. Thus, fecal egg excretion was less well maintained in shunted animals than in previously reported unshunted chimpanzees. The numbers of eggs per worm pair in the tissues

Fig. 10. Chimpanzee No. 204, Liver. The Kupffer cells in the center and on the left are filled with tiny hyaline granules; many leukocytes are seen in the sinusoids; the liver cell trabeculae are intact.

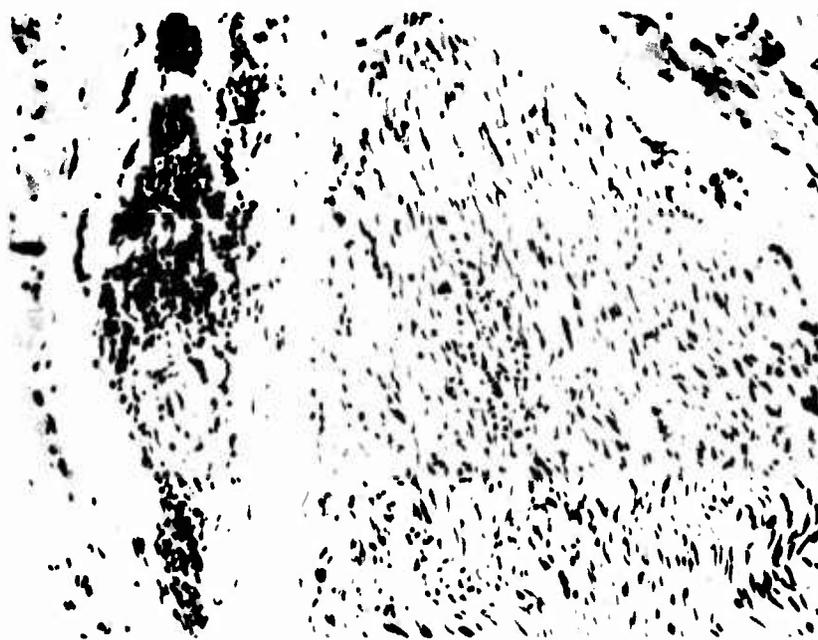
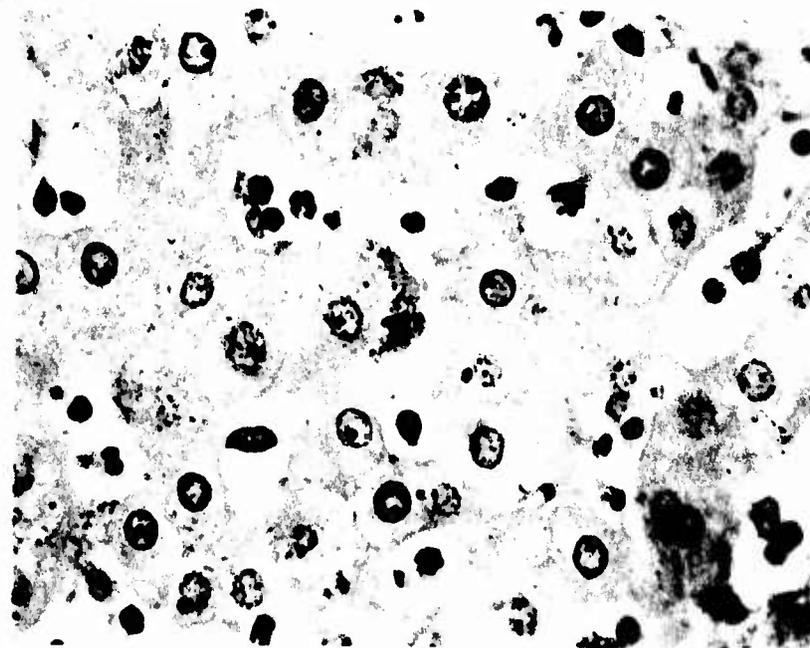


Fig. 11. Chimpanzee No. 204, Peripheral nerve. A lymphocytic infiltrate is inside a nerve fascicle and around an adjacent arteriole.

Fig. 12. Chimpanzee No. 367, Liver. The enlarged, fibrotic middle sized portal field lacks inflammation. This is a typical residual lesion.

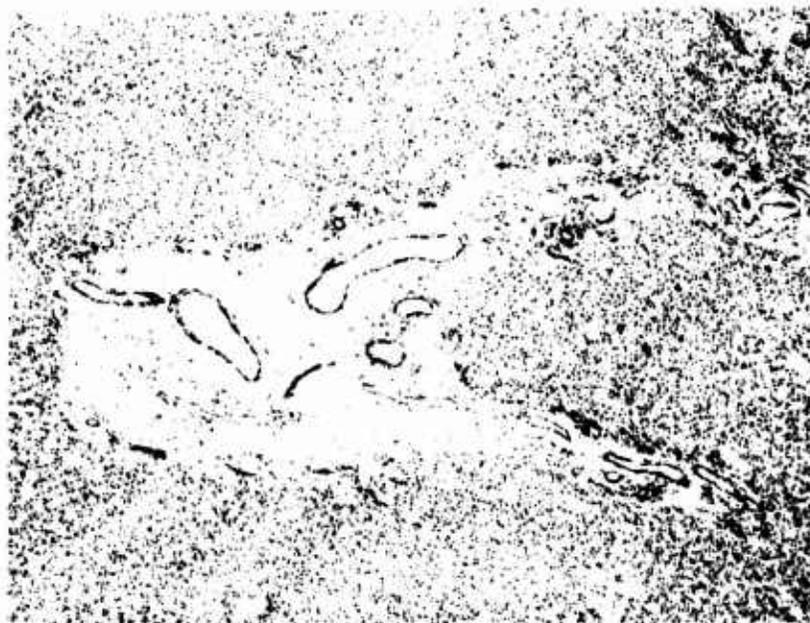
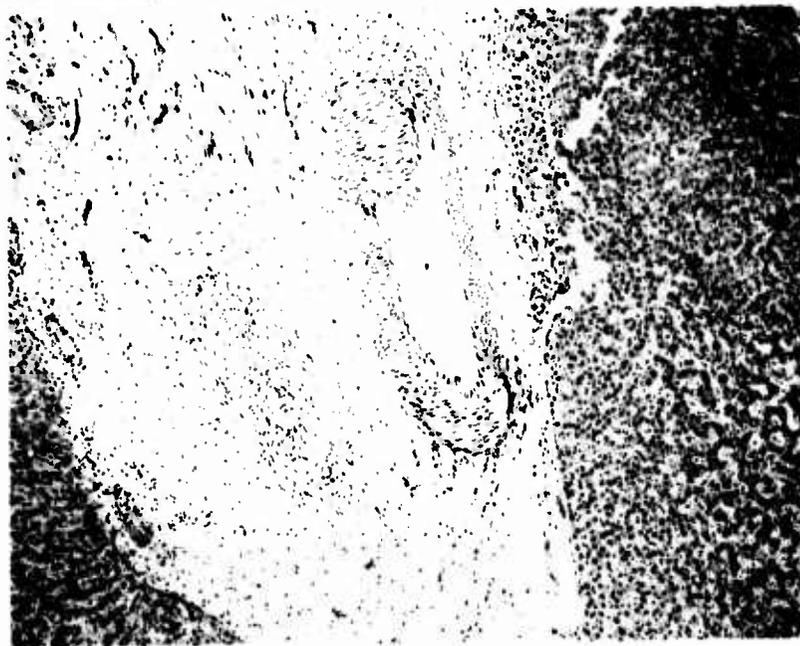


Fig. 13. Chimpanzee No. 761, Liver. A somewhat smaller, fibrosed portal field shows a recanalized, contracted portal radicle contrasting with multiple, widely dilated, arterial branches. The stroma shows mild residual inflammation.

of shunted chimpanzees were approximately three times higher (Table V) than in expected in unshunted chimpanzees which suggests that a larger number of worms had previously been present. Shih et al. recorded marked decreases in worm burdens in dogs subjected to portacaval shunting 7 to 22 days after infection with S. japonicum.

There was evidence of continued egg laying into the liver up to three months, but not at seven months, after shunting. This indicates that some worm pairs may have been trapped above the anastomotic site and that these worms subsequently died. Further worm loss could have resulted from migration of worms into the systemic venous circulation via the shunt or from other unknown factors. Despite the increased proportion of eggs reaching the lungs after shunting, significant pulmonary arteritis and cor pulmonale did not develop. The ratios of pulmonary to systemic vascular resistance did not change and indicates the absence of functional pulmonary vascular obstruction. The pulmonary hypertension noted in chimpanzees No. 204 and No. 759 was associated with systemic hypertension and a high cardiac index and is related to these factors (perhaps caused by anesthesia) rather than to pulmonary lesions.

The shift of egg burden from the liver to the lungs (Table V) proves that the end-to-side anastomosis was functionally and anatomically patent. Therefore, after the stranded intrahepatic worms had ceased laying eggs neither worm products nor eggs could reach the liver except by the arterial route. Concordantly, the liver pathology in all three long-term shunted animals was found to be inactive and stationary although marked inflammatory activity continued in the colon, lymph nodes and lungs. The fibrous portal lesions which were seen in only two of these animals lacked active inflammation, fibroblastic proliferation or basophilic edema as seen in progressive pipe stem fibrosis. These instead showed bland fibrous tissue and evidence of healed portal endophlebitis as seen in chimpanzees successfully treated with SQ 18,506, but with even less inflammatory activity.

The dilation and proliferation of hepatic arteries seen after portacaval shunting exceeded that described in pre-sinusoidal portal block caused by schistosomal pipe stem fibrosis alone. This is consistent with the development of a greater hepatic arterial compensatory flow after total surgical severance of the portal vein. However, the absence of this phenomenon in one of the shunted animals remains unexplained.

Unlike the liver pathology, glomerular lesions in animal No. 367 were found to remain active through the seventh month after portacaval shunting. On the other hand, nephropathy was neither enhanced nor accelerated by shunting and was similar to that of a group of unshunted S. japonicum-infected chimpanzees. The small number of animals available precludes analysis of the relationship between worm and egg burden and nephropathy, or between the degrees of liver and kidney

Fig. 14. Chimpanzee No. 761, Liver. An even smaller triad, demonstrates the multiple lumina of the portal radicle. The liver parenchyma is normal.

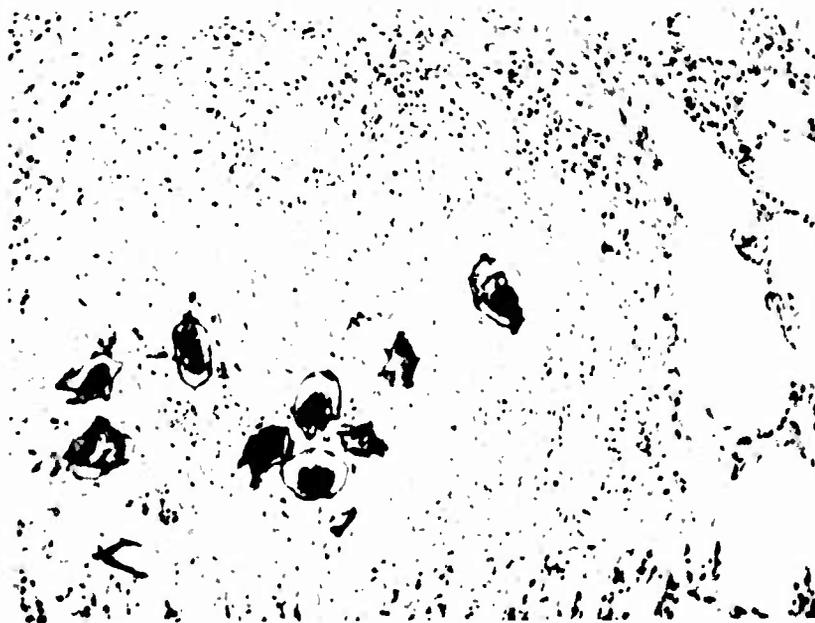
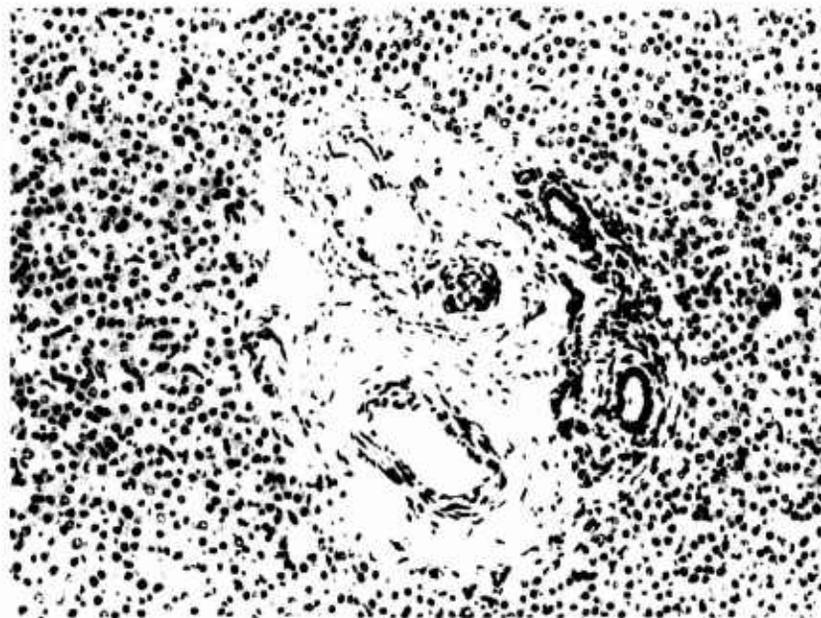


Fig. 15. Chimpanzee No. 367, Lung. The composite active granuloma of histiogrulocyte type contains clustered viable eggs which are sharply contoured by the stain.

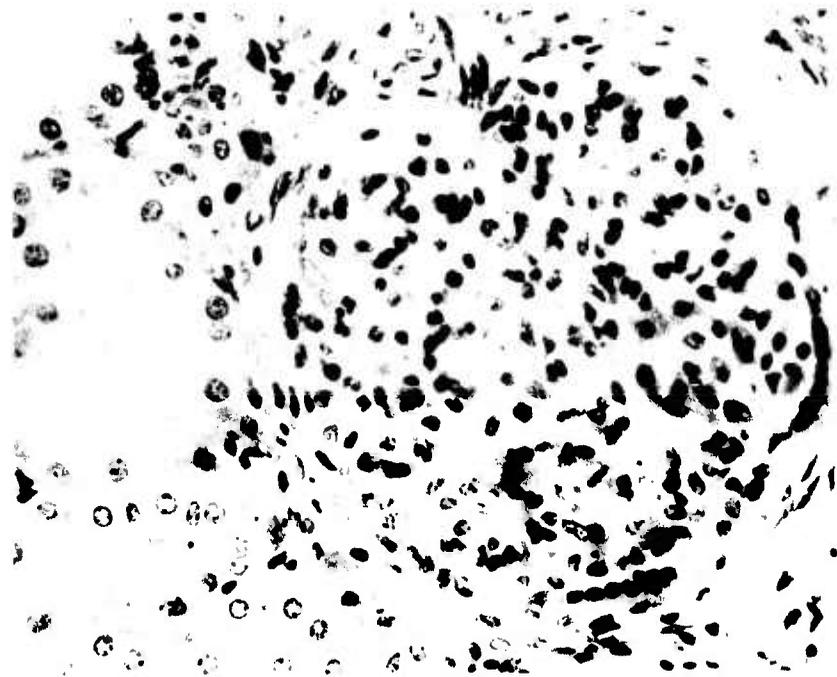


Fig. 16. Chimpanzee No. 367, Kidney. This glomerulus illustrates nephropathy Grade II with mesangial and capsular proliferation and some pericapsular fibrosis.

pathology, but it is clear that schistosomal nephropathy did not depend on the presence of active pipe stem fibrosis nor indeed on any significant portal lesions (note animal No. L364). There was thus neither evidence that any product of hepatic intermediary metabolism was involved nor that modification of schistosomal antigen by liver tissue played any role in the pathogenesis of nephropathy. All of the findings were compatible with the earlier suggestion that nephropathy is related principally to schistosomal infection intensity in a manner not yet adequately understood, but which possibly involves circulating worm antigen and/or host antibody.

The glomerular lesions described here caused no change in glomerular filtration rate; neither was there significant proteinuria noted in previously studied chimpanzees. Similarly, in patients with hepatosplenic schistosomiasis, focal glomerulitis frequently had no evident effect on renal function. In both host species renal tubular structure remained essentially intact.

Clearly there is marked host species variation with respect to schistosomal nephropathy and liver fibrosis. Variation in bilharzial liver pathology has been commented upon previously. Similarly, mice with heavy S. mansoni infections develop only mild glomerulosclerotic change, while human patients and chimpanzees progress to chronic proliferative and sclerosing glomerulonephritis. Other host species, such as hamsters, rabbits, and some monkeys appear to be elicit intermediate glomerular pathology. Although we have shown here that pipe stem fibrosis itself is not required for nephropathy, the frequent association of both lesions in humans and chimpanzees may still provide significant clues in searching for their pathogenesis.

Spontaneous glomerulonephritis occurs in a variety of experimental hosts but less frequently in chimpanzees than in Macaca irus. We must therefore remain cautious in our interpretations of renal lesions in schistosomiasis, especially those which are morphologically minor or noncharacteristic.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable diseases and immunology

Work Unit 165 Parasitic diseases of military importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL <sup>c</sup>	
				DA OA 6441	75 07 01	DD-1498 (AR) 636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY <sup>d</sup>	6. WORK SECURITY <sup>e</sup>	7. REGRADING <sup>f</sup>	8. DMS <sup>g</sup> INSTR <sup>h</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>i</sup>	10. LEVEL OF SUM <sup>j</sup>
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10. NO./CODES <sup>k</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3A161102B710	00	166			
b. CONTRIBUTING							
c. <del>CONTRIBUTING</del>	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>l</sup>							
(U) Viral Infections of Man							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>m</sup>							
002600 Biology 010100 Microbiology 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
63 08		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. FUND (In thousands)	
a. DATES/EFFECTIVE: NA				PREVIOUS			
b. NUMBER: NA				FISCAL YEAR		75	
c. TYPE:				CURRENT YEAR		76	
d. KIND OF AWARD:				PROFESSIONAL MAN YRS		6.5	
e. AMOUNT:				750			
f. CUM. AMT.				6.5		750	
20. RESPONSIBLE OGD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Russell, COL Phillip K.			
				NAME: Brandt, Dr. Walter E.			
22. KEYWORDS (Precede each with Security Classification Code)							
(U) Viruses; (U) Immunology; (U) Respiratory Tract Infections; (U) Arbovirus Infections							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Publish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To define etiology of acute infectious diseases of special hazard to military personnel, to determine and evaluate factors influencing occurrence, distribution, severity and medical result of human virus infections, and to develop means for reducing disability due to virus diseases.							
24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed for specific problems.							
25 (U) 74-07-75 06. To determine the potential contribution of antibody to individual structural and nonstructural dengue proteins in the pathogenesis of dengue hemorrhagic fever, a solid phase radioimmune assay has been developed with a structural hemagglutinin antigen(s) (SHA) and a nonstructural soluble CF antigen (SCF). Methods have been devised for purification of both antigens in acceptable yields. A temperature sensitive clone of Dengue 2 virus has been adapted to fetal Rhesus lung cell which will serve as an acceptable substrate for vaccine production. A basic difference in the mechanism of replication of two arbovirus groups was found in that the cell nucleus was shown to be essential for replication of the flavivirus, Japanese encephalitis virus, but not for replication of the alphavirus Sindbis. Results of immune cytolysis experiments indicate that influenza virus differs from paramyxoviruses by entering cells by a mechanism other than fusion of viral envelopes and cell membranes. Potent and stable adenovirus vaccines resulted in excellent control of adenovirus ARD in basic combat training posts with less than 5 percent of ARD hospitalizations associated with adenoviruses. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 July 1974 - 30 June 1975.							

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DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORM 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable Disease and Immunology

Work Unit 166, Viral Infections of Man

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Description

To define the etiology and ecology of human virus infections, particularly those of military medical significance; to devise and evaluate means for precise diagnosis, control and/or prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to understanding disease caused by respiratory, arthropod-borne and other viruses, the factors influencing transmission among men, other vertebrates and invertebrates, and their survival in nature.

Progress

I. The arthropod-borne viruses

A. Radioimmunoassay of dengue antigens and antibodies

A major goal of this department is protection of military personnel against dengue infection. One approach is immunization with attenuated virus vaccines (see following sections). The safety of this immunoprophylactic approach has been questioned in view of evidence that a severe clinical form of dengue infection, dengue hemorrhagic fever (DHF) may be mediated through immune mechanisms (Bokisch, et al. 1973). Thus, antibody raised by immunization might

participate in an immunologic reaction in dengue infection just as antibody induced by previous dengue infection. Since nonstructural as well as structural antigens are produced by dengue replication, it is possible that a nonstructural antigen may be responsible for DHF. One dengue nonstructural antigen, a soluble complement-fixing antigen (SCF), is of interest in that it contains both group and type reactive antigenic sites and is the predominant antigen found in the blood of suckling mice infected with dengue virus. In preliminary complement-fixation tests employing semipure SCF antigens, some but not all DHF patients developed anti-SCF antibody after illness. Antibody responses were generally low titered. In order to further explore the frequency of and type specificity of antibody to SCF after primary and secondary dengue, a more sensitive assay using highly purified SCF was required. Further, a sensitive and specific test to detect circulating dengue antigens in DHF patients is needed. Described below is the development of an RIA test for assay of antibody to specific dengue antigens which should be a valuable tool to determine differences in the immune response to virulent and attenuated dengue viruses.

#### 1. Preparation of dengue antigens for radioimmunoassay

Tartrate-glycerol gradients as described by Obijeski, et al. (1974) were investigated as a one-step physical purification method due to the gradient's combining effect of rate zonal and equilibrium centrifugation under certain conditions. Gradients were constructed with the aid of a peristaltic pump using 50% potassium tartrate and 30% glycerol; the tartrate decreased and the glycerol increased, proceeding upward from the bottom of the centrifuge tube. Either 14 ml gradients with up to 2 ml samples or 32 ml gradients with up to 6 ml samples were centrifuged in the Beckman 27 or 27.1 rotor at 25,000 rpm for 16 hrs at 40°C. Fractions were collected from the bottom of the tube and numbered accordingly.

Dengue-3 virus, the slowest replicating and lowest titering of the dengue serotypes, was examined on the tartrate glycerol gradients using 20% infected mouse brain suspensions in PBS that were either: 1) crude 10,000 rpm supernatants (SS 34 rotor); 2) crude material clarified with 2 mg/ml protamine sulfate; 3) ultracentrifuge pellets of the protamine sulfate clarified material; and 4) ultracentrifuge pellets of the crude material (40,000 rpm, 40 angle rotor). Results of the HA and CF tests on the gradient fractions are shown in Fig. 1. HA activity (solid line) at a 1:2 dilution in the lower half of the gradients probably represents the nonspecific effects of the tartrate. The tartrate was also anti-complementary (AC) in the lower portion of the gradient. Only CF titers greater than the AC activity are shown in Fig. 1 (dotted lines). A peak of HA antigen could not be observed in gradient 1 (crude 10,000 rpm supernatant) probably due to inhibitors, since an HA peak was observed in the protamine sulfate clarified aliquot in gradient 2. Gradient 3 represents about a 20-fold concentrate of the sample applied to gradient

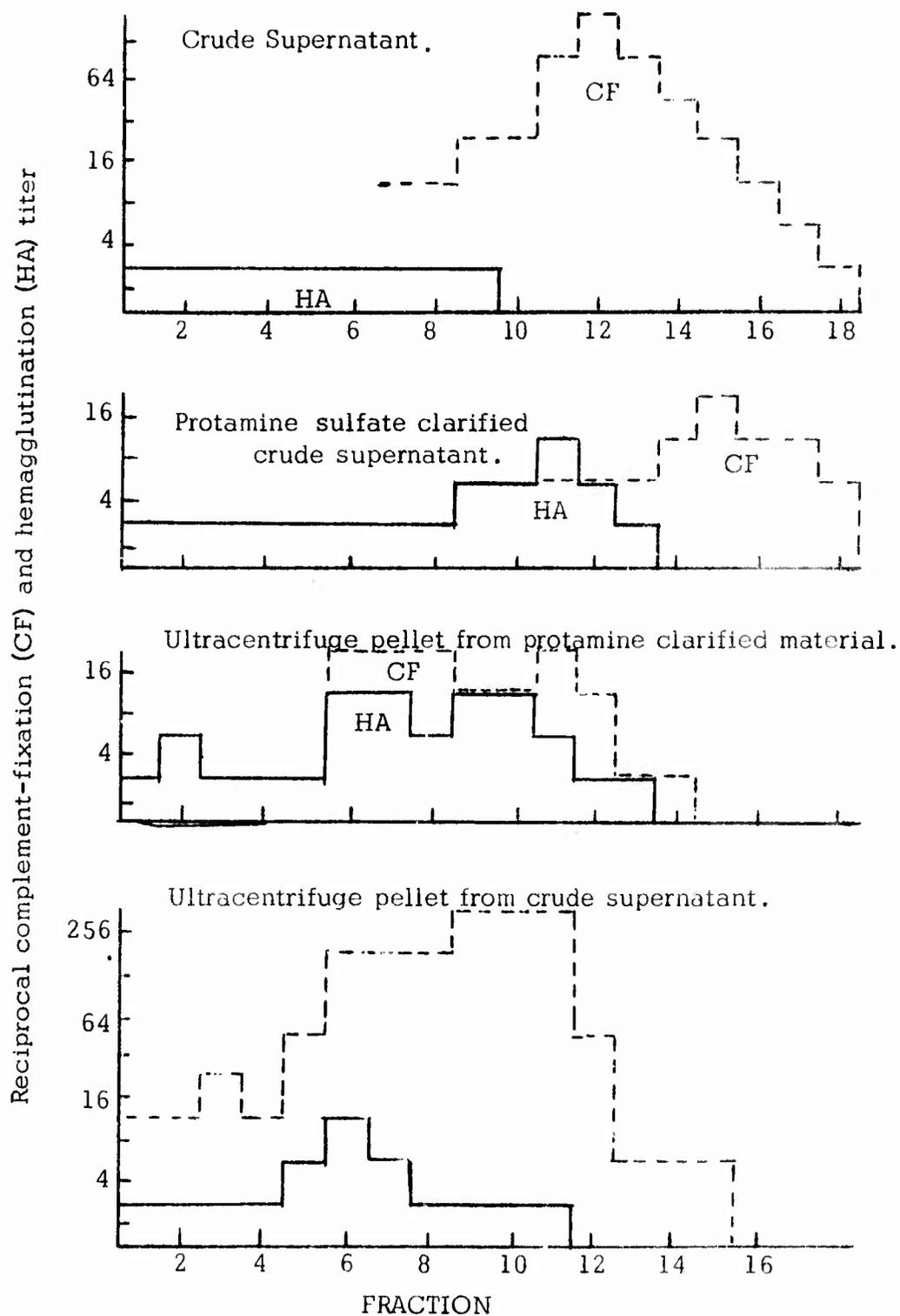


Figure 1. Tartrate-glycerol gradients (see text) of dengue-3 antigens derived from infected mouse brain as indicated in each panel.

2; some additional HA antigen can be seen. HA antigen in gradient 4 indicates that hemagglutinins may have been concentrated somewhat more than the inhibitors by ultracentrifugation of an aliquot of crude mouse brain shown in gradient 1. CF antigen was easier to detect in all of the gradients. It can be observed that the clarification of infected mouse brain suspensions with protamine sulfate, which removes HA inhibitors, also removes large amounts of CF antigen. (Compare gradients 2 and 3 with gradients 1 and 4 in Fig. 1).

Since we wish to measure antibody responses to hemagglutinating and soluble CF antigens independently, hemagglutinins for radioimmunoassays might best be obtained from protamine sulfate clarified mouse brain. Larger sample sizes of dengue-3 infected mouse brain treated in this manner contained 2 peaks of HA antigen on glycerol-tartrate gradients similar to that shown for concentrated HA shown in gradient 3. Further information and characterization of hemagglutinins on tartrate-glycerol gradients was obtained with dengue-2 virus since it replicates faster and to higher titer than dengue-3 virus. A 20% infected mouse brain suspension in PBS was clarified at 10,000 rpm (crude antigen), and a portion was further clarified with 2 mg/ml protamine sulfate. A larger portion was concentrated 10-fold by precipitation with 60% saturated ammonium sulfate. Approximately 4 ml samples were placed on 14 ml gradients which were centrifuged as described above. Microtiter hemagglutination tests on 36 0.5 ml fractions revealed 2 peaks of HA antigen in the crude and protamine sulfate treated dengue-2 infected mouse brain samples (gradients 1 and 2, Fig. 2). The sample concentrated 20-fold by ammonium sulfate (gradient 3) did not appear to have the lighter peak shown in gradients 1 and 2. There was instead a larger amount of HA at the top of the gradient, which may represent disrupted particles. The slightly heavier appearance of the bottom peak may have been due to somewhat larger fractions from the bottom of a faulty centrifuge tube. There were large amounts of contaminating material visible in the gradients of the crude and concentrated samples. The contaminants were greatly reduced by the protamine sulfate treatment which also allowed the detection of more HA antigen compared with the untreated aliquot, gradient 1).

Analysis of the denser or heavier tartrate-glycerol peaks by rate zonal centrifugation through 5-25% sucrose gradients as described in previous annual reports (1968-1973) revealed similarity with the 200S rapidly sedimenting hemagglutinin (RHA) previously shown to contain infectious virions. The lighter tartrate-glycerol peaks sedimented about 70S, suggesting a similarity to the slowly sedimenting hemagglutinin (SHA) described in previous annual reports. "Native" SHA consists of doughnut-like structures about 14 nm in diameter and has a density about  $1.23 \text{ g/cm}^3$ ; this may correspond to the center peak in gradients 1 and 2 in Fig. 2. "Derived" SHA consists of virion fragments and has a density about  $1.19 \text{ g/cm}^3$ ; this may correspond to the very light HA peak in the ammonium sulfate concentrated sample shown in Fig. 2, gradient 3. Additional experiments showed that RHA and SHA from sucrose gradients were

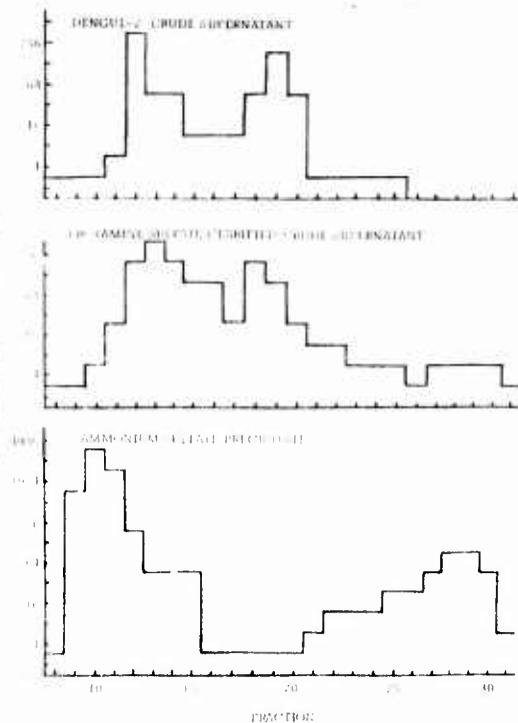


Figure 2. Tartrate glycerol gradients of dengue-2 antigens derived from infected suckling mouse brain as indicated in each panel.

found in the expected position in tartrate-glycerol gradients. Therefore, for the present work, we will refer to the heavier or faster peak, and the lighter or slower peak in tartrate-glycerol gradients, as RHA and SHA, respectively.

Preliminary RIA experiments (see below) indicated that hemagglutinins from tartrate-glycerol gradients were better antigens than hemagglutinins from sucrose gradients, so antigens of the other dengue serotypes were prepared on tartrate-glycerol gradients. Protamine sulfate treatment of infected mouse brain preparations was chosen as the clarification step prior to tartrate-glycerol centrifugation of unconcentrated 20% brain suspensions, since this removed a considerable quantity of CF antigen and increased the yield of detectable HA antigen. Dengue-1 infected mouse brain contained 2 peaks of HA but each was slightly lighter than the corresponding peak from dengue-2 infected mouse brain (compare gradients 1 and 2 in Fig. 3). Dengue-4 infected brain suspensions contained only one discernible HA peak which we suspect is RHA (gradient 3, Fig. 3) since it did not react with primate D-4 antibody in RIA experiments described below, hemagglutinins (HA) were first pelleted from large

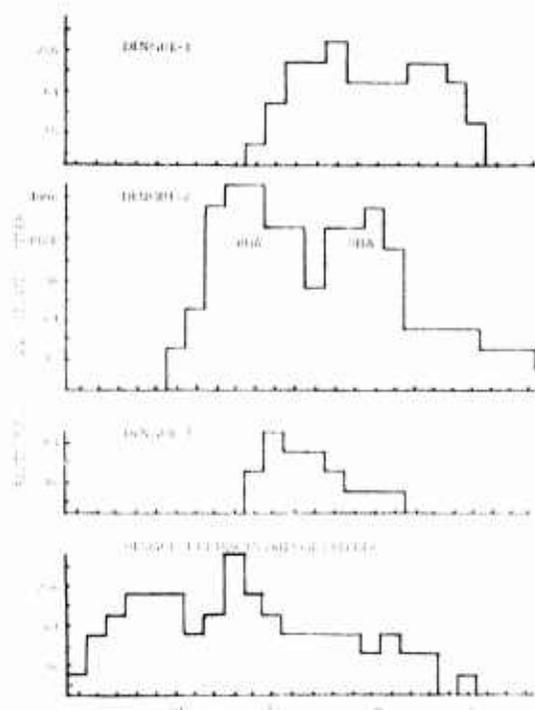


FIGURE 3.

Figure 3. Comparison of dengue types 1, 2 and 4 on tartrate-glycerol gradients. The first 3 panels represent samples of protamine sulfate clarified 20% infected suckling mouse brain suspensions. The last panel represents gradient centrifugation of pooled ultracentrifuge pellets from 334 ml of protamine sulfate clarified mouse brain.

quantities of protamine clarified infected mouse brain (250-350 ml) prior to tartrate-glycerol centrifugation. Panel 4 in Fig. 3 is representative of gradient profiles of dengue-4 pelleted hemagglutinins; the HA in this particular experiment was pelleted from 334 ml of 20% infected suckling mouse brain. HA titers here are probably not indicative of suitable potency for RIA tests; concentrated D-4 hemagglutinins from these gradients (center peak) were much better RIA antigens than the other serotypes having much higher HA titers (see below). Experiments with pelleted hemagglutinins of the other serotypes are underway to increase antigen potency and to determine if the pelleting procedure produces the dense HA peak seen in gradient 4, Fig. 3.

## 2. Method of radioimmunoassay

The following procedure was developed after examining the various aspects of this test. The reagents are dispensed into soft (flexible) microtiter plates (Cooke Engineering 220-24) containing 96 "U" shaped wells, each with a maximum capacity of 0.25 ml.

a. The first reagent, usually purified antigen diluted in Dulbecco's PBS, is added either dropwise with a 0.025 ml microtiter pipette, or injected with a Schwarz-Mann 25 lambda pipette. The microtiter plates are incubated for 1 hr at 36°C.

b. The antigen is aspirated from the microtiter wells with small Tygon tubing attached to a vacuum source; 0.05 ml of 10% fetal bovine serum (FBS) in Dulbecco's PBS is added to each well to simultaneously wash out residual antigen and to start to fill the remainder of the surface on the plastic well not coated with antigen. The 10% FBS is aspirated out and 0.1 ml 10% FBS is added and the plates incubated for 1 hr at 36°C to complete the "filler" activity of the FBS. The "filler" is aspirated from the wells which are now ready to receive antibodies.

c. Antibody, usually in the form of serum dilutions in 10% FBS, is injected in 25 lambda volumes into the bottom of each antigen coated well. The solid-phase reaction mixture is incubated for 2 hrs at 36°C.

d. The serum dilutions are aspirated from the wells and 0.05 ml PBS is added to wash out residual serum. The PBS is aspirated and another 0.05 ml volume of PBS is added and incubated for 30 min to soak off unbound globulins. The PBS is aspirated again and the plates are vigorously washed by filling them 14 times with tap water, or 40 sec in a plexiglass plate washing device which directs multiple jets of water simultaneously into the 96 microtiter wells. Excess water is removed by tapping the plates on lined absorbant paper and the plates are air dried for 30 min.

e. Radioactive ( $^{125}\text{I}$ ) antiglobulin (see following section) is injected in 25 lambda aliquots into the bottom of each well and the plates are gently "tapped" to insure even distribution in the well and then incubated at room temperature overnight in a moist atmosphere sealed plastic box with wet paper towels). The similar concentrations of antiglobulin added to each successive test with the same lot of iodinated globulin, i.e., 12 ng/25 lambda, may initially contain 50,000 cpm, but the radioactivity, of course, decreases with time.

f. The  $^{125}\text{I}$ -antiglobulin is aspirated from the microtiter wells, which then receive 0.05 ml PBS as a washing step. Another 0.05 ml PBS is incubated in the plates for 30 min to soak off nonspecifically attached antiglobulin. The PBS is aspirated and the plates are washed in tap water and dried as described above.

g. The wells are cut from the plate in strips and then individually into counting tubes for assay in a Nuclear Chicago Model 1185 gamma counter.

### 3. Iodination of anti-globulin preparations

Iodination procedures have been varied extensively throughout these studies in attempts to obtain high specific activity iodination without degradation of specific antibody. The following outlines the procedures currently employed for routine iodination of anti-globulins used in the R.I.A.

Antisera prepared in the goat to each of the immunoglobulins used (human IgG, rabbit IgG, and mouse globulins) were purchased from Antibodies, Inc., Davis, CA, at the highest available antibody concentration. Goat antisera were twice precipitated with ammonium sulfate (30% followed by 45%) to obtain the globulin fractions and stored at  $-20^{\circ}\text{C}$  in small aliquots. Protein concentration of these fractions was estimated using adsorbance at 280 m $\mu$  and the conversion factor of 1.4 OD units = 1 mg/ml. An aliquot of 100  $\mu\text{g}$  protein in a volume of 10-50  $\mu\text{l}$  was iodinated in each experiment. All dilutions of anti-globulin to achieve the desired protein concentration were performed using 0.25 M phosphate buffered saline (PBS).

Iodine-125 was obtained as carrier free  $\text{Na}^{125}\text{I}$  in aqueous solution (Ph 8-10) at the highest specific activity available (generally 400-800 mCi/ml) from New England Nuclear, Boston, MA. Isotope was always used within one month of processing and was diluted in 0.25 M PBS to yield a concentration of one mCi/10  $\mu\text{l}$  prior to use.

The actual procedures for iodination were as follows: an aliquot of anti-globulin containing 100  $\mu\text{g}$  of protein was placed in a small glass tube in an ice bath and a 10  $\mu\text{l}$  (1 mCi) quantity of  $^{125}\text{I}$  added. The reaction was initiated in the ice bath by the addition of 70  $\mu\text{l}$  of chloramine T (3.5 mg/ml) and mixing vigorously for 30 sec. The reaction was terminated after exactly 30 sec by the rapid addition of 100  $\mu\text{l}$  sodium metabisulfite (4.8 mg/ml) and further mixing. The following reagents were added to the reaction tube: 100  $\mu\text{l}$  sucrose solution (20% w/v), 100  $\mu\text{l}$  potassium iodide solution (.01 M) and 30  $\mu\text{l}$  phenol red solution (0.5%). The entire mixture was placed on a G-25 Sephadex column prepared in a disposable 5 cc syringe barrel by layering under the buffer above the gel bed. The column had been previously equilibrated in phosphate buffered saline containing sodium azide and 0.01 M KI. Approximate 1-2 ml fractions were collected and the void volume fractions containing peak radioactivity (but no phenol red color) were saved as the iodinated anti-globulin fractions. Samples were diluted 1:2 in Dulbecco's PBS to 0.1% sodium azide and 10% fetal bovine serum and stored at  $4^{\circ}\text{C}$ .

Since variation is common using this iodination procedure, anti-

globulin samples are checked by a variety of methods prior to use. A 5  $\mu$ l aliquot is precipitated with trichloroacetic acid (TCA) 10% to determine the percent radioactivity bound to protein. A lower limit of 70% precipitable counts has been established; however, most preparations generally exceed 90%. Calculations of recovery using estimates of counter efficiency, etc., indicate that 35-40% of the  $^{125}\text{I}$  added to the reaction mixture is recovered bound to protein. Deviations from these values indicate an error in the labeling procedure and samples are discarded.

An estimate of the radioactivity bound to specific antibody is made using a primary radioimmunoassay procedure as described in the previous section. Frozen aliquots of normal serum pools, representing each animal species for which iodinated anti-globulins have been prepared (human, mouse, rabbit), are thawed, diluted (a 0.5  $\log_{10}$  dilution scheme) and added to the radioimmune assay plates as antigens. Following the addition of 10% fetal bovine serum to fill the remainder of the space on the plastic surface dilutions of freshly iodinated anti-globulins are added, incubated overnight at room temperature and counted. Each anti-globulin preparation is tested against all antigen dilutions at two concentrations of approximately 50,000 and 150,000 cpm/25  $\mu$ l (the volume) added to each well. A prozone of reduced binding is always observed; however, maximum binding is generally obtained at antigen dilutions of 1:1000 or greater. The percentage of radioactivity binding varied with the various anti-globulin preparations; however, the lower acceptable limits were adopted as 10-15% for anti-human, 5-10% for anti-rabbit, and any value approaching 5% was considered acceptable for anti-mouse.

Iodinated anti-globulin preparations were used for one month or until exhausted. A dilution was determined that met all of the criteria discussed above, as well as yielding 7-10,000 cpm bound to the globulin in a standard positive radioimmunoassay test, and this dilution was constantly used for a given preparation.

#### 4. Evaluation of dengue hemagglutinins from mouse brain as RIA antigens

RHA and SHA from tartrate-glycerol gradients of protamine clarified dengue-2 infected mouse brain were each diluted to contain 8 units of hemagglutinin/0.025 ml. Following the coating of the micro-titer wells with these antigens according to the RIA test described above, 2-fold dilutions of dengue-2 immune chimp serum (No. 680, which had a CF titer of 1:64) were reacted with each antigen, and then labeled antiglobulin was used to detect the chimp antibody bound to that particular antigen. The results in Fig. 4 suggest that SHA might be a better antigen for RIA tests since more CPM were bound to the antibody attached to SHA than to RHA. For this reason, subsequent studies on required purity of the antigen derived from mouse brain were carried out with SHA. These experiments also established that 2-fold serum dilutions produced more data points than necessary, so that subsequent

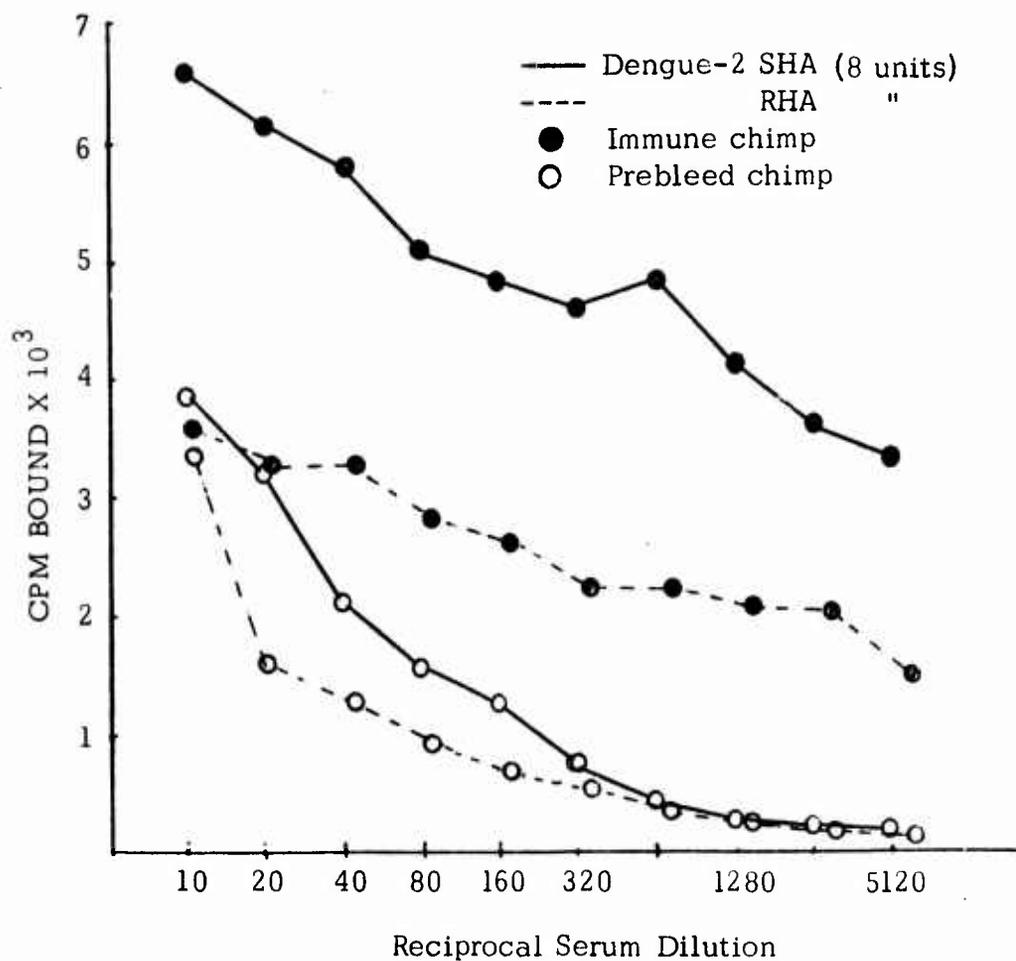


Figure 4. Radioimmunoassay of antibodies in dengue-2 immune chimp (no. 680) serum when tested against the rapid- and slow-sedimenting hemagglutinins, RHA and SHA.

studies utilized a  $0.5 \log_{10}$  serum dilution scheme.

Various concentrations of SHA from protamine-clarified brain suspensions on tartrate-glycerol gradients were reacted with dilutions of the dengue-2 immune chimp serum; the results are shown in Fig. 5. An individual curve represents the CPM bound when successive  $0.5 \log_{10}$  serum dilutions are reacted against a single concentration of antigen, following addition of a single concentration of labeled anti-globulin. While the height of any curve is dependent on the concentration of labeled antiglobulin, it appeared that as the antigen concentration decreases, the height of the curve decreases, indicating antigen saturation has not been achieved with this particular preparation of antigen. HA titration of the antigen added to the microtiter plates after storage at  $-70^{\circ}\text{C}$  revealed only 3 HA units/0.025 ml as the highest concentration of antigen

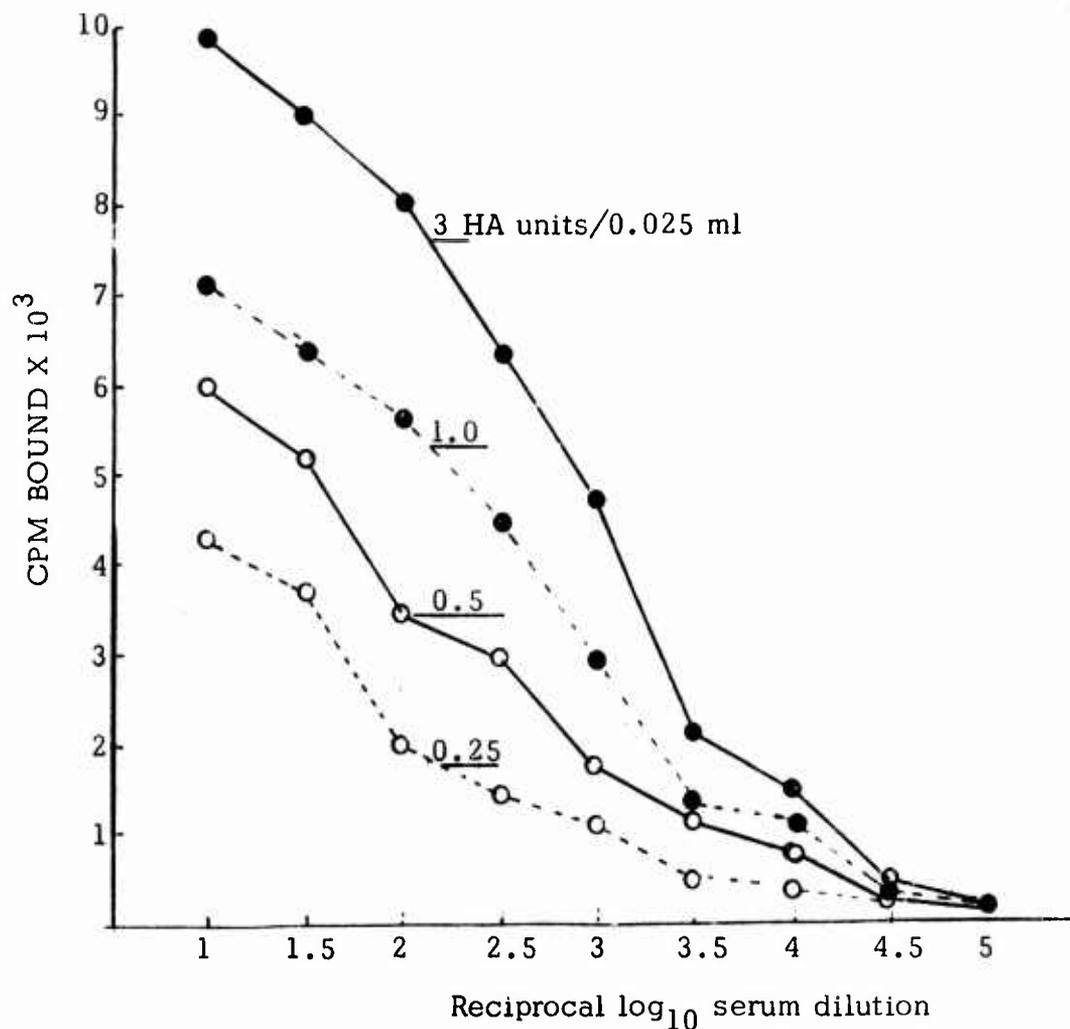


Figure 5. Effect of low concentrations of dengue-2 SHA on the radioimmunoassay of dengue-2 immune chimp serum. Hemagglutinin (HA) units are per 0.025 ml volume placed in the microtiter wells to coat the plastic surface.

in the test.

If dengue-2 SHA was obtained from a tartrate-glycerol gradient of crude dengue-2 infected mouse brain, there were essentially overlapping curves at the two highest antigen concentrations used (8 and 2 HA units/0.025 ml), indicating that antigen saturation under these conditions occurred with 2 HA units (Fig. 6). However, the maximum CPM bound in the reaction mixture was less than that bound in the reaction mixture containing hemagglutinin from protamine-sulfate clarified mouse brain (8,000 compared to 10,000 CPM). The apparent antigen saturation in this

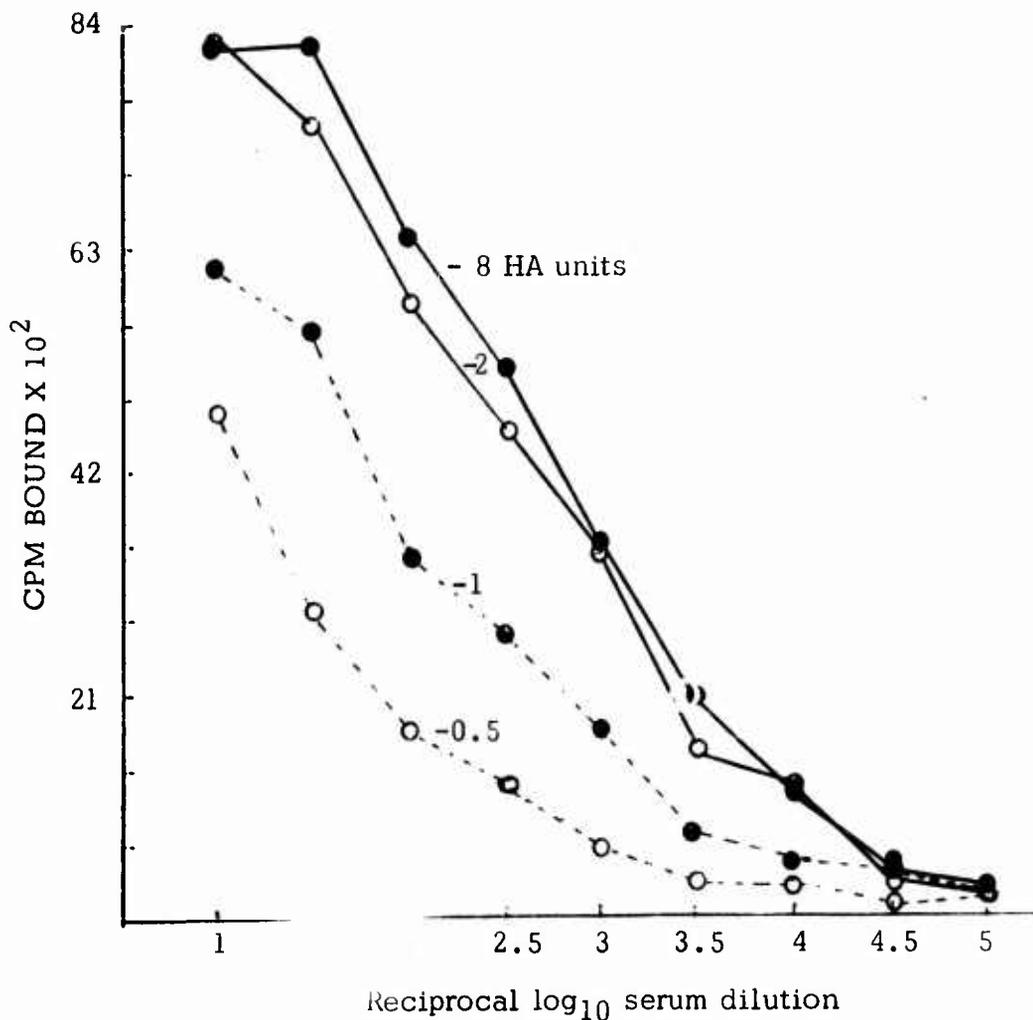


Figure 6 . Radioimmunoassay of dengue-2 immune chimp serum when tested against several concentrations of dengue-2 SHA obtained by gradient centrifugation of crude infected suckling mouse brain supernatants.

case may represent interfering contaminants in the crude mouse brain preparation not treated with protamine sulfate, since in separate experiments, added contaminating protein was shown to block the attachment of antigen to the microtiter wells. In completely evaluating the RIA system, a considerable amount of effort might have to be expended toward documenting the fact that overlap of curves at highest possible antigen concentrations truly represents antigen saturation, if such documentation is even necessary. The use of sufficient antigen to bind a significant number of counts with a reference antiserum may be all that is necessary. The above data suggest that smaller concentrations of a

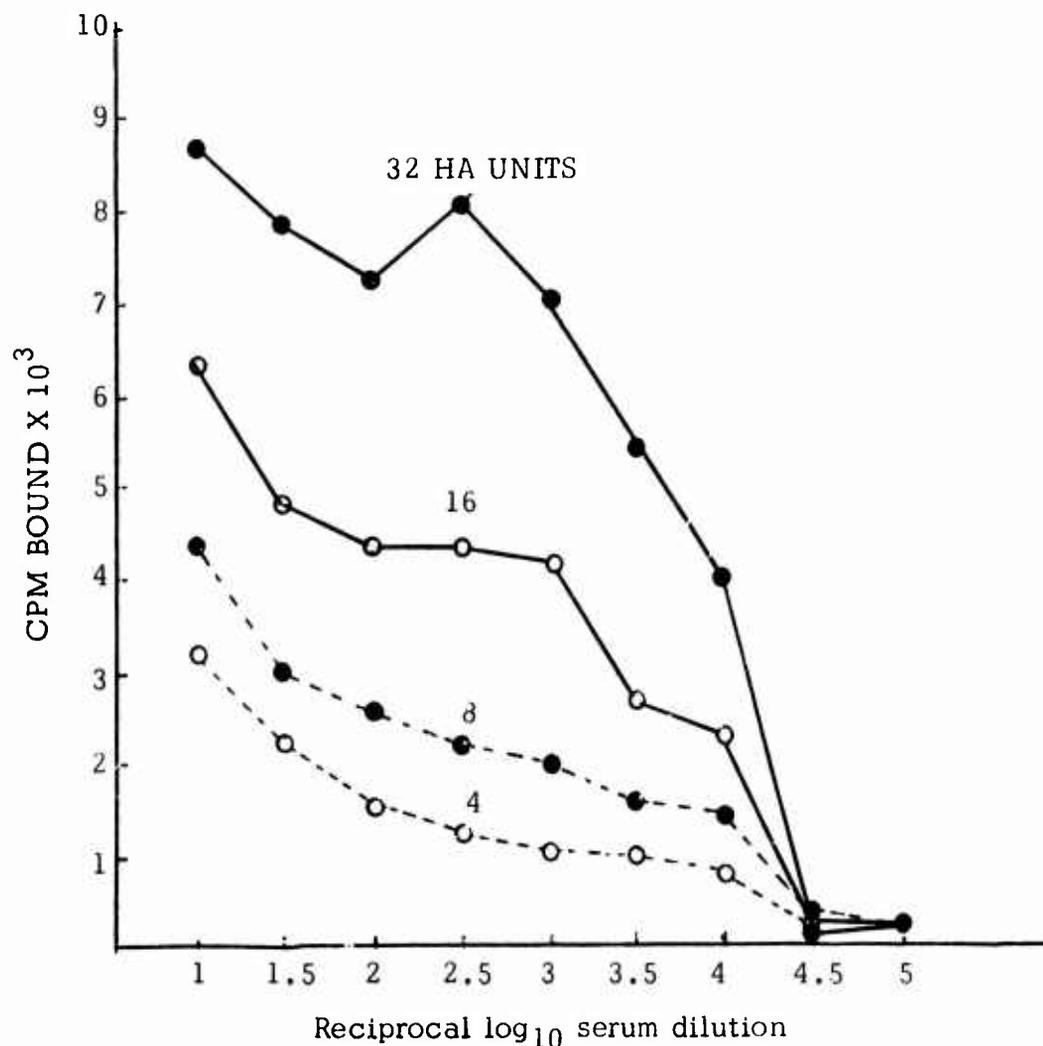


Figure 7 . Radioimmunoassay of dengue-2 immune chimp serum (680) when reacted against higher concentrations of SHA obtained by gradient centrifugation of protamine sulfate clarified suckling mouse brain supernatants.

more purified antigen preparation result in a more sensitive test.

When using another lot of labeled antiglobulin, up to 32 HA units from the cleaner protamine sulfate clarified brain suspensions were used and antigen saturation still had not been reached (Fig. 7). This lends support to the likelihood that contamination in the less pure antigen preparations produced an artifactual antigen saturation level with only 8 HA units. Useable antigens for RIA could be obtained by simply placing protamine-clarified infected brain suspensions on tartrate-glycerol gradients for dengue types 1, 2, and 3. A plateau was produced

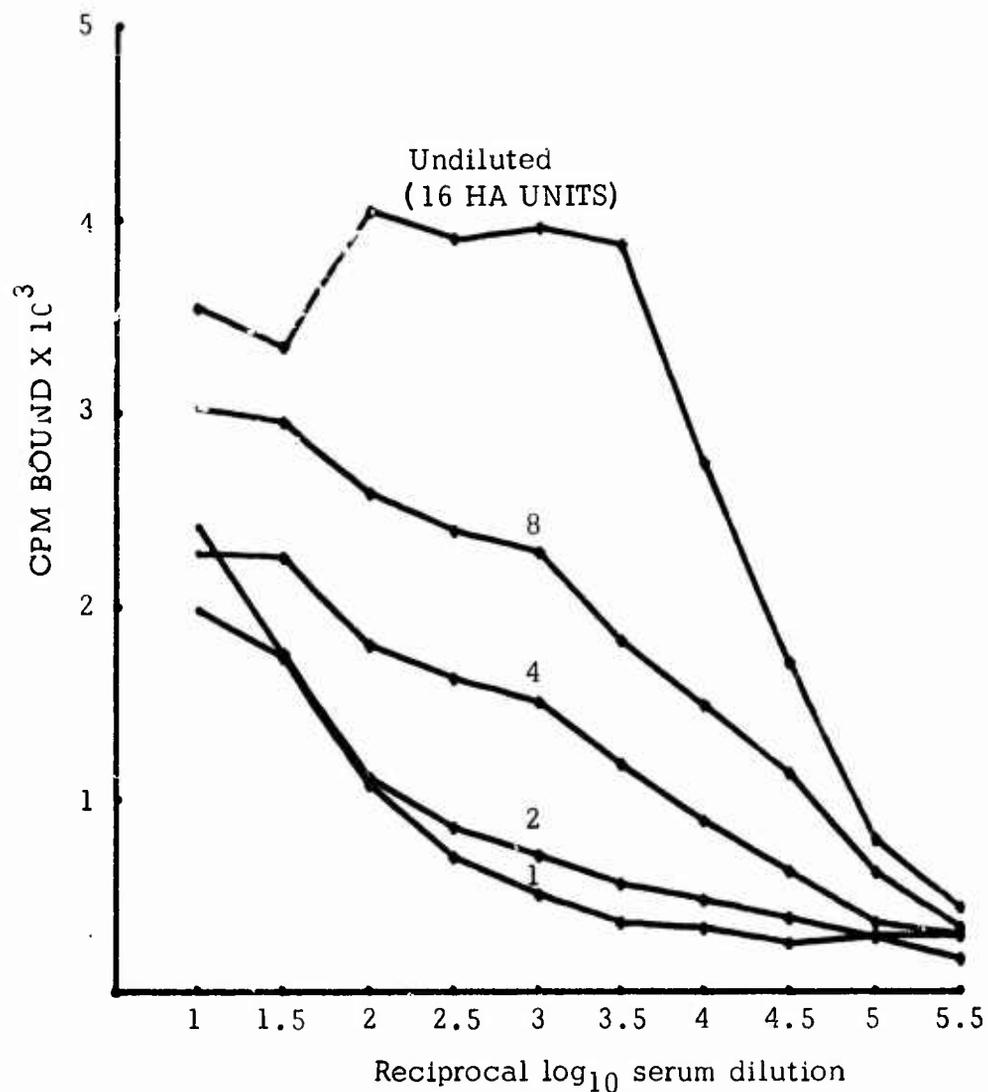


Figure 8 . Radioimmunoassay of dengue-3 immune human serum (Thai 54324) when reacted with various concentrations of dengue-3 SHA. The plaque reduction neutralization titer of the serum was 1:1280 to D-3, and 1:340 or less to the other dengue serotypes.

with undiluted type 3 HA antigen direct from the gradient when it was reacted with Thai human serum 54324 having a dengue-3 plaque reduction neutralization titer of 1:1280 (Fig. 8). These particular results show that the antigen concentration has a critical effect on the shape of the curve produced by the serum dilutions, and that the serum dilution

endpoint is altered whether a 50% value of maximum CPM bound is chosen, or whether a certain CPM above background is used. Using undiluted antigen, the RIA titer of this serum is about 10-fold higher than the PRNT titer, underscoring the potential sensitivity of the test.

Some problems were encountered in preparing dengue-4 hemagglutinins for RIA tests. In the same manner that the other dengue serotypes were prepared, 20% infected brain suspensions clarified with protamine sulfate were placed on tartrate-glycerol gradients. SHA peak titers of 1:8 to 1:16 were insufficient to react with broadly reactive dengue-4 immune chimp serum in the RIA test. We used double infection serum from chimp 682 which received a second infection with type 4 virus following a dengue-2 infection. We then concentrated the hemagglutinins from lots of 250 to 350 ml of protamine-sulfate clarified mouse brain by pelleting them in the ultracentrifuge. The pellets were resuspended in volumes of PBS to effect a 100-fold concentration and then were placed on tartrate-glycerol gradients as described above. Each of the 2 peaks as shown in Fig. 3, panel 4, behaved as potent RIA antigens when reacted with serum from chimp 682. Various concentrations of the less dense peak (probably RHA) were reacted with 0.5 log<sub>10</sub> dilutions of chimp 682 (Fig. 9). Antigen dilutions of 1:2 through 1:16 (8-64 HA units) produced essentially overlapping curves with the serum dilutions. These are shown above the dotted line in Fig. 9. The test was repeated with higher antigen dilutions as shown below the dotted line. About the same overlap of curves was obtained with 8 and 16 HA units at the lower serum dilution as the first time, but less CPM of the same <sup>125</sup>I-antiglobulin were bound; the latter is an unpredictable, but not serious, variable in the test because of the overlap in curves at the higher serum dilutions which produced similar serum endpoints. The type 4 HA still reacted with the chimp serum at lower HA concentrations (Fig. 9), whereas the same HA titers from unconcentrated dengue-4 preparations were not effective as RIA antigens. In general, it appeared that the RIA potency of any given preparation of any dengue serotype could not be predicted from the HA titer. This type 4 HA antigen was the most reactive of the 4 serotype antigens with broadly reactive antibody from humans with second dengue infections, probably due to its greater potency. Effective and/or standardized quantities of antigen may have to be based on a percentage of the antigen concentration that produces an overlapping curve with the next higher concentration of antigen when tested against a reference antiserum. We will attempt to obtain antigens of sufficient potency from all the dengue serotypes and standardize them based on their reactivity with reference primate antiserum.

5. Propagation of dengue virion antigens in LLC-MK<sub>2</sub> cells for use in radioimmunoassays.

Mouse brain derived hemagglutinin antigens used in RIA tests for dengue antibodies have some disadvantages that directed investigations into other methods of antigen production. First, mouse brain

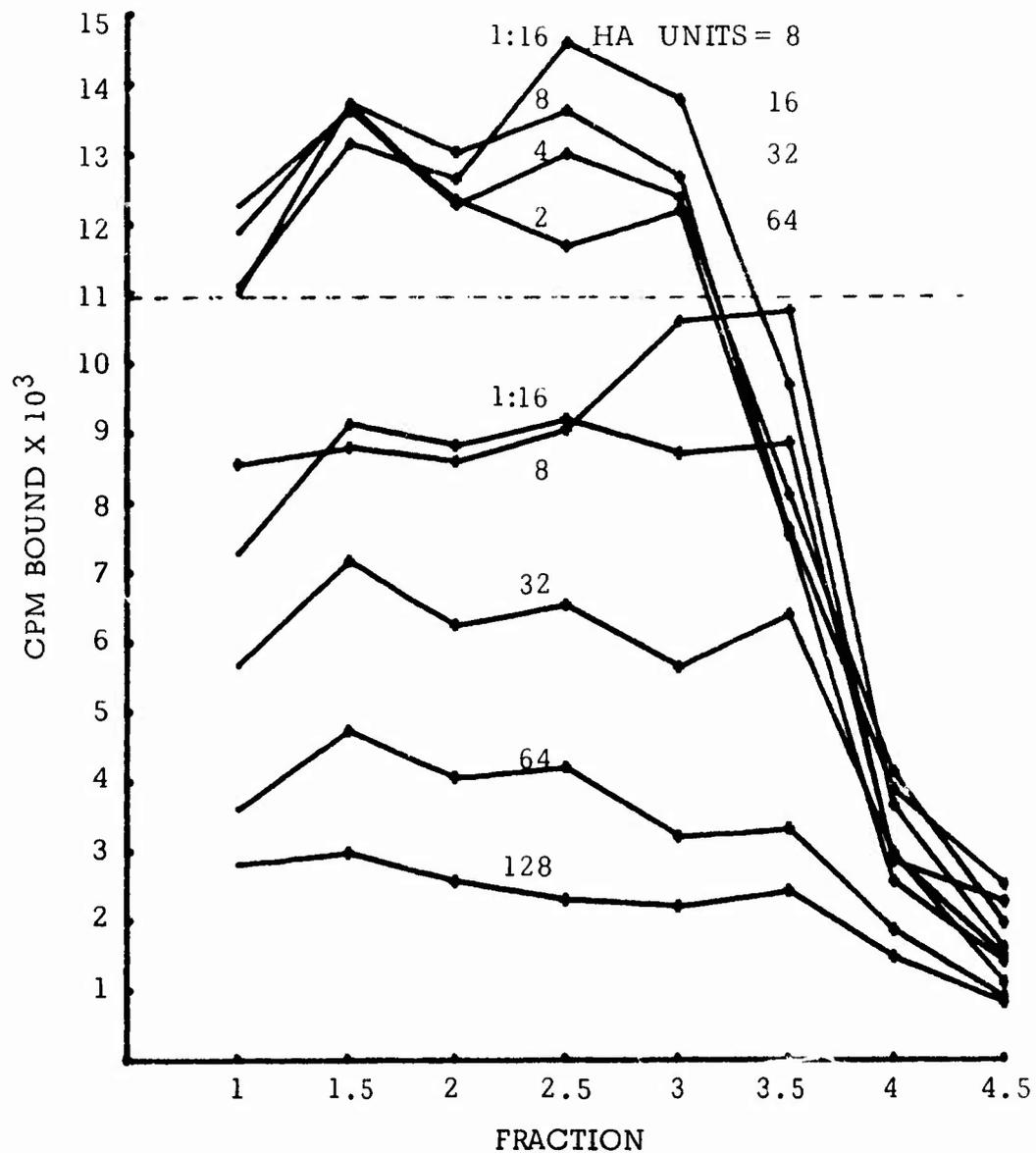


Figure 9 . Radioimmunoassay of double infection chimp serum (682) when reacted against ultracentrifuge-concentrated dengue-4 RHA. The second infection (dengue-4 following dengue-2) raised the dengue-2 CF titer from 1:64 to 1:512; other serology is in progress. The test was repeated with higher dilutions of antigen and the same lot of labelled antiglobulin; less CPM were bound in the repeat test with the two repeated antigen dilutions as shown below the dashed line.

antigen preparations contain a very high non-viral protein concentration which must be removed before the virus antigens will absorb efficiently to the plastic surface used in a solid-phase type of test; non-viral

protein must also be removed if the antigen has to be concentrated to required potencies. Second, there is variation in the antigen titers recovered from the various preparations, and there was no correlation between antigen titer and effectiveness as an RIA antigen. A third disadvantage is the excessive cost of very large numbers of suckling mice required, especially for the production of concentrated antigens.

Propagation of each of the dengue viruses in LLC-MK<sub>2</sub> cells was investigated as an alternative to mouse brain propagation. Confluent monolayers of cells in 32 oz prescription bottles, 6 bottles per virus, were infected with 1 to 3 ml of stock virus suspensions yielding a multiplicity of infection of 0.1 to 10. Following adsorption, the cell monolayers were rinsed and 30 ml maintenance medium added to them. The cells were initially grown in Medium 199 with 20% FBS and maintained after infection with Medium 199 with 5% FBS and 0.075% sodium bicarbonate. Infected cultures were harvested repeatedly at 3 to 5 day intervals and 30 ml maintenance medium replaced until cytopathic effects became so pronounced that very few viable cells remained. The harvested culture fluids were clarified by low speed centrifugation and the antigens precipitated by addition of saturated ammonium sulfate to 60% as described in the previous annual report. The precipitates from 150 ml culture fluid were resuspended in 3 ml PBS and centrifuged on tartrate-glycerol gradients as described above.

Each of the 4 types of dengue virus infected cultures exhibited varying severity of CPE which might restrict the number of possible harvests for certain viruses. Only 3 harvests were possible in one experiment for dengue-2 virus (days 4, 7, and 10) because of extensive CPE, yet 6 harvests were obtained from dengue-1 virus (days 5, 7, 10, 15, 18, 21). Only 2 harvests from dengue types 3 and 4 viruses have been examined thus far (days 6 and 9); however, multiple harvests are expected because CPE was minimal at day 9.

Hemagglutinating activity (HA) was assayed directly from the density gradient fractions with little interference by the tartrate or glycerol. A typical HA profile of a density gradient of dengue-2 virus is shown in Fig. 10. The peak of HA was always observed in the range of fractions 11-15, representing a density of 1.196-1.220. This most probably represents RHA (rapidly sedimenting HA of virions described previously by rate zonal centrifugation) and correlates well with the RHA peaks described above when infected mouse brain suspensions are used. A low titered peak of HA of greater density was observed in fractions 7-9 (density 1.24); this was only observed with dengue types 1 and 2 viruses from cell culture and the titer varied considerably between preparations. In contrast to infected mouse brain preparations, the SHA region of these gradients of cell culture derived virus did not exhibit high titered HA. The same results were obtained when comparisons were made by rate zonal centrifugation through 5-25% sucrose; mouse brain preparations of virus yielded good peaks of SHA, while cell culture preparations did not. The peak HA titers of the RHA fractions

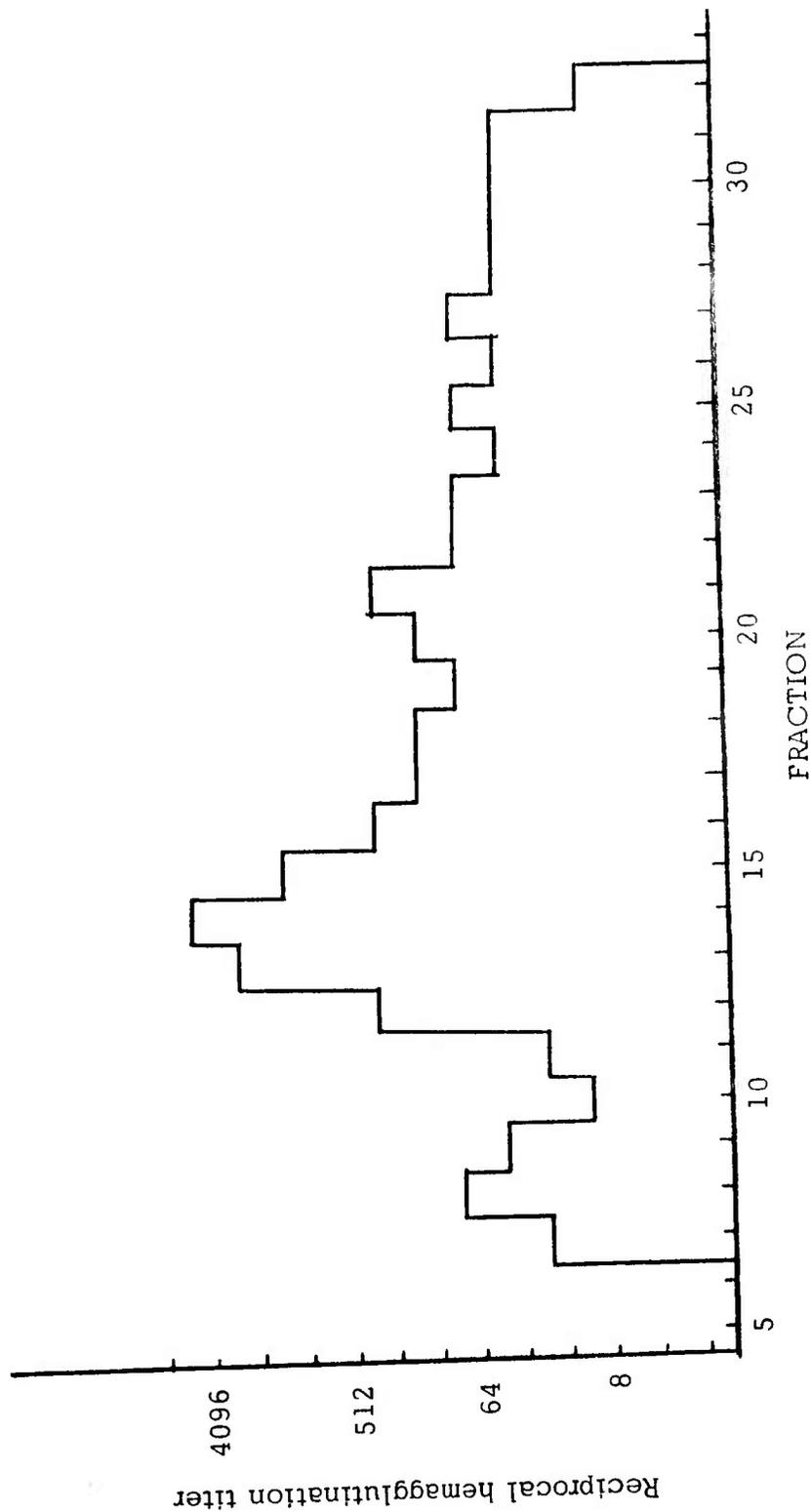


Figure 10. Tartrate-glycerol gradient centrifugation of an ammonium sulfate precipitate of dengue type 2 infected LLC-MK<sub>2</sub> cell culture medium. The gradient was centrifuged for 16 hours at 25,000 rpm in the SW 27 rotor; fractions were collected from the bottom of the tube and assayed for hemagglutinins by the microtiter method.

from successive harvests of each of the four dengue serotypes is shown in Table 1.

Table 1. Hemagglutinin (RHA) titers from successive harvests of dengue virus-infected LLC-MK<sub>2</sub> cells

Harvest	DEN-1		DEN-2		DEN-3		DEN-4	
	Day	RHA titer						
1	5	> 256	4	256	6	512	6	4
2	7	2048	7	> 4096	9	64	9	2
3	10	256	10	2048				
4	15	16						
5	18	256						
6	21	32						

Most of the HA from dengue-1 and 2 virus infected cultures was obtained during the first week and continued harvests in the case of dengue-1 virus were less productive. Dengue-3 and 4 viruses were low titered by comparison; indeed, very little HA could be detected with dengue-4 virus.

Preliminary RIA tests using cell culture propagated virus antigens showed that there was little correlation between HA titer and antigen as detected by RIA. This discrepancy in detection of antigen between HA and RIA was best demonstrated by examining fractions to either side of the peak of HA. Since antigens suitable for use in RIA tests were our ultimate goal, 0.025 ml aliquots of each gradient fraction were applied to microtiter wells and assayed by RIA as described above. The third harvest of dengue-2 virus was assayed in this manner, using dengue-2 virus immune chimp No. 680 at a 1:100 dilution. Comparative HA and RIA antigen curves, each plotted on an arithmetic scale, are shown in Fig. 11. These data indicate that the pooling of fractions for use as RIA antigens should be based on an RIA scan of the gradient fractions rather than on the easier and faster HA assay. The decline in antigen detectable by R.I.A. at the higher fractions may represent either a real decrease in antigen concentration in these fractions or the presence of contaminating protein which would compete for available sites on the plastic well and therefore reduce virus antigen attachment.

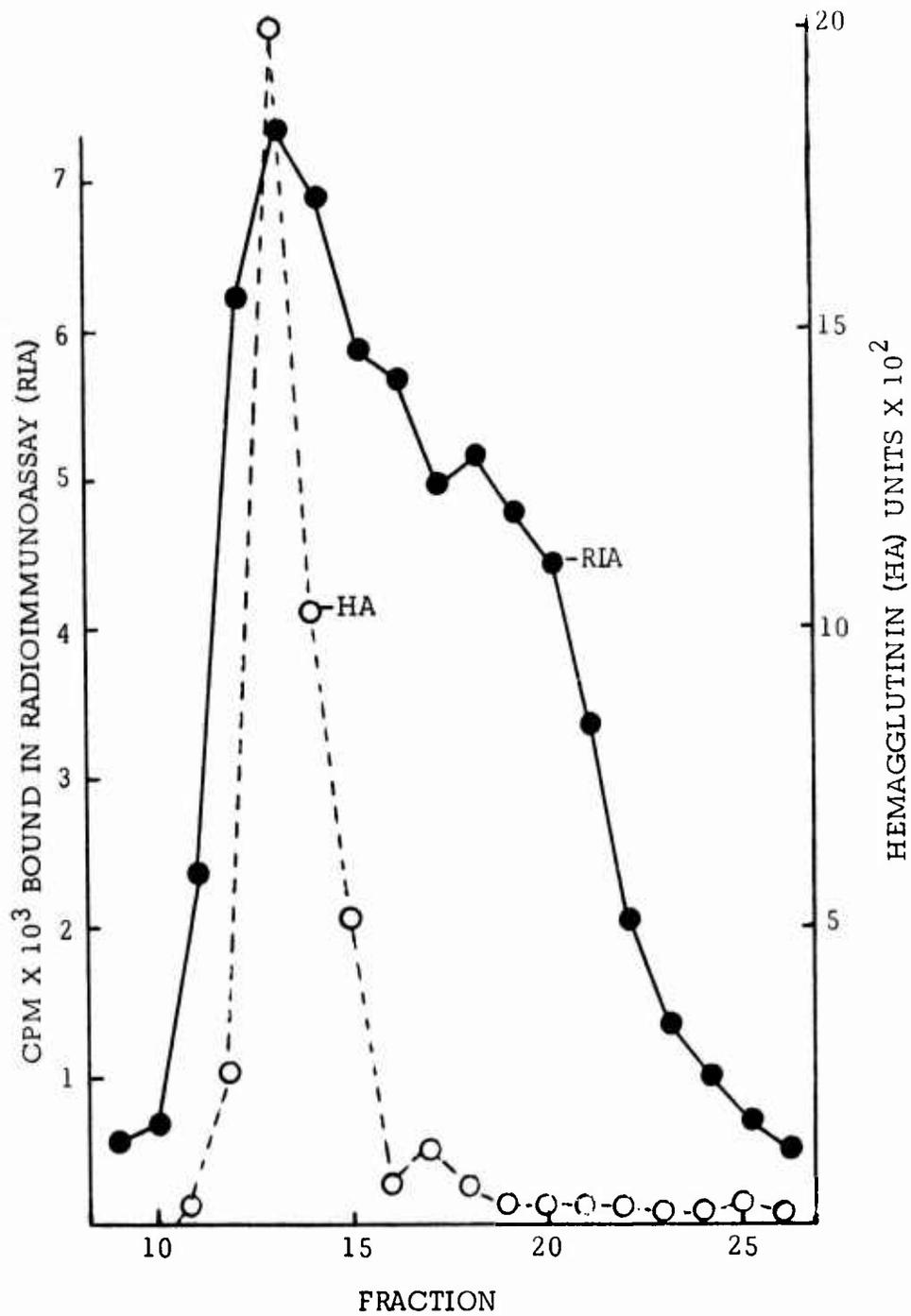


Figure 11. Tartrate-glycerol gradient centrifugation of a third harvest of dengue-2 infected LLC-MK<sub>2</sub> cell culture medium as described in the preceding figure, except that dengue hemagglutinin is shown on an arithmetic, rather than log<sub>2</sub> scale when compared here with a radioimmunoassay.

If this latter is true, less dense and smaller antigen components such as SCF could not be detected using these methods.

Routine examination of tartrate-glycerol density gradients using R.I.A. has been hampered by the lack of available primate antisera to each of the four dengue serotypes. Mouse hyperimmune ascitic fluids (MHAF) are available for use in R.I.A.; however, iodinated anti-mouse globulin is not nearly as high titered as the other reagents (anti-human and anti-rabbit). In spite of these limitations, examination of gradients for R.I.A. antigens from each of the dengue serotypes using MHAF are shown in Fig. 12. Dengue antigens could be detected in all cases even though HA was low or non-existent. Dengue-3 virus antigens appeared unique in that a biphasic antigen peak was consistently observed. This did not appear to be unique to the R.I.A. method but was also reflected by the less sensitive HA titration. Dengue-4 virus also appeared less dense than the RHA of the other 3 viruses; however, these results must be considered preliminary because only two gradients have been tested.

In an attempt to overcome the difficulties encountered by not having high titered specific primate antisera to each of the 4 dengue viruses at the present time, we investigated the possibility of using reference heterologous MHAF for determining the extent of cross reactions on gradients by R.I.A. A dengue-3 virus gradient was selected because of the split-peak that was found; it was examined in duplicate R.I.A. with homologous dengue-3 MHAF and heterologous dengue-2 MHAF (Fig. 13). Both dengue antisera detected the major antigen on the gradient and even yielded the same split peak. We suspect that the R.I.A. method may be far superior to other serological methods (HA, CF) for characterizing antigen preparations even though heterologous antisera may have to be used, as is sometimes the case with serum from other species where antibodies to all 4 serotypes may not be available.

#### 6. Evaluation of SCF antigens for radioimmunoassay

The production and purification of dengue SCF antigens was described in the previous Annual Report. Briefly, 20% infected mouse brain suspensions were clarified at 8000 rpm for 1 hr, treated with 1.5 mg/ml protamine sulfate for 45 min, and centrifuged again at 8000 rpm for 1 hr. A substantial loss of CF antigen occurs in the protamine sulfate precipitate, but the removal of excess contaminating mouse brain proteins that also occurs is necessary for the subsequent purification steps. If desired, the hemagglutinins can be harvested by pelleting in the ultracentrifuge following the protamine sulfate clarification. Ammonium sulfate is added to the protamine sulfate clarified brain suspension to 60% saturation; after 1 hr of mixing the precipitate is collected by centrifugation at 8000 rpm for 1 hr and resuspended in PBS to effect a 10 to 20-fold concentration. The resuspended precipitate is chromatographed through a Sephadex G-100 column and the CF antigen peak eluting in the 39,000 dalton region is concentrated about

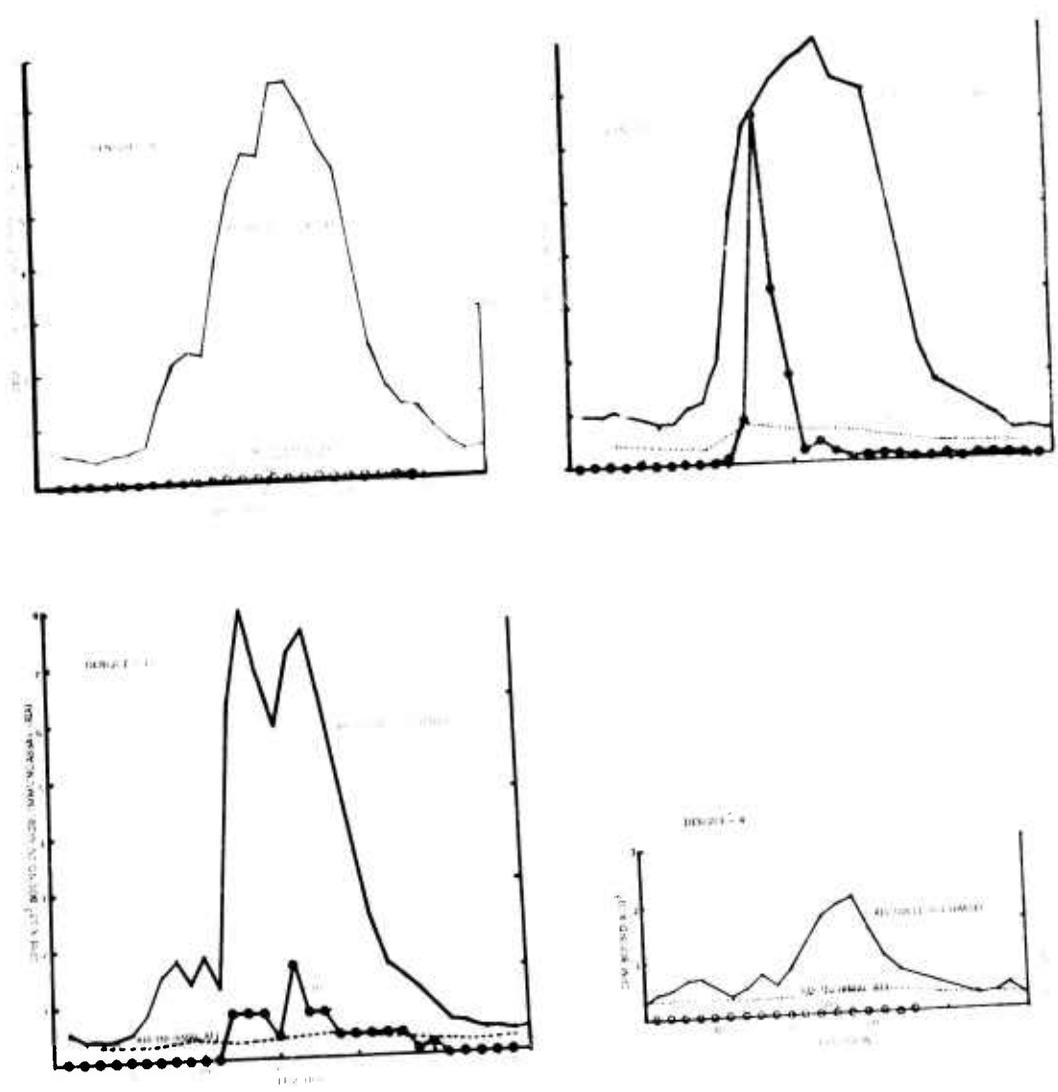


Figure 12. Tartrate-glycerol gradient centrifugation of each of the four dengue serotypes harvested from infected LLC-MK<sub>2</sub> cell culture medium. The gradient fractions were assayed by hemagglutination and by radioimmunoassay using dengue hyperimmune mouse ascitic fluids.

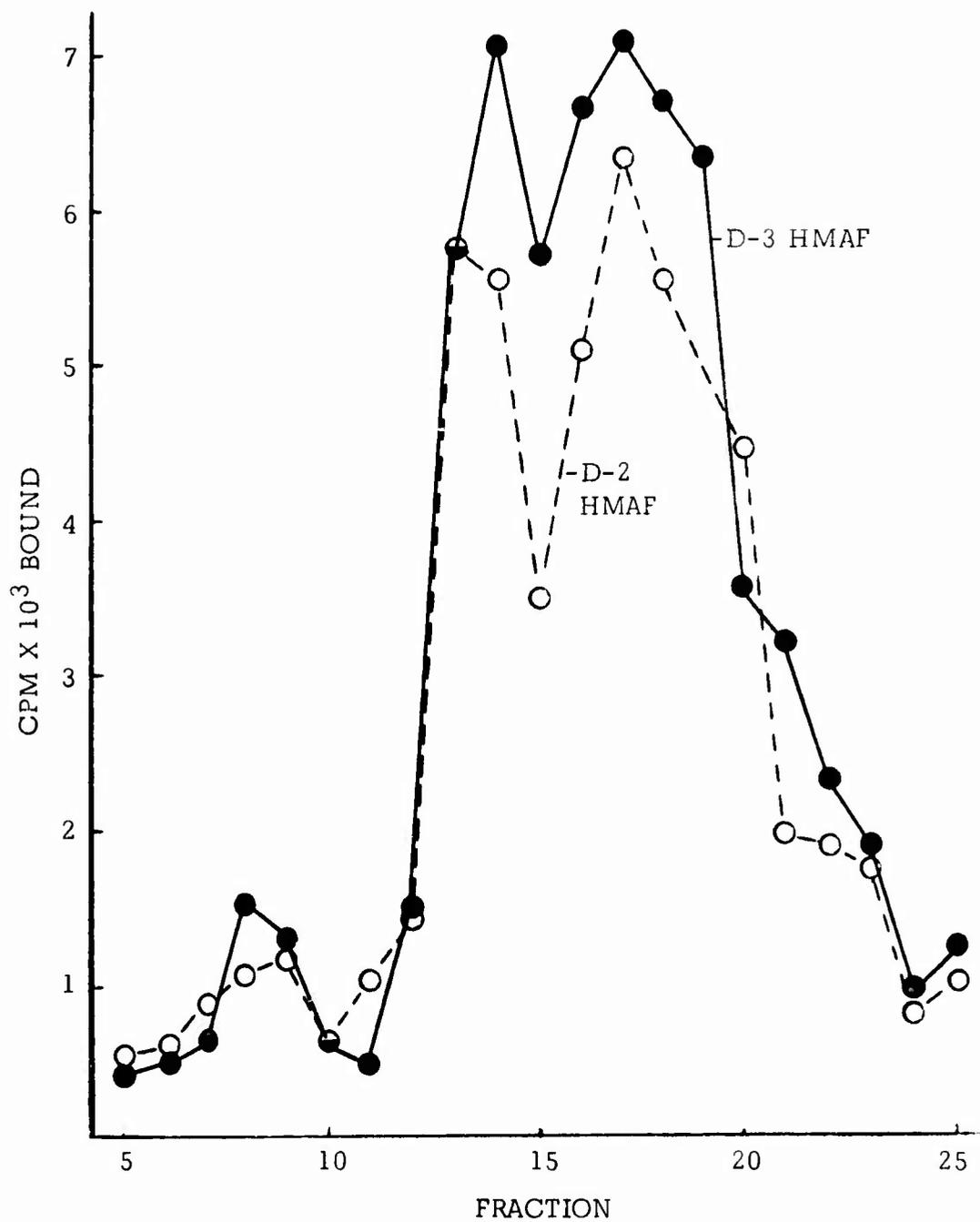


Figure 13 . Tartrate-glycerol gradient of dengue-3 antigens from infected LLC-MK<sub>2</sub> cell culture medium. Gradient fractions were analyzed by radioimmunoassay using homologous hyperimmune mouse ascitic fluid (HMAF) and heterologous dengue-2 HMAF.

100-fold by nitrogen pressure dialysis. Finally, the concentrated Sephadex peak is electrofocused in a pH 3-10 gradient and the CF antigen peak at either pH 5.2, 5.5, 5.8, or 6.2 for dengue SCF types 2, 4, 3, and 1, respectively, are pooled and frozen for use in the R.I.A. tests.

SCF antigens exhibited a prozone effect when used as R.I.A. antigens; that is, when more than 4 CF units (i.e., a 1:8 dilution of an antigen preparation that titered 1:32) were used to coat the microtiter wells, less labeled antiglobulin would bind to the SCF antibody than when only 2 or even 1/2 CF unit was used. This prozone effect was dependent on the serotype and/or the antigen preparation. As shown in Fig. 14, several concentrations of dengue-1 SCF antigen were tested against dilutions of broadly reactive human serum from a dengue hemorrhagic fever case. In this case, one CF unit represents a 1:64 dilution of the pooled antigen peak from an electrofocus column. Two CF units appear to give a more sensitive test than 1/2, 1, or 4 units. When various concentrations of type 2 SCF were tested (Fig. 15), it was found that 1/2 to 1 CF unit was optimal for detecting antibody in human serum. The antigen concentration appears to be most critical for a sensitive test, and this concentration must be determined for each serotype or antigen preparation. Experience will tell us if the CF test can be depended upon to select antigen units. The effect of 4 CF units or more to decrease antibody binding may be due to contaminants competing with the antigen for the available surface in the solid phase assay. However, the purification steps, at least for type 2 SCF, were sufficient enough that rabbit anti-mouse brain antibody did not react with the SCF antigen.

#### 7. Radioimmunoassay of antibodies to SCF antigens in human sera from primary and secondary cases of dengue fever

Acute and convalescent sera from cases of dengue hemorrhagic fever and/or dengue shock syndrome were tested for the presence of antibodies to the nonstructural soluble complement fixing (SCF) antigen. It was reported in the previous Annual Report that the presence of antibodies to SCF was sporadic and of low titer when assayed by the CF test. Fig. 16 shows the rise in titer of paired serum to dengue SCF-1 and SCF-2. Serum curves to SCF-3 and SCF-4 are not shown since there was little, if any, increase in the amount of labeled antiglobulin bound to the SCF-antibody complex. The curves in the graph, produced by dilutions of the sera vs constant SCF 1 or 2 antigen, suggest that more antibody bound to type 1 than to type 2 SCF. Whether or not this indicates that type 1 virus was the most recent infecting agent and type 2 dengue the earlier infecting virus, must await precise standardization of the test. Similar results were obtained from other paired sera from the same epidemic of hemorrhagic fever where the antibody response was broadly cross-reactive by HI and CF tests, but where a tendency toward a higher titer to type 1 SCF by RIA was observed. Reactions with SCF antigens by R.I.A. occurred with sera that did not react with SCF by the CF tests.

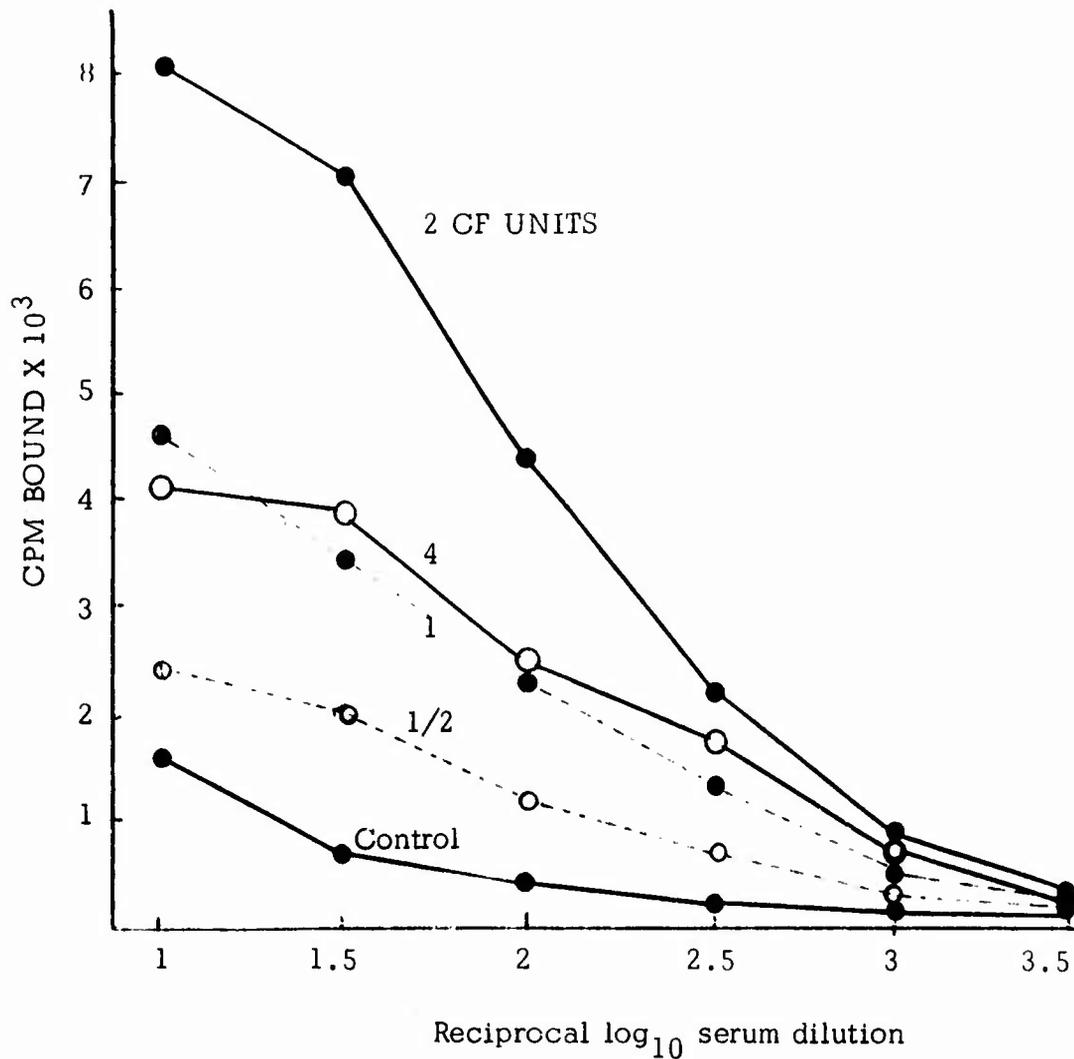


Figure 14. Optimal concentration of dengue-1 soluble complement-fixing (SCF) antigen determined by radioimmunoassay. Various concentrations of the SCF antigen, expressed in units based on its CF titer to D-1 HMAF, were reacted here against dilutions of human serum (51372) from a Thai hemorrhagic fever case having a high and broadly reactive antibody response to the dengue serotypes (CF titer to structural and nonstructural antigens in crude mixtures = 1:1024; CF titer to SCF-1 and SCF-2 in laboratory of S. B. Halstead = 1:32).

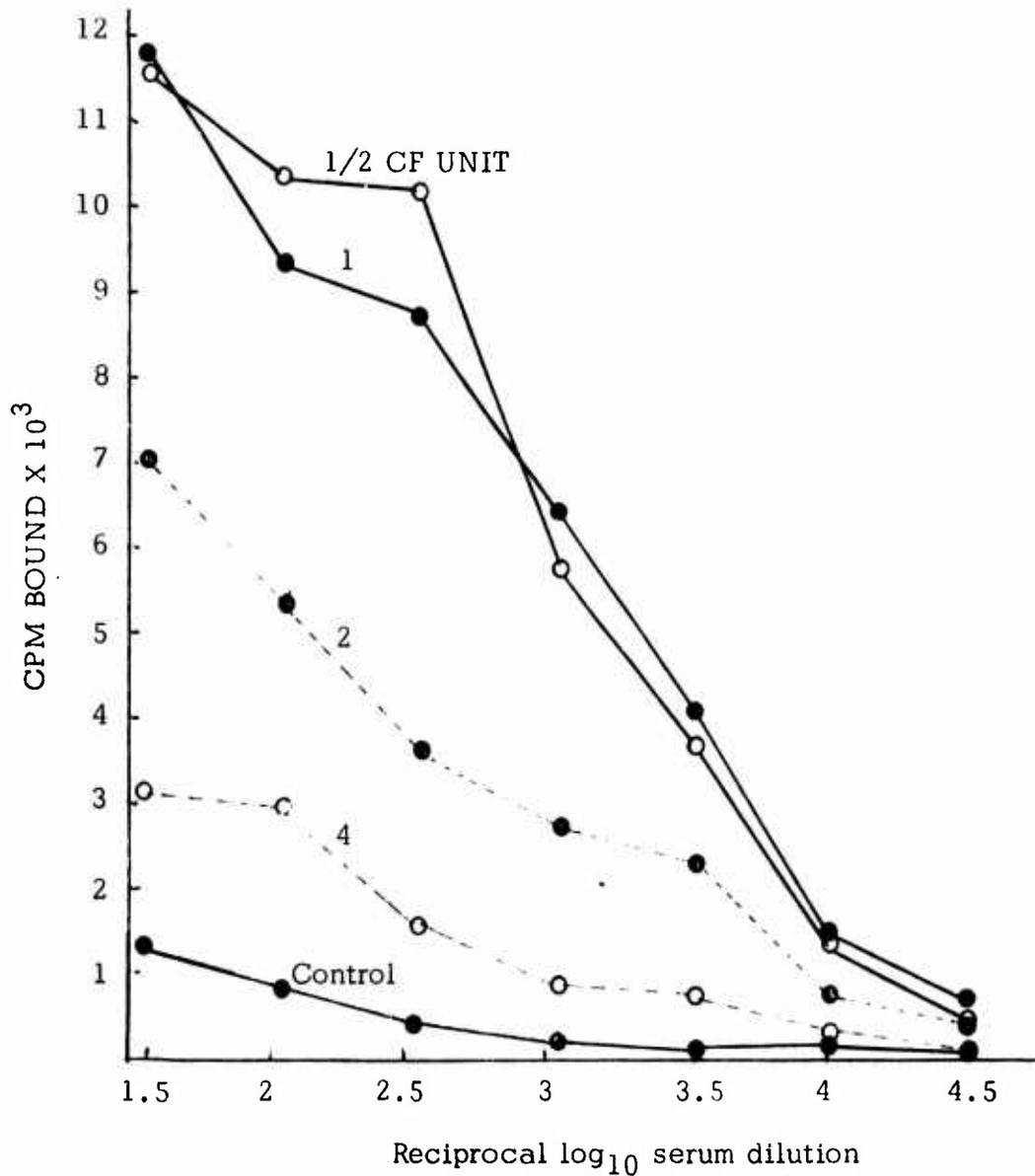


Figure 15. Optimal concentration of dengue-2 SCF antigen by radio-immunoassay as described for type 1 SCF antigen in the preceding figure. Thai serum 49355 used here had a D-2 CF titer of 1:2048 against crude antigen and 1:32 against SCF-2 antigen.

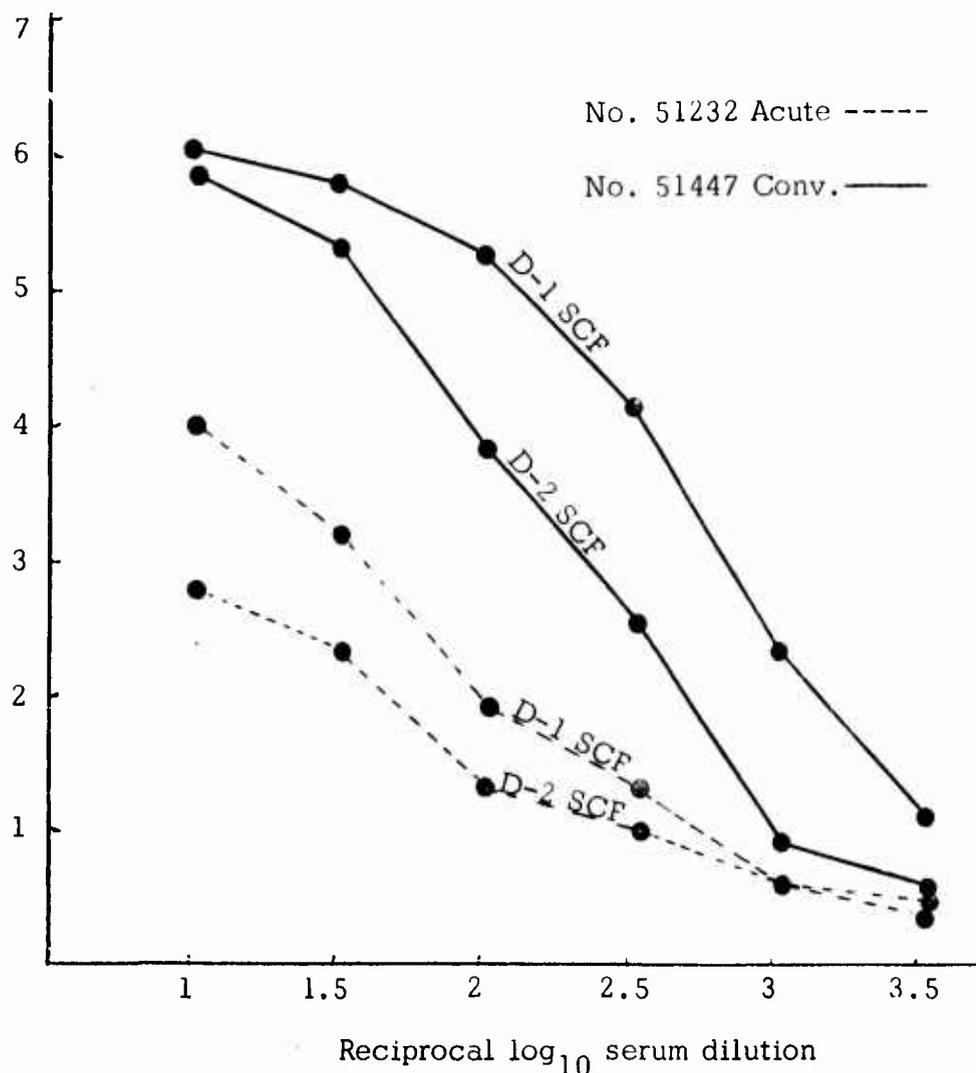


Figure 16. Radioimmunoassay of paired human sera from a case of dengue hemorrhagic fever reacted with dengue type 1 and 2 SCF antigens. No rises in bound counts per minute (CPM) were observed to types 3 and 4 SCF antigens which were deleted from the graph.

Of three secondary cases of dengue suspected to be due to type 3 virus, only 1 reacted with D-3 SCF above background (Fig. 17), the background values being similar to those shown for the serum response to the type 4 SCF antigen. The reaction of the serum to types 1 and 2 SCF antigens was essentially identical, and of much greater magnitude. The lack of response of most of the secondary dengue sera to the type 3 SCF antigen necessitates a reevaluation of this antigen since cross reactions with the other dengue serotypes have been demonstrated with less sensitive tests (Russell, et al., 1970). In the same vein, the response of dengue immune sera to the type 4 SCF generally paralleled the

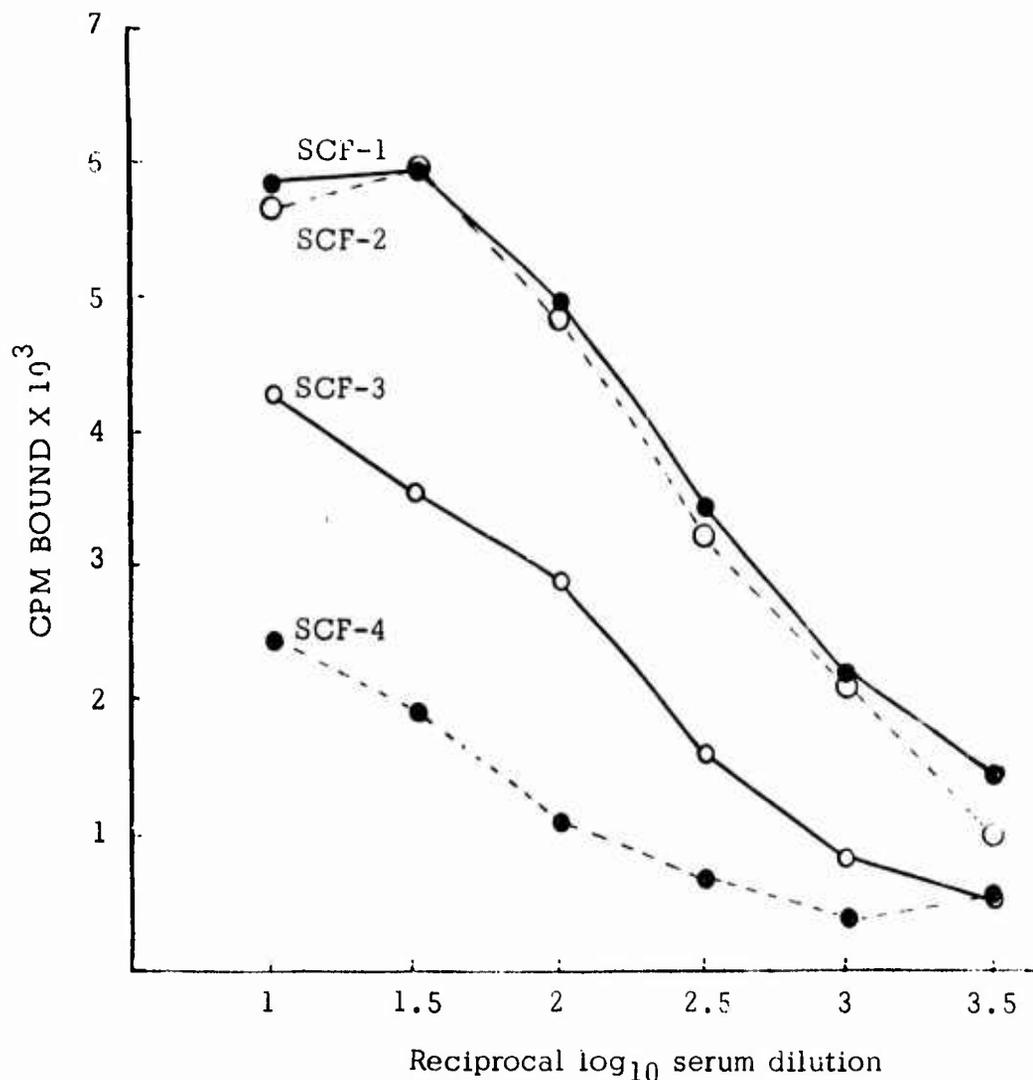


Figure 17. Antibody response of a convalescent, secondary infection Thai serum (9250) to each of the 4 dengue SCF antigens by radioimmunoassay.

response of the serum dilutions when no antigen was used in the microtiter wells. The type 4 SCF antigen potency has to be improved or its optimal concentration redetermined with an appropriate antibody source. Secondary cases of dengue suspected to be due to type 4 virus had no antibody to any of the dengue SCF serotypes. If type 4 SCF antibody exists, it might not be as cross reactive with the other dengue serotypes in this test since the virus appears to fall in between the dengue virus complex and the Japanese encephalitis virus complex antigenically.

With the sera tested up to the present time, we could not detect SCF antibody to any of the dengue serotypes in primary cases of dengue

fever, only in some secondary cases not complicated by hemorrhagic fever or shock, and in most of the hemorrhagic fever cases. Carefully collected and characterized sera will be tested when the test is completely standardized.

#### 8. Radioimmunoassay of dengue-immune human sera for antibodies to structural antigens

Preliminary tests were carried out when only types 1 and 2 HA antigen from tartrate-glycerol gradients were available. Six units of SHA antigen of each dengue type were absorbed to microtiter wells and then reacted with acute and convalescent sera from Bangkok. Plaque reduction neutralization tests (PRNT) with all 4 serotypes were performed about the same time that the R.I.A. tests were done. Representative results are presented using 3 paired sera. In the first case, a low rise in titer mainly to type 1 by PRNT (see data in Fig. 18) was paralleled by a higher rise in titer, and greater CPM bound to the type 1 SHA antigen than to the type 2 antigen. In the second case, a substantial rise in titer to type 2 by PRNT was paralleled by a higher rise in titer and more CPM bound to type 2 SHA than to type 1 (Fig. 19). In the third case where a substantial rise in PRNT titer to dengue 2 was accompanied by significant but lower rises in titer to the other serotypes, the RIA response to types 1 and 2 tended to overlap at the higher serum dilutions, but more CPM were bound to the type 2 antigen at the lower serum dilutions (Fig. 20). Correlation of RIA tests using structural antigens with neutralization tests employing infectious virus thus appears possible; cross reactions perhaps being sorted out on the height of the curve, and the more type specific reactions being identified with a higher serum dilution endpoint. We have occasionally obtained biphasic curves with human serum when reacted against structural antigens, somewhat suggested by the shape of the curves in Fig. 20. This may be similar to the investigations of Dalrymple et al. (1972) who defined several subpopulations of antibody in reference immune ascitic fluids initially on the basis of biphasic curves using the radioimmune precipitation test. Type and group specific reactions may eventually be defined in a similar manner with the group B arboviruses.

Finally, acute and convalescent serum pairs were reacted with dengue-2 SHA and dengue-2 SCF in the same test in order to compare the relative magnitude of the antibody responses. The RIA curves are substantially higher with the structural (SHA) antigens than with the non-structural (SCF) antigens. In the representative case shown in Fig. 21, the structural antigens bound sufficient antibody such that 9000 to 10,000 CPM of antiglobulin were bound; apparently a much lower quantity of antibody bound to the nonstructural antigen since only 3000 to 5000 CPM of antiglobulin were bound. This might explain the difficulty of attempting to find antibodies to dengue nonstructural antigens by conventional serological techniques. These preliminary

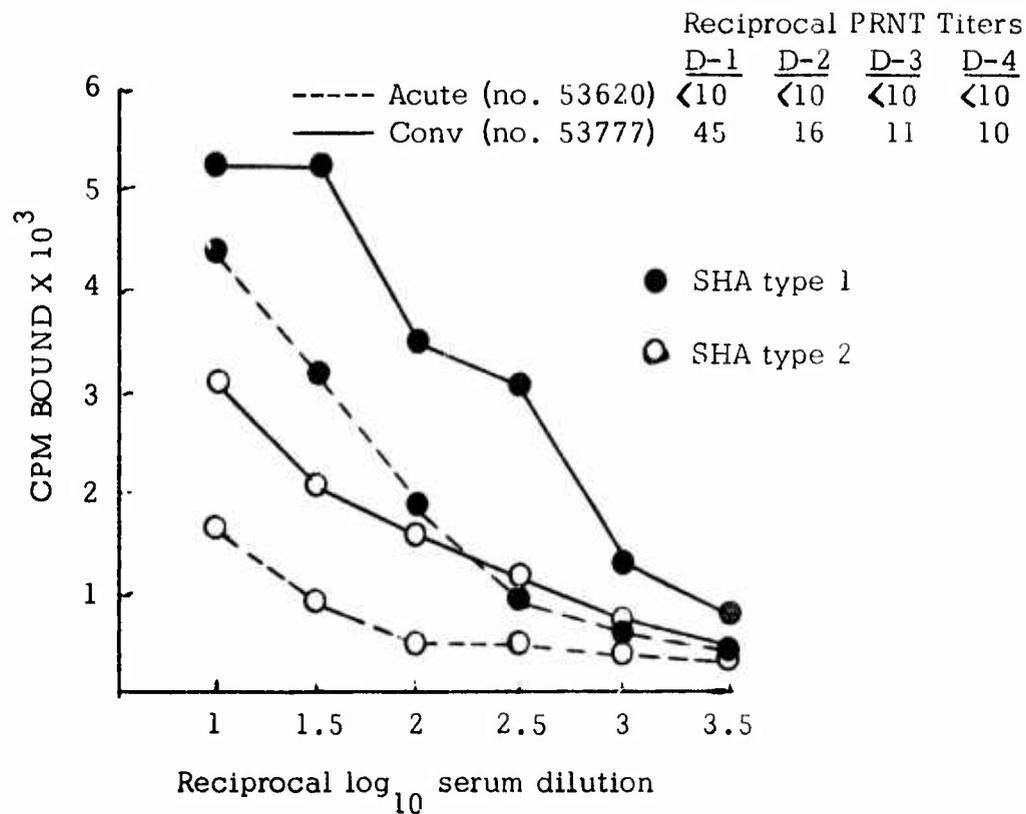


Figure 18. Antibody response in paired sera to dengue virus types 1 and 2 SHA (slow sedimenting hemagglutinin) by radioimmunoassay. The plaque reduction neutralization (PRNT) titers of the acute and convalescent serum are listed above the figure for comparison with the RIA results.

experiments utilizing a solid phase radioimmunoassay, indicate that antibodies to structural and nonstructural antigens can be measured independently, and should therefore assist in characterizing the various stages of the immune response following infection with virulent and attenuated dengue viruses.

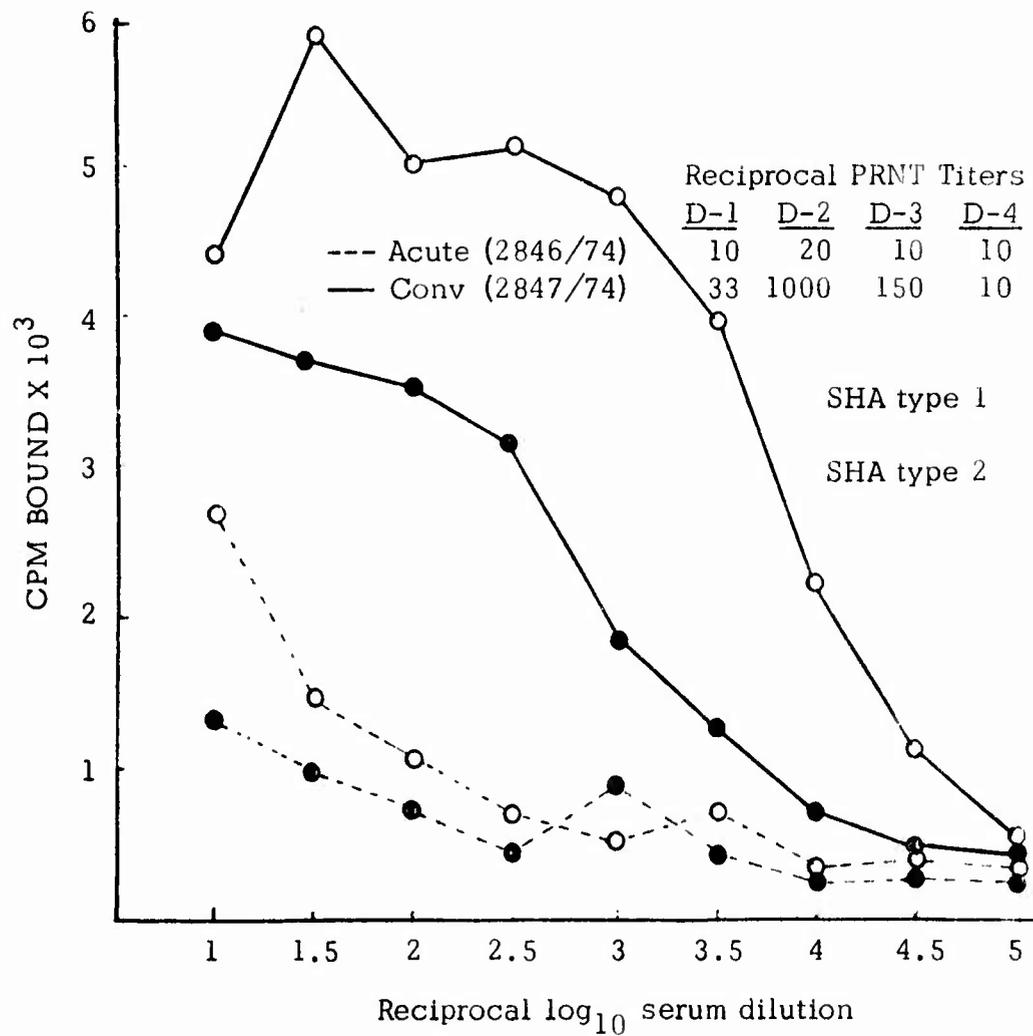


Figure 19. Antibody response of paired sera to dengue virus types 1 and 2 SHA (slow sedimenting hemagglutinin) by radioimmunoassay. The plaque reduction neutralization (PRNT) titers of these sera are listed in the figure for comparison with the RIA results.

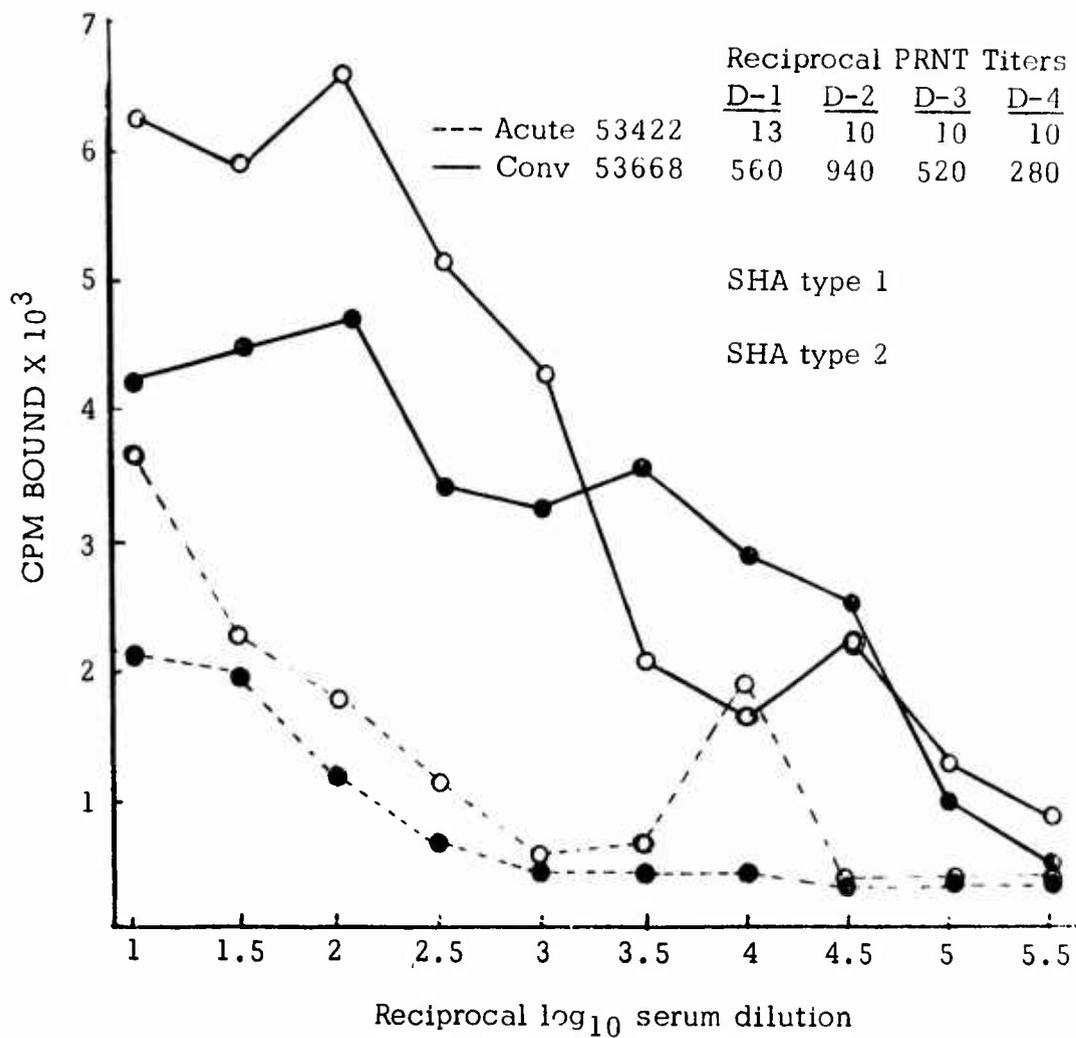


Figure 20. Antibody response of paired sera to dengue virus types 1 and 2 SHA (slow sedimenting hemagglutinin) by radioimmunoassay. The plaque reduction neutralization (PRNT) titers of these sera are listed in the figure for comparison with the preliminary RIA results.

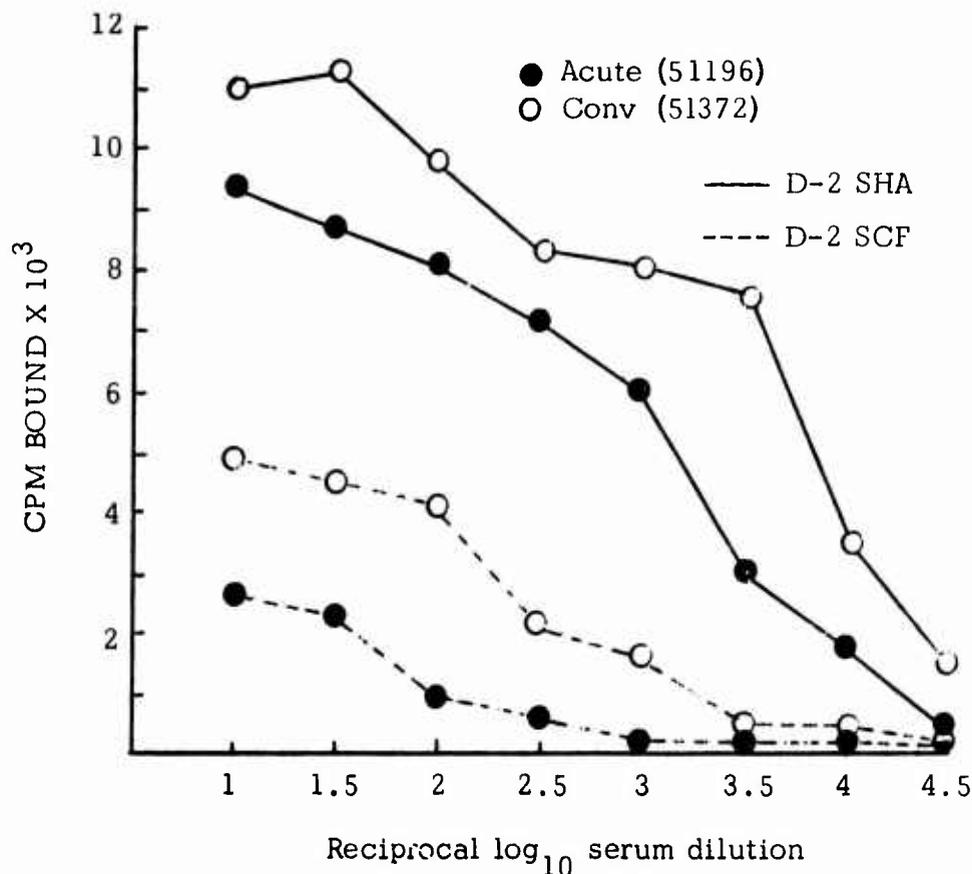


Figure 21. Comparison of antibody response to dengue-2 SHA and SCF antigens in the same radioimmunoassay.

## B. Dengue vaccine progress

### 1. Dengue-2 vaccine strains

#### a. Dengue-2 passage in fetal Rhesus lung (FRhL) cells.

Attempts were made to utilize FRhL cells as a vaccine substrate for dengue-2 (D-2) virus. All virus passages up to this point have been done primarily in primary green monkey kidney (PGMK) cells. Due to concern over parenteral administration of a dengue vaccine prepared in these primary cells, alternate cell lines were tested for dengue replication.

Pre-tested and certified FRhL cells were received from Lederle Laboratories, Pearl River, New York, and held in liquid nitrogen until they were thawed and seeded in suitable cell culture flasks. FRhL cells at passage level 35 were inoculated with D-2, PR-159, passage #1 in GMK cells. Three serial passages of D-2 were done in these cells and

viral harvests taken after 14 to 18 days. Virus titers, listed in Table 2, were still very low after three passages in the FRhL, p-35 cells. Various media and fetal bovine serum concentrations failed to enhance replication of D-2 in these cells.

Table 2. Dengue-2 (PR-159), GMK-1 passage in FRhL (p-35) cells

Virus	Harvest (day)	PFU/0.2 ml
D-2, FRhL/1	18	$5 \times 10^2$
D-2, FRhL/2	18	$4 \times 10^2$
D-3, FRhL/3	14	$3 \times 10^2$

Earlier passage FRhL cells were compared to Lederle, FRhL p-35 cells using two criteria: cell viability and viral replication. Passage 18, FRhL (Fort Detrick/Merrell-National) and passage 21 FRhL (Lederle) both formed monolayers more rapidly than FRhL p-35 cells when all three were seeded in flasks using the same seed concentration. After inoculation with D-2 (FRhL/1), the lower passage FRhL cultures showed an increased yield in a 7-day harvest (Table 3). All virus passage work after this point was performed in FRhL cells either at the 18th or the 21st passage level. The standard media for growth and maintenance of these cells was EMEM (GIBCO F-15 with non-essential amino acids) with added fetal bovine serum and antibiotics.

Table 3. D-2 (PR-159), FRhL/1 passage (2) in FRhL cells at p-18, 21, 35

Cells	Harvest (day)	PFU/0.2 ml
FRhL, p-18 (Detrick/Nat.)	7	$3.2 \times 10^3$
FRhL, p-21 (Lederle)	7	$4.2 \times 10^3$
FRhL, p-35 (Lederle)	7	$1.1 \times 10^1$

D-2, PR-159 passages 3 to 15 in FRhL cells at 35°C are listed in Table 4. The EOP (efficiency of plating) is the ratio of the PFU titer: PFU @ 35°C/PFU @ 39.5°C and is an indicator of the trend of temperature sensitivity during passage of the virus. The suckling mouse LD<sub>50</sub> values for some passages are also given as a marker for virulence. Virus titers in cell culture and suckling mice reached optimal levels after six passages and indicate a degree of viral adaptation was attained in the FRhL cells. An unexpected finding was the increase in temperature sensitivity up to the eighth passage while suckling mouse virulence was on the increase. Mouse virulence has usually correlated with lack of temperature sensitivity. Plaques in LLC-MK<sub>2</sub> cells were faint compared to the parent (D-2, GMK-6) and varied in size from 1 to 3 mm.

Table 4. D-2 (PR-159) serial passage in FRhL cells (35°C).

Passage	PFU/0.2 ml		EOP	SMIC LD <sub>50</sub> /0.02 ml
	35°C	39.5°C		
3 (day 8)	2.6 x 10 <sup>3</sup>	9.6 x 10 <sup>1</sup>	0.037	ND
4 (day 7)	7.9 x 10 <sup>3</sup>	3.7 x 10 <sup>2</sup>	0.047	2.1
5 (day 7)	2.3 x 10 <sup>4</sup>	5.0 x 10 <sup>2</sup>	0.022	2.5
6 (day 7)	1.3 x 10 <sup>5</sup>	1.5 x 10 <sup>4</sup>	0.011	4.5
7 (day 7)	1.1 x 10 <sup>6</sup>	7.8 x 10 <sup>3</sup>	0.007	4.9
8 (day 7)	4.8 x 10 <sup>5</sup>	2.7 x 10 <sup>3</sup>	0.006	4.9
9 (day 4)	1.9 x 10 <sup>6</sup>	6.8 x 10 <sup>4</sup>	0.036	ND
10 (day 6)	6.8 x 10 <sup>5</sup>	3.6 x 10 <sup>4</sup>	0.053	ND
11 (day 4)	1.7 x 10 <sup>6</sup>	6.7 x 10 <sup>4</sup>	0.039	ND
12 (day 4)	1.3 x 10 <sup>6</sup>	1.3 x 10 <sup>5</sup>	0.100	ND
13 (day 4)	1.4 x 10 <sup>5</sup>	2.4 x 10 <sup>4</sup>	0.171	ND
14 (day 4)	1.9 x 10 <sup>5</sup>	5.4 x 10 <sup>4</sup>	0.284	ND
15 (day 4)	6.1 x 10 <sup>5</sup>	3.5 x 10 <sup>4</sup>	0.057	ND

D-2, PR-159 (FRhL/1) was also serially passaged at 31°C with the objective of enriching for temperature sensitive mutants. Table 5 lists four passages of D-2 done at short intervals (7 day). The EOP trend is in the direction of increased temperature sensitivity; however, viral yields never reached those attained from passage at 35°C.

Table 5. Den-2, PR-159, FRhL/1 passage in FRhL cells at 31C.

Passage	Harvest (day)	PFU/0.2 ml		EOP
		35C	39.5C	
1	7	$1.9 \times 10^2$	ND	--
2	7	$1.7 \times 10^4$	$6.0 \times 10^2$	0.035
3	7	$2.9 \times 10^3$	$8.0 \times 10^1$	0.028
4	7	$4.4 \times 10^3$	$9.0 \times 10^1$	0.020

This may still be a promising  $ts^+$  enrichment technique if carried to the point where a persistently infected culture system were attained.

b. Cloning of D-2, PR-159 virus in FRhL and PGMK cells.

Attempts were made to clone D-2, PR-159, FRhL/7 virus in FRhL cells. Well defined plaques were developed under an agarose/BME overlay following the technique of Mr. D. Dubois. One isolated plaque was successfully picked and grown up in fluid culture. After 14 days outgrowth in FRhL cells, the virus harvest contained  $1.5 \times 10^5$  PFU/0.2 ml but was not temperature sensitive ( $1.4 \times 10^4$  PFU/0.2 ml at 39.5°C). Attempts at direct plaque-plaque cloning were unsuccessful. Also, FRhL/9 passage virus was used for plaque picking. None of 4 plaque picks gave virus yields in fluid culture.

D-2, FRhL/7 virus was also plaqued in PGMK cells using techniques similar to those used for plaquing in LLC-MK<sub>2</sub> cells. Six picks were made at a  $10^{-6}$  virus dilution and labeled C-1 through C-6. Each of these picks was inoculated in fluid culture (FRhL cells) or back into PGMK cells for direct plaque-plaque cloning. After three direct clonings, eight clones resulted from the original six plaque picks. The results of two passages of these clones grown in FRhL cells are listed in Table 6. The C-5 clones (subclones a and b) appear not to be temperature sensitive; the C-6 clones (subclones a and b) have some degree of temperature sensitivity; the C-4 clones (subclones a and b) have a higher degree of  $ts^+$  characteristics; and C-2a appears to be the most  $ts^+$  and similar to our standard S-1 clone. Although C-2a has an EOP of  $> 0.00001$ , suckling mouse virulence is high ( $SMICLD_{50} = 3.4$ ) compared to S-1 with an EOP of  $> 0.000003$  and a  $SMICLD_{50}$  of 1.8. These two clones also differ in plaque morphology with S-1 being small (1-2 mm) and faint while C-2a plaques are distinctly larger (2-3 mm) and

Table 6. Den-2, PR-159 clones derived from FRhL/7 pass virus.

The harvests from FRhL cells represent the first and second passages past three cloning passages in PGMK cells.

Passage (past cloning)	Clone	PFU/0.2 ml		EOP	SMIC LD <sub>50</sub>
		35C	39.5C		
FRhL/1	C-2a	$2.0 \times 10^3$	$< 10^1$	$< 0.0003$	ND
(day 13)	C-4a	$1.0 \times 10^4$	$1.0 \times 10^4$	0.005	ND
	b	$1.1 \times 10^5$	$6.6 \times 10^2$	0.006	ND
	C-5a	$7.1 \times 10^4$	$8.5 \times 10^3$	0.120	ND
	b	$2.0 \times 10^4$	$1.8 \times 10^3$	0.090	ND
	C-6b	$6.0 \times 10^3$	$1.1 \times 10^2$	0.018	ND
	c	$5.0 \times 10^4$	$7.1 \times 10^2$	0.014	ND
FRhL/2	C-2a	$9.8 \times 10^4$	0	$< 0.00001$	3.4
(day 7)	C-4a	$2.8 \times 10^5$	$1.0 \times 10^1$	0.000036	3.6
	C-4b	$3.0 \times 10^5$	$6.9 \times 10^2$	0.0023	3.7
	C-5a	$6.4 \times 10^5$	$1.4 \times 10^3$	0.0022	4.6

faint. Both C-2a and C-4a demonstrate leakiness (reversion) with an increase in EOP similar to the S-1 clone when passaged more than two times using inadequately diluted inocula. Attempts are underway to demonstrate complementation between the S-1 and C-2a clones.

c. Passage of D-2, PR-159, S-1 clone in FRhL cells. Earlier attempts (WRAIR, 1974 Annual Report) to adapt S-1, p-19a, ts<sup>+</sup> mutant to FRhL cells resulted in reversion of the mutant to a ts<sup>-</sup>, mixed population with more virulent characteristics. Poor replication of S-1 in non-certified FRhL cells made adaptation difficult and allowed reversion to occur. Using certified (Lederle p-21 or Fort Detrick/Merrell-National p-18) FRhL cells and culturing optimally, S-1 adapted to growth

Table 7. Passage of S-1 clone in FRhL, p-18 (Fort Detrick/Merrell-National) and FRhL, p-21 (Lederle) cells.

Cells	Passage	Inoc	Harvest (day)	PFU/0.2 ml		EOP
				35C	39.5C	
FRhL, p-18	1	Und	7	$1.6 \times 10^4$	0	$< 0.00006$
	2	Und	7	$3.1 \times 10^5$	0	$< 0.000003$
	3	Und	7	$6.2 \times 10^4$	$2.6 \times 10^1$	0.00042
	3	$10^{-3}$	10	$1.6 \times 10^4$	0	$< 0.00006$
FRhL, p-21	1	Und	7	$2.7 \times 10^3$	0	$< 0.0004$
	2	Und	9	$6.0 \times 10^4$	5	0.000083

in these cells with little or no reversion occurring through two passages. As listed in Table 7, above, high titers of S-1 were reached after two passages in either p-18 or p-21 certified cells. A low input multiplicity was required for the third passage in p-18 cells to prevent reversion of S-1 and loss of  $ts^+$  characteristics.

As of this date, the FRhL, p-21 (Lederle) cells have not cleared mycoplasma testing. Further passage of S-1 for seed and vaccine preparation will be carried out in FRhL, p-18 (Fort Detrick/Merrell-National) cells.

## 2. Dengue-3 vaccine strains

a. Cloning of D-3, CH 53489 virus in PGMK cells. D-3, CH 53489, PGMK-4 passage was designated a "parent" seed and used for plaquing and isolation of naturally occurring viral clones. A chemical mutagen, 5-azacytidine (5-AC) was used to enrich for  $ts^+$  mutants as previously reported (WRAIR Annual Report, 1974). D-3 parent virus was grown in the presence of 50  $\mu\text{g/ml}$  of 5-AC and a harvest made on day 4.

Both non-mutagenized and 5-AC mutagenized D-3 seeds were plaqued at terminal dilutions in PGMK cells and well-isolated plaques were picked for outgrowth in PGMK cells. Out of 11 clones, 7 attained acceptable titers after seven days in fluid culture. A large, faint plaque was associated with an intermediate degree of temperature sensitivity while a small, distinct, plaque was isolated with  $ts^-$  characteristics. As listed in Table 8, clones pp-18 (naturally occurring) and clones pp-16, pp-20, and pp-21 (5-AC mutagenized) were more temperature sensitive than the parent seed while pp-19 (naturally occurring) and pp-17 (5-AC mutagenized) were less temperature sensitive than the parent seed which contains a mixture of large and small plaques. While a greater percentage of mutagenized clones were  $ts^+$ , a much larger number of clones must be studied to attribute actual mutagenization to the 5-AC treatment.

Table 8. D-3, CH 53489 clones from non-mutagenized and 5-AC mutagenized seeds.

Mutagen	Clone	PFU/0.2 ml		EOP	Plaque morph
		35°C	39.5°C		
None	pp-18	$5.0 \times 10^2$	0	< 0.002	large, faint
	pp-19	$3.0 \times 10^4$	$1.1 \times 10^3$	0.027	small, distinct
5-AC	pp-16	$3.0 \times 10^2$	0	< 0.0033	large, faint
	pp-17	$1.6 \times 10^4$	$1.5 \times 10^3$	0.094	small, distinct
	pp-18	$2.5 \times 10^4$	$3.7 \times 10^1$	0.0015	large, faint
	pp-20	$1.5 \times 10^4$	$2.1 \times 10^1$	0.0014	large, faint
	pp-21	$5.0 \times 10^3$	$1.5 \times 10^1$	0.0030	large, faint
--	parent	$1.0 \times 10^4$	$3.2 \times 10^2$	0.032	mixture of large & small plaques

b. Passage of D-3 clones and parent viruses in FRhL cells.

Original attempts to grow D-3 in FRhL, p-35 (Lederle) cells were unsuccessful. As with D-2, virus replication in these cells was poor. Repassage of D-3 in lower passage FRhL cells (p-18, Fort Detrick/Merrell-National or p-21, Lederle) was more successful and resulted in virus titers comparable to those found in PGMK cells. Initial growth curves of clones pp-18 and pp-19 (naturally occurring) and parent D-3 viruses showed that growth at 31°C or 35°C was comparable (Table 9).

Table 9. Growth curves of Den-3, CH53489 clones and parent viruses in FRhL cells (p-18, Fort Detrick) at 31C and 35C

Virus	Temperature	PFU/0.2 ml on day		
		3	7	10
Den-3, parent	31C	$1.4 \times 10^2$	$1.3 \times 10^3$	$6.0 \times 10^3$
	35C	$4.0 \times 10^2$	$8.0 \times 10^2$	$7.0 \times 10^2$
Den-3, pp-18	31C	$3.0 \times 10^1$	$2.0 \times 10^2$	$7.0 \times 10^2$
	35C	$1.3 \times 10^2$	$2.0 \times 10^2$	$1.3 \times 10^3$
Den-3, pp-19	31C	$4.0 \times 10^2$	$2.2 \times 10^3$	$6.0 \times 10^3$
	35C	$1.0 \times 10^3$	$7.0 \times 10^2$	$1.6 \times 10^3$

Due to slow growth and low yields of D-3 virus, frequent media changes and harvests were made of the pp-18 clone during a 15 day growth curve at 31°C and 35°C. The results shown in Table 10 indicate that after a fresh media change, virus is released at a more rapid rate with the result being a higher yield of virus within 24 hrs after the change. On the basis of maximum release and yield of D-3 virus after a medium change, passages 4 to 7 of D-3 parent, and clones pp-18 and pp-19 were carried out with media changes on day 3, 7, and 11, and a final harvest on day 12. Table 11 lists the results from passage of these viruses through 7 passages in FRhL cells. The pp-18 clone seemed to retain an intermediate temperature sensitivity as well as a large, faint plaque morphology. Also, clone pp-19 retained a small, distinct plaque morphology along with a  $ts^-$  marker. Passage of the parent, PGMK-4 virus resulted in emergence of a small, faint plaque, first noticeable at the fifth passage in FRhL cells. A significant increase in temperature sensitivity also accompanied the change in plaque morphology.

Intracerebral virulence in suckling mice for the two D-3 clones and parent virus was very low when the 4th passage was tested. Only occasional deaths were observed for any of these viruses using undiluted seed preparations given IC. A better test for in vivo virulence

Table 10. Growth curve of Den-3, pp-18 clone at 31C and 35C with frequent harvests.

Day of harvest	Temp	PFU/0.2 ml	Temp	PFU/0.2 ml
4*	31C	$<1.0 \times 10^1$	35C	$<1.0 \times 10^1$
5		$1.0 \times 10^1$		$2.7 \times 10^2$
7*		$3.0 \times 10^1$		$5.1 \times 10^2$
8		$1.3 \times 10^2$		$2.2 \times 10^3$
11*		$1.0 \times 10^2$		$6.0 \times 10^2$
12		$2.0 \times 10^2$		$1.1 \times 10^3$
14*		$1.0 \times 10^2$		$3.0 \times 10^2$
15		ND		$3.6 \times 10^3$

\* Complete media change

was found to be Rhesus monkeys inoculated subcutaneously. From the results listed in Table 12, the parent D-3 (PGMK-4) produced a viremia in all of four monkeys inoculated with this virus, with the viremia lasting as long as 4 days. Also producing viremia were the clones pp-18 and pp-19. The highly  $ts^+$  passages 6 and 7 of the parent in FRhL cells were much less virulent when judged by the inability to detect circulating infectious virus in monkeys for more than one day. Antibody responses in these monkeys are also listed in Table 12. The  $ts^+$  parent virus will be characterized further after cloning to determine its efficacy as a D-3 vaccine candidate seed virus.

### 3. Effects of sequential challenge with selected strains of dengue-2 and dengue-3 virus in Rhesus monkeys

This study was divided into 3 parts: (a) a group comprising 10 young adult Rhesus monkeys was initially infected with a selected strain of dengue-3 virus (D3/PGMK-4) and challenged 60 days later with

Table 11. Passage of Den-3 parent (PGMK-4) virus and clones pp-18 and pp-19 in FRhL cells.

Virus	Passage	Harvest (day)	PFU/0.2 ml		EOP
			35C	39.5C	
parent (PGMK-4)	1	10	$6.3 \times 10^2$	$1.9 \times 10^1$	0.030
	2	7	$5.3 \times 10^2$	$9.0 \times 10^1$	0.170
	3	7	$2.8 \times 10^2$	$2.0 \times 10^1$	0.071
	4	12	$3.8 \times 10^3$	$5.0 \times 10^1$	0.013
	5	12	$4.2 \times 10^3$	2	0.00048
	6	12	$8.1 \times 10^3$	0	<0.00012
	7	12	$8.8 \times 10^3$	$1.1 \times 10^1$	0.0013
clone pp-18	1	10	$3.3 \times 10^2$	4	0.012
	2	14	$3.0 \times 10^2$	2	0.006
	3	11	$6.5 \times 10^2$	4	0.006
	4	12	$4.0 \times 10^3$	$5.2 \times 10^1$	0.013
	5	12	$4.7 \times 10^2$	4	0.0085
	6	12	$1.6 \times 10^3$	$1.5 \times 10^1$	0.0094
	7	12	$1.7 \times 10^3$	$5.6 \times 10^1$	0.033
clone pp-19	1	10	$5.3 \times 10^2$	$5.8 \times 10^1$	0.109
	2	7	$1.3 \times 10^2$	$2.3 \times 10^2$	0.17
	3	7	$8.1 \times 10^3$	$1.0 \times 10^2$	0.123
	4	12	$6.7 \times 10^3$	$9.0 \times 10^2$	0.134
	5	12	$4.0 \times 10^3$	$1.5 \times 10^2$	0.038
	6	12	$2.8 \times 10^3$	$2.6 \times 10^2$	0.093
	7	12	$6.7 \times 10^3$	$6.6 \times 10^2$	0.099
Control: parent (PGMK-4)	(no pass in FRhL; 4 passes in PGMK cells)		$4.9 \times 10^3$	$1.8 \times 10^2$	0.037

a selected strain of the dengue-2 virus (D2/PGMK-6); (b) a similar group of Rhesus monkeys was initially infected with a selected strain of dengue-2 virus (D2/PGMK-6) and 60 days later challenged with the dengue-3 strain (D3/PGMK-4); and (c) a third group, comprising 4 Rhesus monkeys, was initially infected with the dengue-2 strain (D2/FRhL-4) and challenged 180 days later with the dengue-3 strain (D3/PGMK-4).

Table 12. Relative virulence and antigenicity of selected clones of dengue-3 virus in Rhesus monkeys

Monkey No.	Inoculum and dose (PFU/0.5 ml)	No. viremic		1/CF		1/HI		
		No. inoculated		15	30	15	30	45
908	D3/pp-18 2.3 X 10 <sup>4</sup>	2/2	32	128	64	320	160	80
909			32	64	32	160	80	40
910	D3/pp-19 6.0 X 10 <sup>4</sup>	2/2	64	128	32	640	160	80
914			128	64	32	640	160	160
917	D3/PGMK-4(6a) 4.0 X 10 <sup>4</sup>	0/2	64	32	32	80	80	80
920			64	64	32	160	80	80
921	D3/PGMK4(7a) 4.3 X 10 <sup>4</sup>	1/2	128	64	16	160	80	80
935			64	128	32	80	80	80
944			32	64	32	320	160	80
946	D3/PGMK4 (Parent Strain)	4/4	32	64	32	320	160	80
948	3.3 X 10 <sup>4</sup>		64	64	64	320	160	80
950			64	64	64	320	160	80

Sixty days after this second challenge this group was given a third challenge with a different strain of the dengue-2 virus than that used in the initial challenge, namely (D2/PGMK-6).

After each sequence of inoculations, the monkeys from each group were bled on 10 consecutive days for the determination of viremia levels and at intervals of 15, 30, and 45 days post-inoculation for complement-fixation (CF) and hemagglutination inhibition (HI) antibody levels. The data pertaining to these groups are summarized in Table 13 and Fig. 22.

### C. Japanese encephalitis (JE) virus antigens and antibodies

#### 1. Immunodiffusion tests

Immunodiffusion (ID) analysis of JE antigens, including purified virion, major envelope, and soluble CF (SCF) antigens has been complicated by multiple precipitin lines in tests of these antigens with hyperimmune mouse ascitic fluid (HMAF) (WRAIR Annual Report: 1972-1974). Briefly, JE virion preparations react with JE HMAF in ID tests to give a minor precipitin which is highly cross reactive with other group B virion antigens. A major precipitin band is also formed which is relatively more type specific than the minor precipitin. Since these precipitins were seen with highly purified JE envelope antigens, the hypothesis was raised that the precipitins were the result of two separate immunoglobulins reacting with two distinct determinants on a common antigen molecule.

#### 2. Purification of immunoglobulins

Preliminary separation of crude JE HMAF was accomplished by gel filtration chromatography on Sephadex G-200 with a PBS, pH 7.2 buffer (Fig. 23, top panel). The void peak fractions were pooled and concentrated approximately 50 X using a XM-300 membrane in an Amicon pressure cell. Before concentration, bovine albumin was added to the void pool at a final concentration of 1% to stabilize the IgM present.

The second Sephadex peak containing IgG was concentrated approximately 100X using a PM-30 membrane and further purified by DEAE-Sephadex ion exchange chromatography (Fig. 23, bottom panel). The IgG concentrate was washed into the gel with 0.13 M NaCl/PBS buffer and stepwise elution with 0.2 M, 0.3 M, 0.4 M and 1.0 M NaCl/PBS buffer followed. The first CF antibody peak eluted in 0.13 M NaCl and was not adsorbed by the ion exchanger. Two more CF peaks were eluted with 0.2 M and 0.3 M NaCl as shown in Fig. 23. All three peaks were pooled and concentrated over PM-30 Amicon membranes. Concentrations of immunoglobulins M, A, G-1, and G-2 in the final preparations were quantified by radial immunodiffusion tests. Agarose ID plates containing specific immunoglobulin antibody and standard reference sera were purchased from

Table 13. Summary of serological data on rhesus monkeys inoculated sequentially with selected strains of dengue 2 and dengue 3 virus.

No. of monkeys	Inoculum PFU/dose	No. viremic		Complement-fixation		Hemagglutination-Inhibition		
		No. inoculated	15d	30d	45d	15d	30d	45d
GROUP 1								
10	(1st chall) D3/GMK-4 <sup>4</sup> 1.4 X 10 <sup>5</sup>	6/10	130	68	60	78*	55	44
10	(2nd chall) D2/GMK-6 <sup>5</sup> 3.4 X 10 <sup>5</sup>	3/10	1450	110	97	280	230	210
GROUP 2								
10	(1st chall) D2/GMK-6 <sup>5</sup> 3.4 X 10 <sup>5</sup>	10/10	215	145	98	64	70	115
10	(2nd chall) D3/GMK-4 <sup>4</sup> 3.0 X 10 <sup>5</sup>	4/10	470	280	105	1050	510	300
GROUP 3								
4	(1st chall) D2/FRh <sub>1</sub> -4 <sup>5</sup> 6 X 10 <sup>5</sup>	3/4	64	76	38	380	460	47
4	(2nd chall) D3/GMK-4 <sup>4</sup> 1.4 X 10 <sup>5</sup>	4/4	2048	1020	360	1520	540	380
4	(3rd chall) D2/GMK-6 <sup>5</sup> 3.4 X 10 <sup>5</sup>	0/4	860	182	430	460	320	134

\* Geometric mean for 10 monkeys.

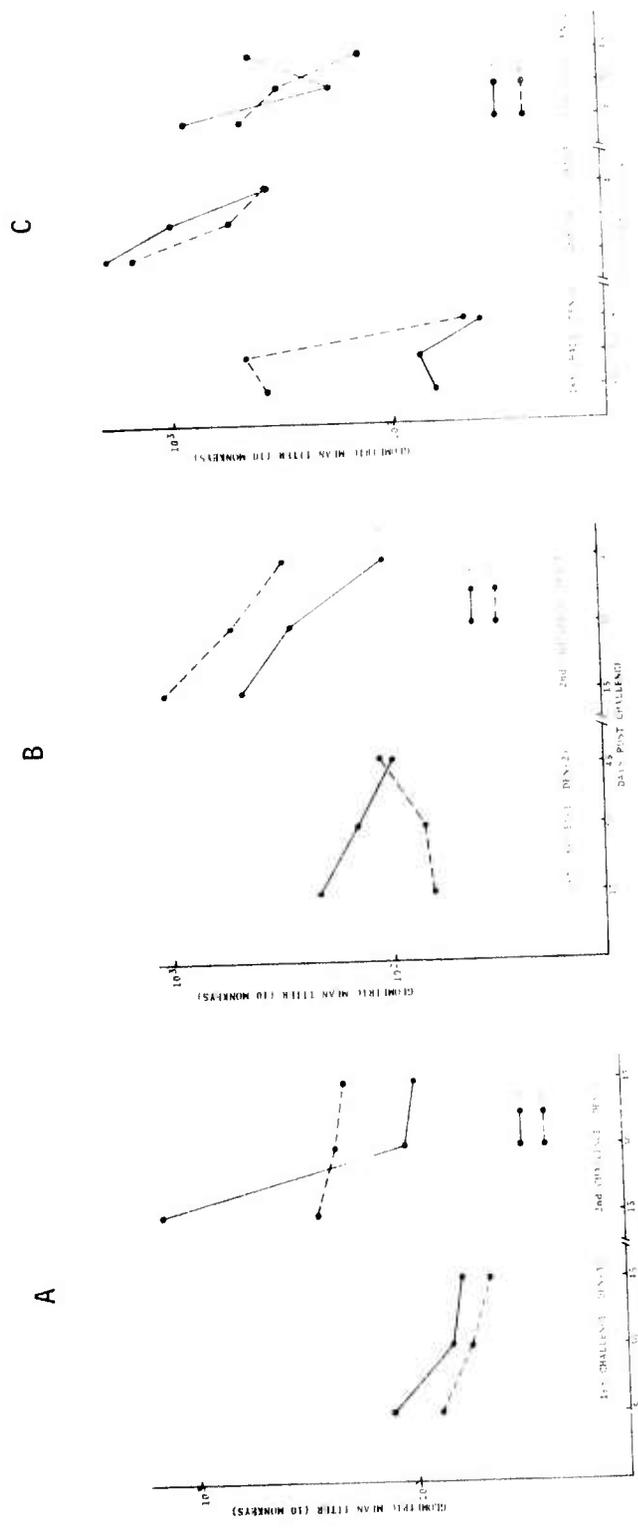


Figure 22. Geometric mean antibody titers of a group of A. ten monkeys receiving an initial inoculation with D-3 and a second inoculation with D-2 virus 2 months later; B. ten monkeys receiving an initial inoculation with D-2 and a second inoculation with D-3 virus 2 months later; C. four monkeys receiving sequential inoculations with D-2, D-3 6 months later, and D-2 again 2 months after the D-3 virus.

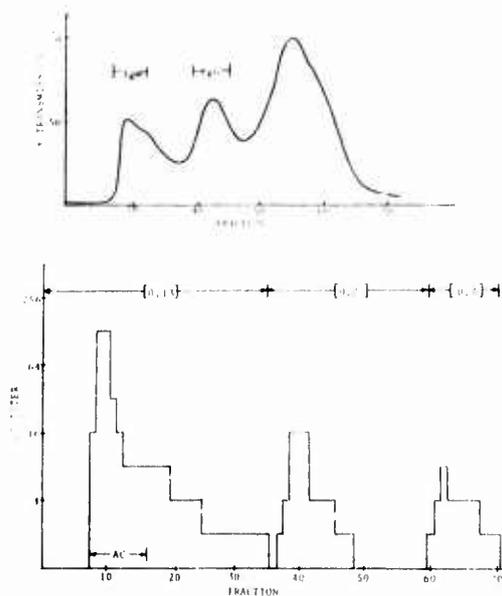


Figure 23. Upper panel depicts Sephadex gel filtration (G-200) of Japanese encephalitis (JE) virus hyperimmune mouse ascitic fluid; lower panel depicts ion exchange chromatography (DEAE-Sephadex, A-50M) of a Sephadex purified JE IgG preparation (from center peak in the upper panel).

Meloy Labs. Table 14 compares immunoglobulin levels in crude HMAF and in the purified IgM and IgG preparations. In crude HMAF, the major immunoglobulin species was IgG-2, comprising 85.8% of total immunoglobulin protein.

The concentrated void preparation from Sephadex columns showed enrichment of both IgM and IgA and still contained small amounts of IgG-1 and IgG-2. The three eluted peaks from DEAE-Sephadex contained IgG-1 and IgG-2 and no detectable IgA or IgM. The mg/% ratio of IgG-1/IgG-2 increased with increasing NaCl molarity. In crude HMAF, the IgG-1/IgG-2 ratio was 0.12 and the three peaks from DEAE-Sephadex in order of their elution was 0.06, 0.36, and 0.63.

The immunoglobulin preparations were also tested for CF, HI, precipitating, and neutralizing activity against JE virion and JE seed preparations (Table 15). The IgM preparation gave a minor precipitin band with JE virion antigen that appeared to be free of IgG (Fig. 24-A). The minor contamination of this preparation with IgG-1 and IgG-2 may account for the HI and neutralization reactions that were seen. The IgG

Table 14. Concentrations of immunoglobulins from JE HMAF.

Immunoglobulin	Concentration (mg %)			
	IgG-1	IgG-2	IgA	IgM
Crude HMAF	95	120/800*	26	12
IgM	17	35	34	25
IgG (DEAE 0.13 M)	95	170/1500*	0	0
IgG (DEAE 0.2 M)	17	35/48*	0	0
IgG (DEAE 0.3 M)	12	19	0	0

\* double precipitin rings

Table 15. Immunologic activity of immunoglobulins from JE HMAF

Immunoglobulin	CF <sup>1</sup>	HI <sup>2</sup>	Ppt	Neut <sup>3</sup>
Crude HMAF	128	160	2 lines	ND
IgM	< 8	10	1 line	88
IgG (DEAE 0.13 M)	512 <sup>4</sup>	160	1 line	100
IgG (DEAE 0.2 M)	32 <sup>5</sup>	ND	0	88
IgG (DEAE 0.3 M)	16	10	0	92

<sup>1</sup> 8 units JE virion CF antigen

<sup>2</sup> 8 units JE virion HI antigen

<sup>3</sup> percent plaque reduction of 26 PFU of JE seed virus

<sup>4</sup> AC to 128

<sup>5</sup> AC to 8

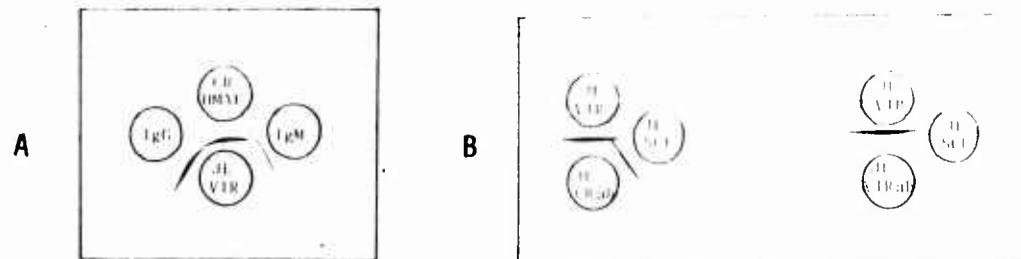


Figure 24. Agar immunodiffusion studies of (A) purified Japanese encephalitis (JE) virus IgG and IgM immunoglobulins against the JE virion antigen; (B) Japanese encephalitis virion and soluble complement-fixing (SCF) antigens against crude JE HMAF and anti-virion antibodies.

preparation from DEAE columns, which was eluted with 0.13 M NaCl and contained mostly IgG-2, was the major precipitin with JE virion antigen and also neutralized JE seed virus and reacted in CF and HI tests with JE antigen. The other two DEAE peaks, although not responsible for precipitin formation, did neutralize JE seed virus and fixed complement in the presence of JE virion antigen.

Purification of IgM, if this is the cross reactive immunoglobulin for precipitin formation with group B antigens, must be complete before conclusions can be drawn concerning the nature of this immunoglobulin. The fact that it is thermolabile (60°C/30 min) makes it a peculiar immunoglobulin which could be easily dismissed as a non-specific precipitin if it were not for specific activity with group B antigens. Serologically active IgM has not been obtainable in the past from ascitic fluid (Brandt et al., 1967). The fact that it is broadly cross reactive also makes it unique; reports of IgM specificity after primary and secondary arbovirus infections have been documented (Scott et al., 1972; Westaway, 1968).

### 3. JE virion and soluble antigens

The JE major envelope antigen appears to be biophysically and antigenically distinct from the JE soluble CF (SCF) antigen (Eckels et al., 1975). Further immunological evidence was needed to label the SCF antigen as virion or non-virion. Formalin inactivated JE virions were used to stimulate antibody production against virion structural proteins in mice. Crude JE HMAF contains antibodies against all JE proteins produced during replication of JE virus in the mouse. Immunodiffusion tests using crude JE HMAF demonstrates precipitin reactions against both JE virion and SCF antigens (Fig. 24B). The seeming reaction of partial identity (spur formation) between virion and SCF antigens may very well be a reaction of non-identity where it is hard to distinguish

an actual intersection of precipitin bands. Antibody against formalin inactivated JE virions, which would not contain non-virion antibodies, reacts strongly with a virion preparation but not with SCF (Fig. 24-B).

The results of CF tests using these antigens correlate with the results of ID tests. Although both virion CF and SCF antibodies are found in crude JE HMAF, only virion CF antibodies are found in virion-specific HMAF, and no complement fixation occurred with JE SCF antigen (Table 16). The preparation of JE SCF specific antisera and reaction of these antibodies with JE virions in ID, CF, and neutralization tests will complete the characterization of these antigens.

Table 16. Complement fixation reactions of Japanese encephalitis (JE) and SCF antigens

Antigen <sup>1</sup>	Hyperimmune mouse ascitic fluid	
	JE crude extract	JE purified virion
JE virion	128 <sup>2</sup>	512
JE SCF	128	< 8

<sup>1</sup>8 units

<sup>2</sup>reciprocal of CF titer per 25  $\mu$ l

#### 4. Antigenic variation in strains of Japanese encephalitis virus

This laboratory has used the M 1/311 strain of Japanese encephalitis virus in plaque reduction neutralization tests (PRNT) for JEV antibody. This strain was isolated from *C. tritaeniorhynchus* in Japan in 1951 and was utilized in neutralization tests in studies defining the epidemiology of JEV in birds, swine, and humans in Japan (Buescher et al., 1959; Scherer et al., 1959 a and b). The M 1/311 strain was originally selected for these early studies because of its specificity in neutralization (N) tests, a characteristic necessary to separate JEV infections from infections by other flaviviruses indigenous to Asia.

Recent experience in this laboratory has re-emphasized that the price of increased specificity is often decreased sensitivity. Sera from 4 marines stationed in Thailand who developed Japanese encephalitis were tested for JEV antibody. All 4 patients had rises to JEV (Nakayama) antigen in HAI and CF tests, but had minimal or no rise in N antibody against the M 1/311 strain (Table 17). Using the Nakayama strain, however, all 4 patients had greater than 4-fold rises in JEV N antibody.

Table 17. JEV antibody titer (reciprocal) in sera from JE patients

Patient	Serum	JEV antibody titer (reciprocal)			
		HAI	CF	Neutralization	
		(Nakayama)	(Nakayama)	M 1/311	Nakayama
PM	acute	320	< 8	< 10	59
	conv.	5120	32	< 10	720
PK	acute	160	8	< 10	< 10
	conv.	640	32	< 10	190
JM	acute	< 20	< 8	< 10	10
	conv.	320	8	16	320
SP	acute	160	16	< 10	16
	conv.	5120	64	19	115

In order to explore this discrepancy, mouse hyperimmune ascitic fluid (MHAF) raised to the M 1/311 strain and rabbit antisera and MHAF (obtained from YARU) to the Nakayama strain, were used in PRNT against M 1/311, Nakayama, and the Peking JEV strains. Results are shown in Table 18. Antibody induced by M 1/311 recognizes Nakayama and Peking identically to M 1/311. Antibody to Nakayama recognizes Peking as identical to Nakayama, but neutralizes M 1/311 to a significantly lower titer.

Similar findings have been documented by Japanese workers. Yoshioka et al. (1972), have described identical antigenic relations in PRNT between Nakayama (NIH) and JaGAR-01 (4). From inspection of their data, it is likely that M 1/311 is antigenically similar to JaGAR-01; however, we have not tested this hypothesis. It would seem essential to use broadly reacting JEV strains like Nakayama, rather than M 1/311, for serologic diagnosis of human infections.

Table 18. Cross neutralization tests (PRNT) with JEV strains

Virus	S e r u m		
	M 1/311	Nakayama	
	(MHAF)	(MHAF)	Rabbit
M 1/311	<u>900</u> *	90	70
Nakayama	700	<u>390</u>	<u>470</u>
Peking	1100	420	1000

\* reciprocal of PRNT titer

#### 5. Viral polypeptide composition of Japanese encephalitis virus-infected cell membranes

Electron microscopic studies of cells infected with flaviviruses suggest that viral maturation occurs in intimate association with internal cellular membranes (Matsumura et al., 1971; Murphy et al., 1968; Ota, 1965). Early in infection the cytoplasm of infected cells shows no prominent signs of viral replication, but during the exponential phase of virus growth the cytoplasm becomes filled with vesicles containing seemingly mature virus particles, which have been shown to differ in polypeptide composition from extracellular virus (Shapiro et al., 1972-a). On the outside of the vesicles are electron dense particles about 27 nm in diameter that have been suggested to be viral precursors, but budding of these particles through the vesicle membrane has never been convincingly demonstrated (Ota, 1965). The vesicles are thought to migrate to the surface of the cell where the virions are released through reverse pinocytosis. This study was undertaken with the hope of elucidating which cytoplasmic membranes were involved in the morphogenesis of Japanese encephalitis virus.

a. Infected cell culture conditions. Methods for infecting chick embryo cells (CEC) with mouse-brain seed of the M 1/311 strain of Japanese encephalitis (JE) virus at high multiplicity of infection (> 50) in closed 32 oz bottles at 35.5°C, have been previously described (Shapiro et al., 1971). Monolayers of primary chick embryo cells were prelabeled for 24 hrs with <sup>14</sup>C-choline (1 μCi/ml), infected with JE virus, treated with actinomycin-D (1 μg/ml) from 9 to 24 hrs post infection, pulsed with cycloheximide (300 μg/ml) from 18-18½ hrs

post infection and labeled with  $^3\text{H}$ -amino acids (20  $\mu\text{Ci/ml}$ ) from 19 to 24 hrs post infection.

b. Cellular fractionation. Cells were fractionated by a procedure that is a modification of several previously described schemes (Bosmann et al., 1968; Caliguiri and Tamm, 1970; Spear et al., 1970; Uhr and Schenkin, 1970). Most of the growth media was decanted, the cells were scraped into residual media and centrifuged at 480 x g for 10 min. All subsequent operations were performed at 4°C. The packed cells were resuspended in 1.0 ml of reticulocyte standard buffer (RSB), (0.01 M tris (hydroxymethyl)-aminomethane, 0.01 M NaCl, 0.0015 M  $\text{MgCl}_2$ , pH 7.4) and allowed to swell for 10 min. They were homogenized in a Sorvall omnimixer for 30 sec and monitored by phase contrast microscopy to assure that all cells were broken but that nuclei remained intact. The homogenate was centrifuged at 480 x g for 2 min and the supernatant, which contained 50-60% of the total acid-insoluble radioactivity, was adjusted to 1.3 M sucrose by the addition of solid sucrose. Molarities were adjusted by reference to the refractive index of sucrose solutions.

The homogenate was then loaded in the middle of a discontinuous gradient constructed by sequentially layering the following molarities of sucrose in RSB: 0.2 ml of 2.0 M, 0.7 ml of 1.75 M, 0.7 ml of 1.55 M, 0.7 ml of 1.35 M, 0.8 ml of 1.3 M (load zone), 0.7 ml of 1.2 M, 0.7 ml of 1.0 M, 0.6 ml of 0.7 M, and 0.2 ml of 0.25 M. The gradient was centrifuged in a SW 65 L Beckman rotor for 90 min at 65,000 rpm (300,000 x g), after which 0.1 ml fractions were collected from the bottom of the tube and assayed for radioactivity. Visible bands, which coincided with peaks of radioactivity, occurred at the interface of each two adjoining sucrose concentrations and were numbered sequentially from the top to the bottom of the gradient. Band 1, at the 0.25-0.7 M interface, contained 3% of the acid-insoluble radioactivity; band 2, at the 0.7-1.0 M interface, contained 2% of the acid-insoluble radioactivity; band 3, at the 1.0-1.2 M interface, contained 4% of the acid-insoluble radioactivity; band 5, at the 1.35-1.55 M interface, contained 22% of the acid-insoluble radioactivity; and band 6, at the 1.55-1.75 M interface, contained 18% of the acid insoluble radioactivity. Band 4, at the interface between 1.30-1.35 M sucrose, was not analyzed because of its proximity to the load zone.

Bands 1, 2, 3, 5 and 6 were adjusted to 1.3 M sucrose and each was layered at the appropriate place in the following separate discontinuous sucrose-RSB gradients. Bands 1 and 2: 0.4 ml of 2.0M, 1.2 ml of 1.3 M (load zone), 1.2 ml of 1.0 M, 1.2 ml of 0.7 M, and 0.8 ml of 0.25 M. Band 3: 0.4 ml of 2.0 M, 1.2 ml of 1.3 M (load zone), 1.2 ml of 1.2 M, 1.2 ml of 1.0 M, and 0.8 ml of 0.7 M. Bands 5 and 6: 0.4 ml of 2.0 M, 0.8 ml of 1.75 M, 1.2 ml of 1.55 M, 1.2 ml of 1.35 M, and 1.2 ml of 1.3 M (load zone).

All gradients were centrifuged in a SW 50.1 rotor for 110 min at 50,000 rpm (234,000 x g), after which 0.2 ml fractions were collected from the bottom of the tube and assayed for radioactivity. Each gradient had a single sharp peak of radioactivity, corresponding to a single visible band, which had rebanded at the same interface as in the original gradient. The ratio of  $^3\text{H}$ -amino acids:  $^{14}\text{C}$ -choline increased according to the density of the band: band 1, 0.2; band 2, 1.0; band 3, 1.5; band 5, 3.5; and band 6, 5.0. The peaks were pooled and centrifuged in the SW 50.1 rotor for 45 min at 50,000 rpm (234,000 x g).

c. Electron microscopy. The individual pelleted membrane fractions were prefixed with 2% phosphate buffered glutaraldehyde, pH 7.2 at 4°C for 30 min and then washed three times with 1% phosphate buffered saline pH 7.2. They were then postfixated with 1% phosphate buffered osmic acid at 4°C for 2 hrs, washed again with the buffer, dehydrated in ethyl alcohol and embedded in epoxy resin (Epon 812). The sections were stained with uranyl acetate followed by lead citrate and photographed in a Hitachi 11-A electron microscope at an accelerating voltage of 75 KV.

d. Polyacrylamide gel electrophoresis (PAGE). The procedures used here have been previously described (Shapiro et al., 1971). Briefly, the pelleted bands were boiled in 0.01 M phosphate buffer, pH 7.0, containing 1% sodium lauryl sulfate and 1% 2-mercaptoethanol for 10 min and electrophoresed on 8% polyacrylamide gels for approximately two hrs at a constant voltage of 60 V.

e. Chemical assays. Protein determinations were made by the method of Lowry et al. (1951). Glucose-6-phosphatase was assayed by the method of Heppel and Hilme (1951) and 5'-nucleotidase was assayed by the method of Weaver and Boyle (1951).

f. Results. Electron microscopic (EM) examination of the pelleted membrane bands revealed that band 1 (Fig. 25-A) contained only smooth membranes. Band 2 contained smooth membranes with some cytoplasmic debris, some rough membranes and occasional free ribosomes. Band 3 was still predominately smooth but ribosomes were clearly seen in every field; membranes morphologically similar to the type seen around maturing virions in thin sections were also numerous. Virus particles were easily detectable. Band 5 (Fig. 25-B) consisted predominately of rough membranes with some cytoplasmic debris; e.g., dense chromatin. Virus particles were rare relative to band 3. Band 6 contained rough membranes, debris, and some mitochondria. Virus particles were again rare relative to band 3. In general, only band 1 membranes were completely smooth; as the density of the bands increased, so did the heterogeneity.

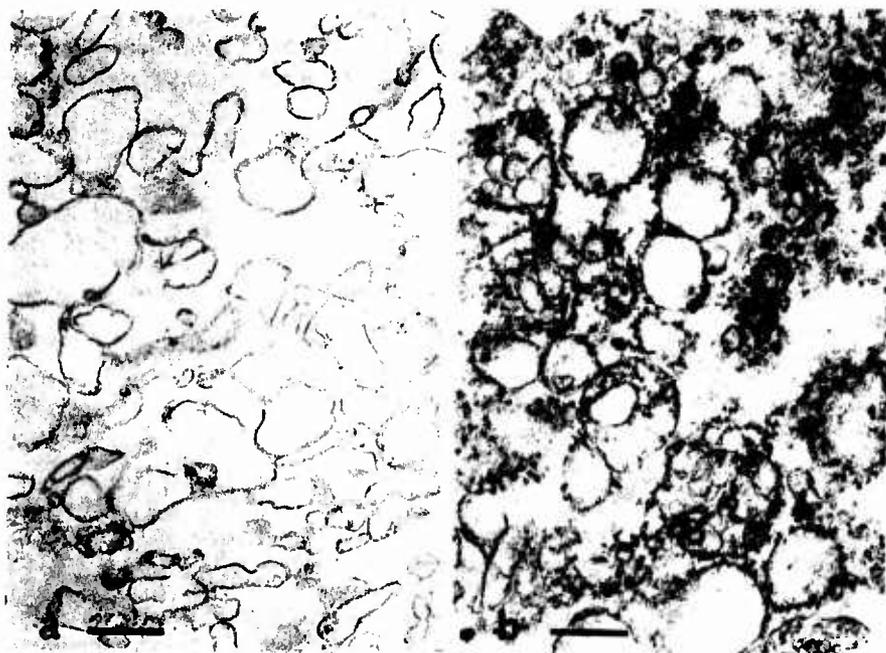


Figure 25-A and 25-B. Electron-micrographs of pelleted membrane fractions (magnification 40,000 X); on both micrographs, bar equals 250 nm; a) Band 1; b) Band 5.

Each of the purified membrane bands was assayed for the presence of glucose-6-phosphatase, an internal membrane marker (Bosmann et al., 1968) and 5'-nucleotidase, an enzyme found predominately on plasma membranes (DePierre and Karnovsky, 1973), as well as for total protein (Lowry et al., 1951). The specific activity ( $\mu\text{g Pi}$  liberated per hr/ $\mu\text{g}$  protein) of 5'-nucleotidase was highest in bands 1 through 3 (2 to 3-fold higher than bands 5 and 6). Glucose-6-phosphatase had a similar specific activity in all bands with the highest concentration being found in bands 2 and 3. We therefore concluded that the less dense bands which were relatively free of ribosomes by EM examination contained some plasma membrane as well as smooth endoplasmic reticulum.

PAGE of the pelleted membrane bands revealed the presence of all of the viral specified polypeptides in all of the bands; representative patterns are shown in Fig. 26. However, the relative proportion of each polypeptide varied among the membrane fractions (Table 19). The most significant difference was the increased amount of NV-5 (non-virion, molecular weight 94,000 daltons) in smooth membranes (19% of the total viral count; in band 1 and only 7% in band 6 (Table 19, Fig. 26). Another large structural polypeptide NV-4 did not have the same distribution as NV-5. In previous studies on release of viral

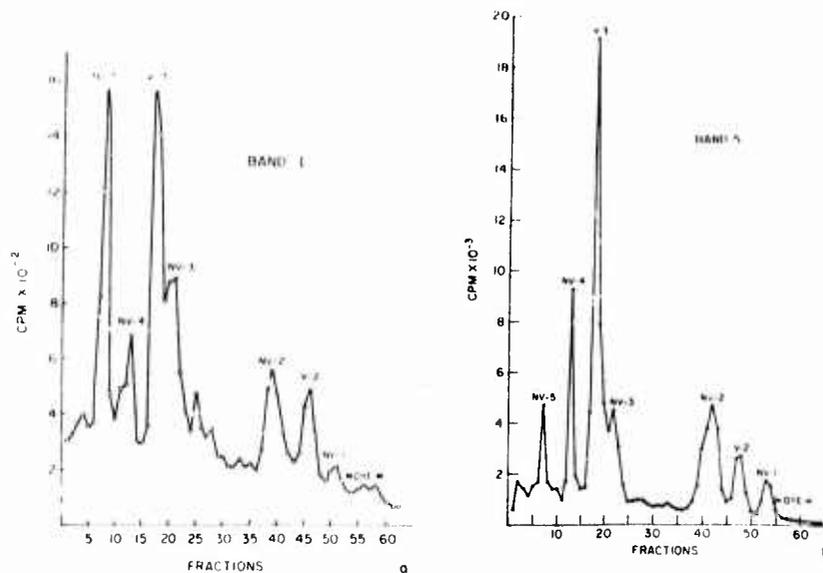


Figure 26. Polyacrylamide gel electropherograms of pelleted membrane fractions; A, Band 1; B, Band 2.

Table 19. Relative amount of each virus-specific polypeptide expressed as a percentage of the total virus specific radioactivity within each band.

Band	NV-5	NV-4	V-3	NV-3	NV-2	V-2	NV-1
1	19	9	26	16	14	12	4
2	15	14	32	10	16	9	4
3	12	14	34	11	13	8	4
5	8	13	37	11	19	7	5
6	7	12	42	10	19	7	4

polypeptides from membranes by trypsin (Shapiro et al., 1972b), these two polypeptides behaved similarly, suggesting that they may have been two subunits of a single membrane bound protein. This possibility now seems unlikely.

Smaller changes in the distribution of some of the other polypeptides were noted: V-3 (the large membrane glycoprotein of the virion) was enriched in the rough bands (26% of the total viral counts in band 1, 42% in band 6); NV-2 (a membrane glycoprotein of the intracellular form of the virion) was slightly enriched in the rough bands (14% in band 1, 19% in band 6); V-2 (the viral nucleocapsid polypeptide) exhibited opposite behavior (12% in band 1, 7% in band 6). Since intracellular virus is composed of V-3, NV-2 and V-2 (Shapiro et al., 1972a), and since the proportions of these three polypeptides did not vary coordinately, it is not probable that a specific distribution of intracellular virus accounted for the differences in polypeptide composition of the membrane fractions.

g. Discussion. Electron microscopic studies of flavivirus infected cells indicate that viral maturation occurs on internal membranes of the cell (Murphy et al., 1968; Ota, 1965). Seemingly complete virus particles are seen in vesicles within the cytoplasm. The vesicles are lined by 27 nm particles that have been suggested as viral precursors (Ota, 1965), although budding of these particles has not been convincingly demonstrated. It seems unlikely that NV-5 is a predominant component of these particles since this polypeptide is present in highest concentration in smooth membranes lacking particles.

In contrast to flaviviruses, alphaviruses mature by budding through the plasma membrane (Acheson and Tamm, 1967, 1970). These different routes of morphogenesis are reflected in the distribution of viral polypeptides in infected cell membranes. JE-infected cells evidence structural and nonstructural viral polypeptides on both smooth and rough endoplasmic reticulum, but internal membranes of cells infected with Sindbis, an alphavirus, do not contain any viral polypeptides. Both the envelope and nucleocapsid polypeptides were found on plasma membranes isolated from Sindbis-infected cells (Bose and Brudige, 1972).

The fractionation scheme used in the present experiments did not differentiate between smooth and plasma membranes of the cell and we cannot rule out the possibility that the enrichment of NV-5 in band 1 may be due to its presence on plasma membranes. However, preliminary studies involving PAGE of purified CEC plasma membranes, obtained at 24-hr post infection by the method of Perdue and Sneider (1970), suggest that they are free of viral polypeptides at this stage of virus replication (unpublished data). This result is consistent with that obtained with dengue-2, another group B arbovirus, where virus-specific antigens in the plasma membrane were not detected until 36-hrs post infection (Catanzaro et al., 1974).

The enrichment of V-3 and NV-2 in the rough membrane fractions

suggests that they may be accumulating at the site of maturation. The nucleocapsid polypeptide V-2 appears to be decreased in the rough membranes and may represent the rate limiting polypeptide in viral assembly with the membrane polypeptides being produced in excess. This hypothesis is compatible with the production of SHA, a subviral particle found late in infection, consisting of the virion membrane polypeptides but lacking V-2 (Shapiro et al., 1971). It has been suggested that SHA may, in fact, represent the residue after virion membrane maturation (Shapiro et al., 1972a), since the intracellular form of the virus differs from the extracellular form in the amount of virion membrane protein present relative to V-2.

Earlier studies on whole CEC extracts have shown that NV-5 is not a precursor of the viral structural polypeptides, since the synthesis of NV-5, NV-4, and NV-1 is inhibited by treatment with puromycin, whereas the structural polypeptides are unaffected (Shapiro et al., 1973), and NV-5 has been shown to be immunologically distinct from the virion structural polypeptides (Qureshi and Trent, 1973). Thus the decrease of NV-5 in rough membranes is not due to its cleavage into V-3 or NV-2. However, the possibility exists that NV-5 is in some way consumed during viral maturation on rough membranes. This possibility cannot be ruled out without pulse-chase experiments. Such experiments are not feasible with the current methodology of cell inhibition using both actinomycin-D and cycloheximide, since these drugs also decrease the efficiency of labeling viral polypeptides (Shapiro et al., 1973). Currently, we are developing drug treatments to effectively inhibit cellular protein synthesis while allowing better incorporation of radioactivity into viral proteins. Such methods would make kinetic studies possible.

#### D. Studies on the involvement of the nucleus in flavivirus replication

##### 1. Inhibition of flavivirus antigen production and replication in cells enucleated with cytochalasin B.

An examination of the replication patterns of alphaviruses and flaviviruses reveals a number of differences between the two groups. A comparative examination of the kinetics of replication has shown that the replication time of the flaviviruses is considerably longer than those of alphaviruses (Pfefferkorn, 1968). In chicken embryo cells (CEC), Japanese encephalitis (JE) virus, the representative flavivirus group used in these studies, has a latent period of about 10 hr (Shapiro et al., 1971), twice as long as that of alphaviruses such as Sindbis (SIN) virus (Pfefferkorn and Hunter, 1963). The difference suggests that early processes in flavivirus replication may be of a more complex nature. Little information exists on the intracellular events occurring during the latent period of flavivirus replication. Ultrastructural studies of infected cells have revealed no morphological changes until the appearance of cytoplasmic virus-filled vesicles during the logarithmic phase of viral replication (Matsumura et al., 1971; Murphy et al., 1968; Ota, 1965). However, the electron

microscope studies of Yasuzumi and Tsubo (1965) and Yasuzumi et al., (1964), describe viral precursors in the nucleus during the latent period, as well as degenerative nuclear changes during the course of infection. In addition, a few biochemical studies also suggest the possibility of nuclear involvement (Takeda et al., 1965; Brawner et al., 1973).

Using the recently developed technology for mass enucleation of populations of cells by treatment with cytochalasin B (Prescott et al., 1972), we have inquired into the necessity of a nucleus for the replication of JE. Furthermore, by enucleation at various times subsequent to the time of infection, we can address the question of how long a nucleus must be present for this flavivirus to replicate. Since there is no indication that alphaviruses require a nuclear step in their replication, a comparative study using SIN virus has been conducted as a control.

Cells were enucleated by a modification of the procedure developed by Prescott et al., (1972). Plastic discs on which secondary cultures of CEC had been grown were inverted into 50 ml plastic centrifuge tubes which contained 8 ml of Dulbecco's modified Eagle medium containing 10% fetal calf serum (DME-10% FCS) with 10  $\mu$ g of cytochalasin B per ml (Aldrich Chemical Co., Milwaukee, WI). They were centrifuged at 37°C for 15 min at 12,000 rpm in the SS-34 rotor in a Sorvall RC2B centrifuge. The discs of cells were then washed with a balanced salt solution and placed at 37°C in the 35 mm petri dishes containing 2 ml of DME-10% FCS. Recovery was accomplished within 15 to 30 min with > 95% enucleation.

After recovery, if the cells were to be used in the immunofluorescence assay, the discs were washed once with Puck saline G (Puck et al., 1958), a phosphate-buffered saline-containing glucose (GPBS) from which we omitted calcium and magnesium, and placed into small beakers containing 10 ml of 0.25% trypsin (GIBCO) for 5 min at room temperature. Using a rubber policeman, the cells were scraped off into the trypsin, 15 ml of DME-2% FCS was added and the cells were centrifuged at 2,000 rpm for 10 min. Cells were resuspended in DME-10% FCS and plated into chamber slides (Lab-Tek, Westmont, IL). Cells from four discs were used for each chamber slide.

Cells were infected at a multiplicity of infection (MOI) of 50 using either JE (M1/311) virus or SIN (AR339) virus with DME-20% added.

Cells were washed twice with GPBS, fixed for 5 min in absolute methanol, rinsed again with GPBS, and frozen at -70°C until used. Hyperimmune mouse ascitic fluid (HMAF), prepared against JE or SIN by previously described methods (Brandt et al., 1967), or normal mouse ascitic fluid was placed on the cells and incubated for 30 min at 37°C. The hyperimmune mouse ascitic fluid (or normal mouse ascitic fluid)

was drained off and the cells were washed on a shaker at 37°C for 1 hr with three changes of 0.01 M phosphate-buffered saline, pH 7.2. Fluorescein-conjugated goat anti-mouse gamma globulin (Antibodies, Inc., Davis, CA) was added to each slide and incubated for 30 min at 37°C. The conjugated antiserum was removed and once again the cells were washed on a shaker at 37°C for 1 hr with three changes of phosphate-buffered saline. Glass coverslips were mounted on the slides with 90% glycerol 10% phosphate-buffered saline. Slides were then viewed using an American Optical Microstar fluorescent microscope equipped with a mercury lamp (HBO-200 W), a Corning No. 5840 exciter filter, an opaque glass heat-absorbing filter, and a Schott OG-1 barrier filter. Photographs were taken with a Nikon Dark Box Model 35S equipped with a Nikon AFM microflex body, using Kodak Tri-X film ASA 400.

In the assay of virus infectivity samples of culture fluid from infected cells were diluted and used to infect LLC-MK<sub>2</sub> cells, an established line of monkey kidney cells. After adsorption for 1 hr at 37°C, 7 ml of an overlay of the following composition was added to the cells: 1% purified agar (Difco), 10% FCS, 10% (10X) Medium 199, 0.3% NaHCO<sub>3</sub>, 0.5% (100 X) vitamins, 0.5% (100 X) BME amino acids, 0.2% DEAE-dextran, 100 U of penicillin, and 100 U of streptomycin. The cells were then incubated for 4 days if infected with SIN or 7 days if infected with JE, at which time 0.5 ml of a 1:1,500 dilution of neutral red (GIBCO) in normal saline was added to each flask. After incubation at 37°C for 2 hr, the cells were left covered at room temperature overnight and plaques were counted the next day.

Results: Nucleate and enucleate CEC were infected with either SIN virus or JE virus and were assayed for virus-specific antigens using the indirect fluorescent antibody technique. Cells were enucleated before infection and fixed and examined at 18 hr postinfection. The cells were counted and the percentage which showed virus-specific fluorescence was determined. Of the cells infected with SIN virus, 83% of the nucleate cells fluoresced as compared to 81% of enucleate cells. The viral fluorescence was of the same intensity in both types of cultures and involved the entire cytoplasm (Fig. 27-A & B). These results indicated that production of group A togavirus antigen does not require the nucleus.

Repetition of the above experiment with JE virus produced opposite results. No enucleate cells (Fig. 27-D) exhibiting JE-specific fluorescence were observed after scanning 1,000 cells, whereas 69% of the nucleate cells showed JE-specific fluorescence. This indicates the requirement for a nucleus-associated function.

In addition to assaying for the synthesis of viral antigens, cultures of nucleate and enucleate togavirus-infected cells were tested for the production of infectious virus. Samples of infected culture fluid were removed during the latent period and late in the virus replication cycle, i.e., at 2 hr and 10 hr postinfection in the case of

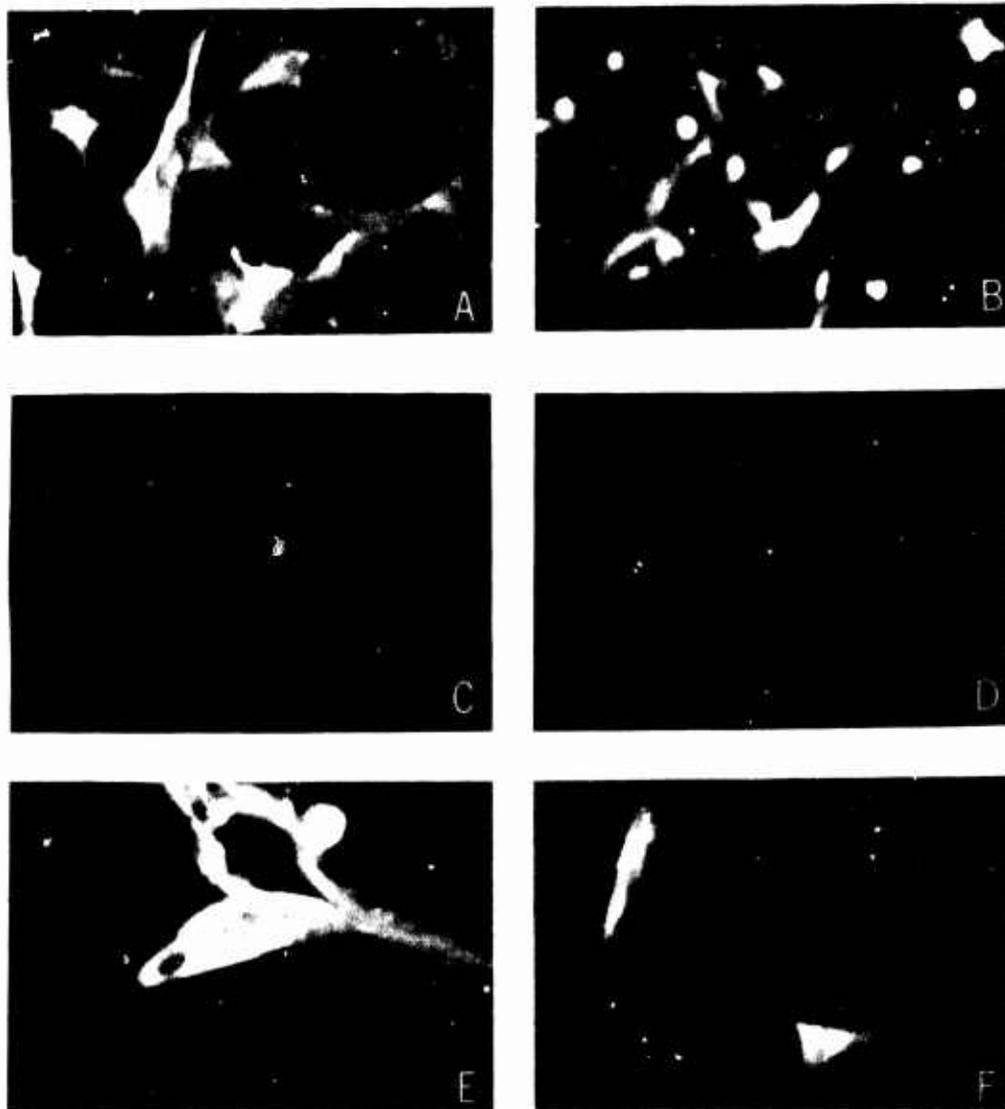


Figure 27. Immunofluorescent patterns of chicken embryo cells observed using specified hyperimmune mouse ascitic fluids (HMAF) and fluorescein-conjugated goat anti-mouse gamma globulin. A. SIN virus-infected, nucleated cells treated with SIN HMAF; B. SIN virus-infected enucleated cells, treated with SIN HMAF; C. Uninfected, enucleated cells, treated with JE HMAF; D. JE virus-infected, enucleated cells, treated with JE HMAF; E. JE virus-infected, nucleated cells, treated with JE HMAF; F. JE virus-infected cells, enucleated at 10 hours post infection and treated with JE virus-specific HMAF.

SIN infected cells (Pfefferkorn and Hunter, 1963) and 2 and 24 hr postinfection in JE-infected cells (Shapiro et al., 1971). The samples were titered by plaque assay in LLC-MK<sub>2</sub> cells and the results are shown in Table 20. In agreement with the immunofluorescent data, SIN-infected enucleate cells produced infectious virus, whereas JE-infected enucleate cells did not. The slight rise in titer in the JE-enucleate cultures may be accounted for by the 1% of nucleate cells remaining after cytochalasin B treatment. Both JE- and SIN-infected cells which had been treated with cytochalasin B but not enucleated gave yields of virus comparable to nucleate-infected cells.

Table 20. Comparison of the production of infectious virus in togavirus infected nucleate and enucleate chicken embryo cells

Virus	Expt	PFU/ml of culture fluid (log <sub>10</sub> )					
		2 hr post-infection <sup>a</sup>		10 hr post-infection		24 hr post-infection	
		N <sup>b</sup>	E	N	E	N	E
SIN	1	4.4	4.3	6.7	5.1		
	2	4.3	4.5	6.1	5.5		
JE	1	4.0	3.8			5.7	4.0
	2	4.3	4.3			6.3	4.6
	3	3.3	3.6			6.0	4.0

<sup>a</sup>An inoculum of 10<sup>8</sup> PFU of virus was used in all cases.

<sup>b</sup>N, nucleate; E, enucleate.

Kinetics of the nuclear phase of JE replication: To determine the temporal relationship of nuclear involvement of JE replication, cells infected with JE virus were enucleated at 2, 4, 6, or 10 hr postinfection. All cultures were assayed for virus-specific immunofluorescence at 18 hr postinfection and samples of culture fluid were taken at 10 hr (during the latent period) and 24 hr postinfection. Results are shown in Table 21. These data indicated that the nucleus-requiring

phase occurs during the latent period, being completed in all cells by 10 hr postinfection, with a minimum of 4 hr being required.

Table 21. Production of infectious virus by JE-infected chicken embryo cells enucleated at various times after infection

JE-infected chicken embryo cells	Expt	PFU/ml of culture fluid (log <sub>10</sub> )		% cells showing virus specific immuno- fluores- cence
		10 hr post- infection	24 hr post- infection	
Nucleate cells	1	2.6	4.0	71
	2	2.8	3.8	
Enucleated 4 hr postinfection	1	2.8	3.5	03
	2	2.7	3.3	
Enucleated 6 hr postinfection	1	2.8	3.5	34
	2	2.6	3.6	
Enucleated 10 hr postinfection	1	2.6	4.0	68
	2	2.6	4.0	

In the nucleated JE-infected CEC, the fluorescence was confined to the cytoplasm and was most intense in the perinuclear area (Fig. 27-E). Infected cells enucleated after the nuclear phase showed fluorescence throughout the cell. Often the most intensely fluorescing region appeared in the center of the cell, the space normally occupied by the nucleus (Fig. 27-F). This suggests that the perinuclear area is an important site for viral antigen synthesis, in agreement with immunofluorescent data on cells infected with another group B arbovirus (togavirus), dengue-2 (Cardiff et al., 1973).

Discussion: The data presented indicate a major basic difference in the mechanism of replication of alphaviruses and flaviviruses. Synthesis of viral antigens and production of infectious virus in cells

infected with SIN was clearly not dependent on the presence of the cell nucleus, whereas the presence of the nucleus was essential for JE virus replication.

Enucleate cells infected with SIN virus produced infectious virus, thus demonstrating that not only the direction of the synthesis of virus-specific antigens, but also the completion of all of the biosynthetic and morphogenetic steps necessary for the production of infectious progeny virus, proceeds in the absence of a nucleus. However, the yield of infectious virus from enucleate cells was reduced compared to the nucleate controls. This reduction in yield may be attributable to several factors. Fewer cells were plated in the cultures to be enucleated and some additional cell loss ( $\sim 10\%$ ) occurred during centrifugation. In addition, enucleate cells are considerably smaller than nucleate cells. Since a significant portion of the plasma membrane is lost during enucleation, the total area available for maturation of this budding virus is proportionally reduced. All of the above factors, as well as the extensive disruption of cellular organization that must also occur (Follet et al., 1974), may be responsible for the decreased efficiency of infectious virus production.

In contrast, it was clear from yield and immunofluorescent studies that the nuclear phase of JE replication in most of the cells of the population was completed by 10 hr postinfection. Our data are congruent with observations made by others during studies with metabolic inhibitors. Cordycepin, as well as actinomycin D and daunomycin, prevents the replication of Saint Louis encephalitis, another flavivirus, in BHK 21/13 cells when added during the first 9 hrs after infection (Trent, personal communication). However, reports of both sensitivity and insensitivity of flavivirus replication to actinomycin D exist (Nishimura and Tsukeda, 1971; Shapiro et al., 1971; Stollar et al., 1966; Takeda et al., 1965; Trent et al., 1969; Zebovitz et al., 1972, 1974). These can be explained on the basis of variations in time of addition and dose of the drug, as well as variation in the response of the host function in different cell types. The fact that conditions can be determined where group B replication is sensitive to inhibitors of nuclear functions does suggest the existence of a nuclear phase of virus replication. In contrast, replication of alphaviruses is insensitive to actinomycin D under all conditions and, under the appropriate circumstances, the yield of infectious virus is even enhanced (Heller, 1963; Purifoy et al., 1968). The sensitivity of the replication of Saint Louis encephalitis to cordycepin, combined with the existence of a nuclear-associated viral induced 45S RNA with the same sedimentation coefficient as virion RNA but a higher poly (A) content (5.6 to 7.8% versus 2.4% for virion RNA) (Brawner et al., 1973), suggests that post-transcriptional addition using a host-specified nucleus-associated enzyme occurs. Other support for nuclear involvement in flavivirus replication includes autoradiographic studies indicating that early in infection viral RNA synthesis occurs in the

nucleus (Takeda et al., 1965). Studies also exist to suggest the involvement of the perinuclear area in virus replication. Using 1% Triton X-100, Zebovitz (1974) could remove virus-specific RNA from the nuclear fraction of group B togavirus-infected cells without disruption of the nucleus. Hence, Zebovitz's suggestion that the site of viral RNA synthesis is not the nucleus per se, but the outer nuclear membrane, cannot be ruled out and must be considered in formulating a scheme of replication for group B togaviruses. Likewise, immunofluorescent data indicate the involvement of the perinuclear area of the cell in viral antigen production (Cardiff et al., 1973). Nuclear fluorescence has never been reported (Bhamarapravati et al., 1964; Cardiff et al., 1973; Eldadah and Nathanson, 1969). However, all of the above studies were conducted on cells during the logarithmic phase of virus replication and hence do not conflict with a nuclear involvement during the latent period, as we suggest.

One possible interpretation of our data is that some early essential step in the replication of JE, a representative group B togavirus, occurs in the nucleus. The most probable basis for nuclear participation in group B arbovirus replication is a host-mediated process involving modification of the viral genome by post-transcriptional addition of a poly (A) sequence.

## 2. Studies on the infectivity of flavivirus RNA

The hypothesis that nuclear participation in flavivirus replication involves a host mediated modification of the incoming viral genome could be tested by comparing the infectivity for enucleated cells of virion RNA extracted from Japanese encephalitis (JE) virus, with that of virus-specified intracellular RNA extracted from cells 10 hrs post infection, - i.e., when enucleation of the cells no longer affects virus replication. The expected result would be that virion RNA would be incapable of infecting enucleated cells, whereas virus specified RNA would be modified in such a way as to initiate infection in the absence of the cell nucleus. If this be the case one could then proceed to analyze the two species of RNA to determine the nature of the modification and possibly effect the modification in vitro using isolated nuclei. In order to proceed with such a project the following technologies must be developed: 1) extraction of virion and intracellular virus specified RNA in undegraded form; 2) development of optimal conditions for the assay of RNA infectivity; and 3) application of these in a system involving single cells in which the enucleated state of the infected host cell is apparent.

a. Extraction of virion RNA from purified preparations of <sup>3</sup>H-uridine labeled Japanese encephalitis virus. Roller bottles of chicken embryo cells (CEC) were infected with the M1/311 strain of JE at a multiplicity of infection (MOI) of about 10. After incubation at 37°C for 1 hr for viral adsorption, the inoculum was rinsed off and

50 ml of Medium 199, with 2% fetal bovine serum (FBS) and containing 2  $\mu$ Ci/ml of  $^3$ H-uridine was added to each bottle. After incubation at 37°C for 40 hrs, virus was purified from the culture fluids by standard methods (Annual Reports, 1971, 1973). Final gradient purification of pelleted virus was performed on a 4.8 ml linear 5-25% sucrose gradient with a 0.2 ml pad of 70% sucrose (all sucrose solutions were w/v and were prepared in a buffer of 0.02 M tris(hydroxymethyl) amino methane, 0.15 M NaCl, 0.001 M EDTA, pH 8.2 (TNE 8.2). The gradient was centrifuged at 50,000 rpm for 35 min in the Beckman SW 50.1 rotor. Fractions of 0.2 ml were collected from the bottom and 5  $\mu$ l of each fraction was assayed for radioactivity. The results of a typical experiment are shown in Fig. 28-A. Peak fractions were pooled and stored at -20°C until they were used for the extraction of RNA.

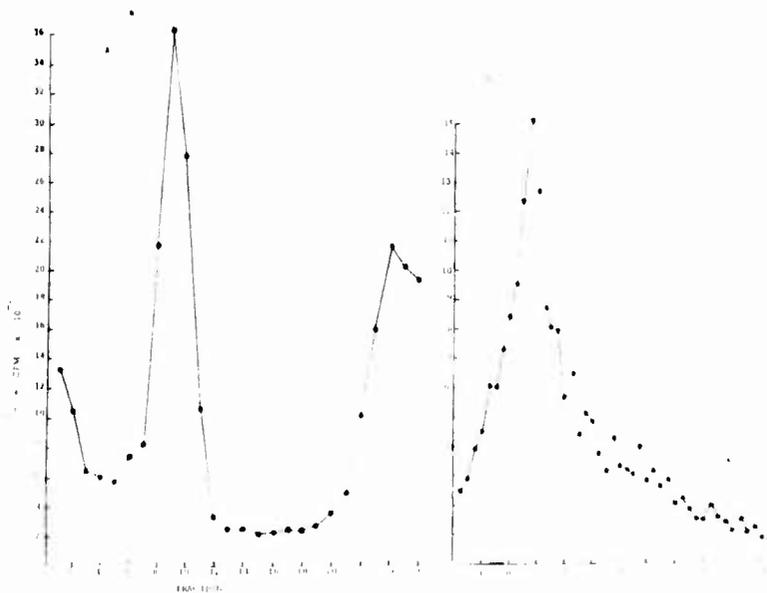


Figure 28-A & B. Rate zonal centrifugation of (A) radioactive Japanese encephalitis (JE) virus ( $^3$ H-uridine) through a 5-25% sucrose gradient at 50,000 rpm for 35 min; (B) JE virus RNA through a 5-25% sucrose gradient at 50,000 rpm for 2 hrs, both gradients in the Beckman 50.1 rotor.

Successful extraction of the RNA was accomplished by first making the pooled gradient fractions 1% with respect to SLS and 0.05 M with EDTA, to help minimize nuclease activities. In addition, all solutions and glassware were sterilized. The sample was then shaken at room temperature with one volume of liquified phenol and one volume of

chloroform. The phases were separated by centrifugation at 1500 rpm for 10 min at room temperature. The aqueous phase was saved and the organic phase was reextracted with one volume of TNE 8.2. The aqueous phases were combined and extracted twice more with chloroform and then carefully removed and precipitated overnight at -20°C by the addition of 1/10 vol of 1.0 M NaCl and 2.5 volumes of 95% ethanol. The precipitate was collected by centrifugation at 10,000 x g for 15 min. The supernatant was decanted and residual ethanol was removed under vacuum. The precipitate was dissolved in sterile TNE 8.2 and analyzed on a gradient of the same composition as that used for viral purification (Fig. 28-B). However, the gradient was centrifuged for 2 hr at 50,000 rpm in the Beckman SW 50.1 rotor. Under these conditions the RNA sedimented as a single peak with a sedimentation value of approximately 40-45 S.

b. Assay of viral RNA infectivity. Since healthy cells are not readily infectable by nucleic acids, it is necessary to use some means to aid in their uptake. Non-physiologic media such as hypertonic and hypotonic salt solutions and sucrose (Colter and Ellem, 1961) have been employed in the past; however, these often damage cells irreversibly and have largely been superseded by treatment with other substances prepared in isotonic media. The most widely accepted among these is the polycationic substance diethyl aminoethyl dextran (DEAE-D). The method we employed in this preliminary attempt to demonstrate the infectivity of our viral RNA preparation is basically that of Pagano (1970). Briefly, the cells are exposed to DEAE-D in an isotonic tris buffer at the time of adsorption with the purified RNA. Since we will eventually have to perform these experiments with enucleated cells, whose 24-hr life span is too short to permit plaque formation, we used an indicator layer of LLC-MK2 cells even in this preliminary experiment with normal CEC.

Primary CEC were grown on 23 mm plastic discs of the type used in enucleation experiments. RNA extracted from purified virions was diluted to contain 600 counts per minute (CPM) per 0.1 ml and virus from the same pooled gradient fractions that had not been extracted were used at one-hundredth that concentration, i.e., 6 cpm per 0.1 ml. Each disc was washed and inoculated with 0.1 ml of virus or RNA which contained one of the following concentrations of DEAE-D: 0, 100, or 1000 µg/ml. The inoculum was allowed to adsorb at room temperature for 15 min before being washed off. The discs then were seeded with  $5 \times 10^5$  LLC-MK2 cells in a volume of 0.5 ml of Medium 199 containing 20% FBS. The cells were allowed to attach and spread overnight at 37°C and were then overlaid with 3.5 ml of standard plaquing overlay, as described in the previous section. After incubation at 37°C for 3 days the discs were stained with a 1:1500 dilution of neutral red in normal saline. The results are shown in Table 22.

The concentration of DEAE-D used did influence the number of plaques produced, with increasing concentrations seeming to favor

plaque formation by RNA and inhibit that by virus. When comparing the optimum yields of each, infection with RNA appears to be about 100 fold less efficient than that of virus.

Table 22. Assay of infectivity of RNA extracted from purified JE virions

	Total CPM plated	DEAE-D( $\mu\text{g/ml}$ )	PFU*	PFU/CPM
RNA	600	0	0	0
	600	100	24	0.04
	600	1000	55	0.09
-----				
VIRUS	6	0	49	8.1
	6	100	60	10.0
	6	1000	2.5	0.41

\* Average of two samples

RNA used in the experiment was gradient analysed and shown to sediment with a coefficient of about 40-45 S. No radioactivity was found to sediment faster, as would be expected if residual virus remained. Due to the limited amount of RNA available this criterion alone was taken as evidence that the infectious material was indeed RNA. Other appropriate controls such as susceptibility to ribonuclease, inability to be neutralized by specific viral antiserum, and sensitivity to chloroform will be included in later experiments. Other parameters such as dose response to DEAE-D, influence of time and temperature of adsorption as well as the use of other substances to enhance RNA infectivity are under investigation.

c. Quantification of the fluorescent antibody technique to assess its feasibility as an assay for infectious RNA. A small percentage of nucleated cells is always present after mass enucleation of populations of cells with cytochalasin B. The necessity of a single cell assay for the interpretation of enucleation experiments is

therefore obvious. The feasibility of the indirect fluorescent antibody technique as an assay system for infectious RNA in enucleated cells has been evaluated in some preliminary studies of its sensitivity.

As a first step toward quantification of the technique, reagents were titrated. Primary CEC were infected with JE virus at an MOI of about 100. After incubation at 37°C for 1 hr for viral adsorption, the cells were washed, trypsinized and plated onto chamber slides. At 18 hrs after infection the cells were washed with phosphate buffered saline (PBS); fixed in methanol for 5 min and frozen at -70°C until the time the test was performed. Methods used for fluorescent staining have been previously described (Cardiff, 1973). Dilutions of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse gamma globulin and hyperimmune mouse ascitic fluid (HMAF) prepared against the M1/311 strain of JE used in the block titration are given in Table 23. Cells were observed for both intensity of fluorescence and percentage of positive cells.

Table 23. Titration of reagents used in indirect fluorescent antibody technique.

Anti-JE HMAF	FITC - goat anti-mouse gamma globulin					
	1:1	1:10	1:20	1:40	1:80	1:160
1:10	+4/60%*	+4/78%	+4/54%	+4/50%	+3/ND	+2/ND
1:20	+4/61%	+4/60%	+4/41%	+4/34%	+2/ND	+2/ND
1:40	+4/51%	+4/55%	+4/50%	+3/20%	Tr/ND	+1/ND
1:80	+4/30%	+4/67%	+4/50%	+2/25%	-/0	Tr/ND
1:160	+4/25%	+4/74%	+4/50%	+1/48%	-/0	Tr/ND
1:320	Tr**/22%	+4/24%	+2/15%	+1/16%	-/0	-/0
1:640	Tr/20%	+4/37%	+1/10%	Tr/ND	-/0	-/0
1:1280	Tr/3%	Tr/2%	Tr/2%	-/0	-/0	-/0

\* Intensity of fluorescence/percentage of cells fluorescing

\*\* Tr = Trace; ND = not determined

When undiluted FITC-goat antimouse gamma globulin was used, the fluorescence observed was spotty and not confined to any one area of the cell. Dilutions of the conjugate from 1:10 to 1:40 were all well suited for the differentiation of positive cells. Fluorescence was diffuse and involved the entire cytoplasm. The highest percentage of positive cells was obtained when both reagents were used at dilutions of 1:10. These optimal concentrations were used in all future experiments.

Next, the effect of MOI on percentage of cells showing fluorescence was determined. Primary CEC were infected and treated as in the block titration, using undiluted 20% mouse brain seed of JE and 10-fold dilutions thereof to give MOIs of 1000, 100, 10, 1, 0.1 and 0.01. The cells were fixed at 18 hrs after infection and stained for immunofluorescence. The intensity of the fluorescence was +4, but the percentage of cells involved increased with increasing MOI (Fig. 29). At an MOI of 10, 15% of the cells were positive at 18 hrs after infection, an easily detectable fraction, but in all future experiments undiluted JE mouse brain seed was used as inoculum.

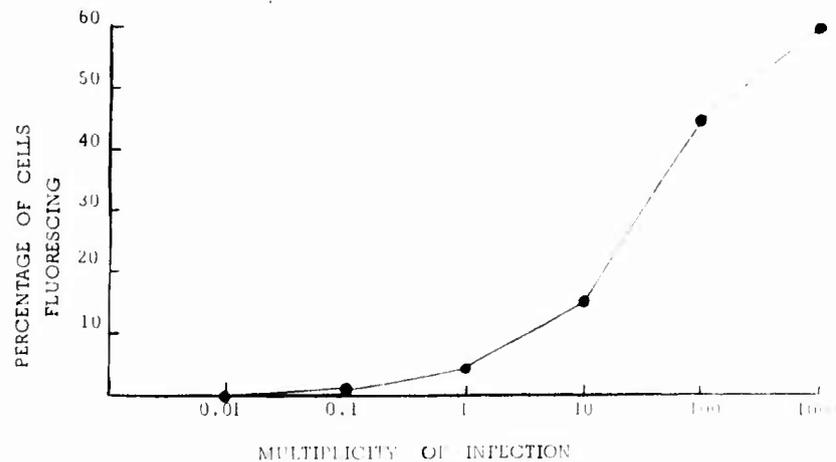


Figure 29. Effect of multiplicity of infection with Japanese encephalitis virus on the percentage of virus-specific fluorescence of chicken embryo cells.

Having optimized conditions to give maximum intensity of fluorescence as well as the highest percentage of positive cells, the time of

appearance of fluorescence was determined. One 32 oz bottle of primary CEC was infected with undiluted JE mouse brain seed. After allowing the virus to adsorb at 37°C for 1 hr, the cells were washed and trypsinized and  $1 \times 10^6$  cells were plated into each of 9 chamber slides and incubated at 37°C. One slide was fixed immediately and one each at 4, 8, 12, 16, 18, 20, 24, and 28 hrs after infection. Cells were stained for fluorescence and the percentage of positive cells was determined; the culture fluid from the chamber slides was also collected and assayed for infectivity by plaquing on LLC-MK2 cells (Fig. 30). Even at 28 hrs after infection, beyond the life span of the enucleated cells and after the peak titer of released virus had been achieved, the percentage of positive cells continued to rise in a linear manner. This indicates that susceptible cells in the culture were being infected by virus released from previously infected cells. Thus, for our experiments, the criterion governing harvest of infected enucleated cultures should be the viability of the enucleates.

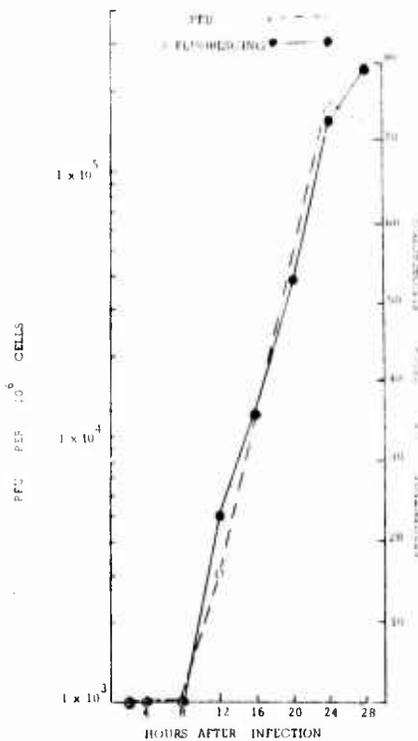


Figure 30. Comparison of Japanese encephalitis virus maturation and percentage of cells exhibiting virus-specific fluorescence at the indicated times post infection with a high multiplicity of infection.

In view of the preceding data, in order to make use of immunofluorescence as an assay for infectious RNA, optimal conditions to be employed are: 1) 1:10 dilutions of both FITC conjugated goat anti-mouse gamma globulin and anti-JE HMAF; 2) as high an MOI as possible - at least 1, but preferably greater; and 3) harvest of cells as late as possible after infection, but before the death of the enucleate culture.

In light of the fact that our preliminary assay for infectivity of viral RNA appears to be 100 times less efficient than that of virus, with a PFU/CPM ratio of about 0.1; and, assuming about a constant efficiency of radiolabeling, it would appear that to demonstrate infectivity by immunofluorescence on a disc containing approximately  $10^9$  CEC, at least sufficient RNA to give  $10^6$  CPM in an effective volume of 0.1 ml, would be required. Preparation of this amount of purified RNA is not feasible. Current investigations are directed at improving the efficiency of the infectious RNA assay by the use of substances such as DEAE-D, cytochalasin B (Koch and Oppermann, 1975), and dimethyl sulfoxide (Tovell and Colter, 1967). An increase in the PFU/CPM ratio of our preparations would make immunofluorescence a feasible single cell assay for infectivity of viral RNA.

#### E. Structural proteins of California encephalitis (BFS-283) virus

Introduction. Knowledge of the molecular structure of members of the Bunyamwera supergroup is limited. Published reports are few and somewhat conflicting as to biochemical and biophysical properties (Bouloy et al., 1974; Pettersson et al., 1974; Rosato et al., 1974). Electron microscopic observations show that the virion has a diameter of approximately 98-100 nm, and contains an electron-dense core with spiked outer projections (Murphy et al., 1968). Recently Rosato et al. (1974a & b) reported that several California encephalitis (CE) virus complex members, including BFS-283, contained three structural proteins. McLerran and Arlinghaus (1973) reported that LaCrosse virus contained three proteins and had a total virion molecular weight of  $2.8 \times 10^8$  daltons. However, uncertainty remains as to the number of polypeptides present in BFS-283 virion, because of the difficulty in getting adequate levels of isotope incorporation. In the present study, the polypeptide compositions of purified BFS-283 virions produced in BHK<sub>21</sub>-Clone 13 and LLC-MK<sub>2</sub> cell cultures, were analyzed by polyacrylamide gel electrophoresis (PAGE). Evidence is presented suggesting a fourth structural protein that may be part of the virus envelope.

Virus strains used, methods of cell culture, virus assay, preparation of sucrose gradients, and polyacrylamide gel electrophoresis are essentially similar to those described in last year's report.

To prepare purified virus for analysis of proteins, BHK<sub>21</sub> and

LLC-MK<sub>2</sub> cells were inoculated at a multiplicity of 50-100 pfu/cell with seed virus which was allowed to adsorb for 60 min at 36°C. Unadsorbed virus was then removed and cells were washed twice with Hank's balanced salt solution (HBSS) and fed with medium consisting of one part normal Medium 199 (or basal medium) and three parts of the same medium from which the metabolites to be used (amino acids or glucosamine) as radioactive precursors were omitted. Four to six hrs after infection, cells were labeled with 60 µCi/ml of either <sup>3</sup>H(6)-L-amino acid mixture containing 15 amino acids (specific activity 23.6 Ci/mmol), or 10-20 µCi/ml of <sup>3</sup>H(6)-D-9 glucosamine hydrochloride (sp. act. 7.3 Ci/mmol), 8 µCi/ml of <sup>14</sup>C-L-amino acid mixture (sp. act. 3.5 Ci/mmol) or 5-10 µCi/ml (5-C<sup>14</sup>)-uridine (sp. act. 20 Ci/mmol). Labeled amino acid mixtures and uridine were obtained from New England Nuclear Corporation, and glucosamine from Amersham/Searle Company. The pH of media to which both amino acids and/or uridine labeled compounds were added, were adjusted to approximately 7.3 with bicarbonate when necessary. Culture medium containing released virus was harvested 18-24 hrs after infection in BHK<sub>21</sub> cells and 40-48 hrs in LLC-MK<sub>2</sub> cells. The culture fluid was centrifuged at 3,000 × g for 60 min. The virus was then pelleted at 205,000 g for 2 hrs at 4°C. The resulting pellet was resuspended in 1-2 ml of TSE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA), pH 8.2. The virus suspension was sonicated by two 1-min cycles in a 10 kc Raytheon Sonic Oscillator and purified through a sucrose gradient. Medium from uninfected cells was prepared in the same manner except that no virus was added at the time of mock-infection.

Results. During these experiments, it was found that maximal incorporation of radioactively labeled precursors in BHK<sub>21</sub> grown virions was obtained at 21-24 hrs after infection and into LLC-MK<sub>2</sub> grown virions at 40-48 hrs post infection. The hemagglutinin and radioactivity coincided in the sucrose gradient fractions. Virus grown in BHK<sub>21</sub> or LLC-MK<sub>2</sub> cells banded in the same broad peak (Fig. 31). The rather broad peak of radioactivity and hemagglutinin may be due to the presence of defective particles.

Proteins of BFS-283 virions. The peak fraction from Fig. 31 was used for protein analysis. Four polypeptides were resolved which migrated identically regardless of whether or not virions were grown in BHK<sub>21</sub> or LLC-MK<sub>2</sub> cells (Fig. 32). Since the newly resolved protein is a minor polypeptide, the possibility of it being a host cell protein existed. To rule this out, purified labeled virions grown in BHK<sub>21</sub> and LLC-MK<sub>2</sub> cells were coelectrophoresed with fluids of mock-infected BHK<sub>21</sub> and LLC-MK<sub>2</sub> cells, and four polypeptides were detected in purified virion preparations (Fig. 33-A & B). By contrast, in the uninfected cell extracts, only a low, generally flat background was present (Fig. 33-A & B).

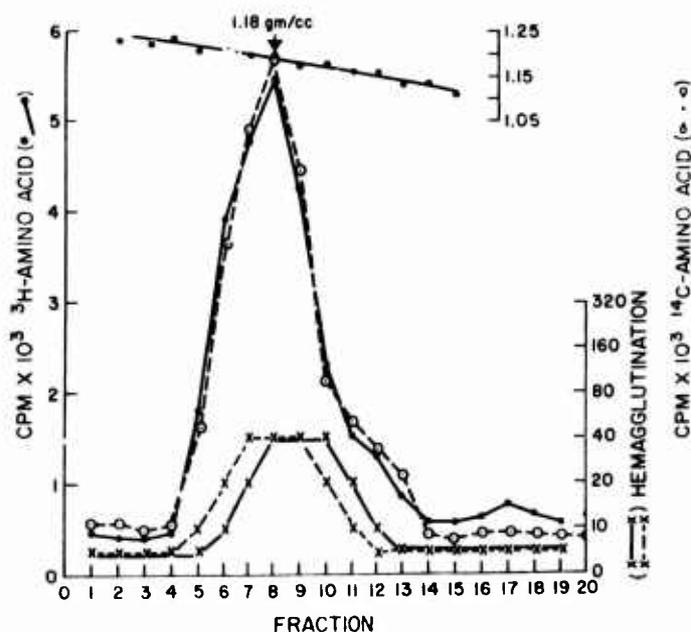


Fig. 31. Isopycnic centrifugation of radioactive BFS-283 CE virus; rate zonal peak fractions of  $^3\text{H}$ -amino acid labeled virus from BHK-21 cells and  $^{14}\text{C}$ -amino acid labeled virus from LLC-MK<sub>2</sub> cells were layered over a 15-50% linear sucrose gradient, with a cushion of 0.1 ml 70% sucrose in D<sub>2</sub>O containing TSE, pH 8.2. The gradient was centrifuged in a SW-50L rotor at 205,000 x g for 18 hrs; fractions were collected and assayed for radioactivity. Hemagglutination (HA) was plotted from separate parallel gradients where the peak of HA from BHK-21 cells consistently sedimented slower than the peak of radioactivity.

To determine which of the viral proteins were glycoproteins, virus was doubly labeled with amino acids and sugars. Electrophoretic migration patterns of BHK-21 grown virus labeled with  $^3\text{H}$ -glucosamine and  $^{14}\text{C}$ -amino acid mixture, and of LLC-MK<sub>2</sub> grown virus labeled with  $^3\text{H}$ -glucosamine and  $^{14}\text{C}$ -amino acid mixture (Fig. 34) show that three of the four viral proteins contained radioactivity due to glucosamine. Therefore, VP-2, VP-3, and VP-4 are probably glycoproteins.

To estimate the relative molecular weights of BFS-183 virion proteins, polypeptides from Sindbis virus with known molecular weights, 53,000 and 30,000 daltons, as well as the polypeptides from BFS-283 virions were mixed and coelectrophoresed in 8% polyacrylamide gels, containing 1% SDS (Shapiro et al., 1967). Fig. 35 shows a typical

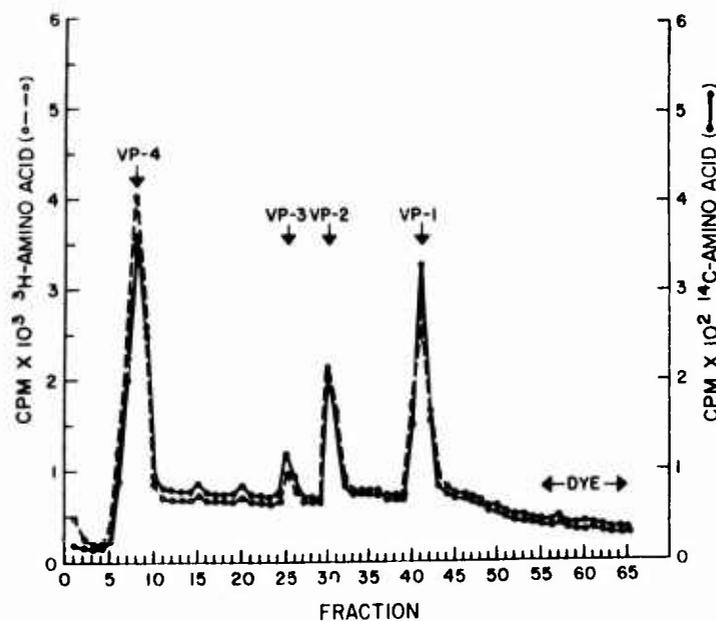


Figure 32. Coelectrophoresis of polypeptides of BFS-283 CE virus grown in BHK-21 cells, labeled with  $^3\text{H}$ -amino acid mixture and BFS-283 CE virus grown in LLC-MK<sub>2</sub> cells, labeled with  $^{14}\text{C}$ -amino acid mixture. Arrows indicate the positions of four virion polypeptides.

migration pattern (Sindbis virus proteins indicated by arrows); molecular weights of 17,500, 30,000, 38,000, and 82,000 were calculated for VP-1, VP-2, VP-3, and VP-4, respectively.

Isolation of nucleocapsid protein. Virions which were doubly labeled with  $^{14}\text{C}$ -amino acids and  $^3\text{H}$ -uridine were centrifuged through a sucrose gradient and were shown to have a density of 1.18 gm per cc (Fig. 36-A). When the peak fraction of this material was treated with the nonionic detergent NP-40 (1.0% for 15 min at 40°C), and rebanded on a 15-50% sucrose-D<sub>2</sub>O gradient, two radioactive peaks were obtained. One was a dense, uridine-rich material, the other a light amino acid-rich material (Fig. 6-B). Coelectrophoresis of the dense uridine-rich  $^{14}\text{C}$ -amino acid "core" material with  $^3\text{H}$ -amino acid labeled virions revealed an electrophoretic pattern which contained principally one polypeptide, VP-1 (Fig. 37).

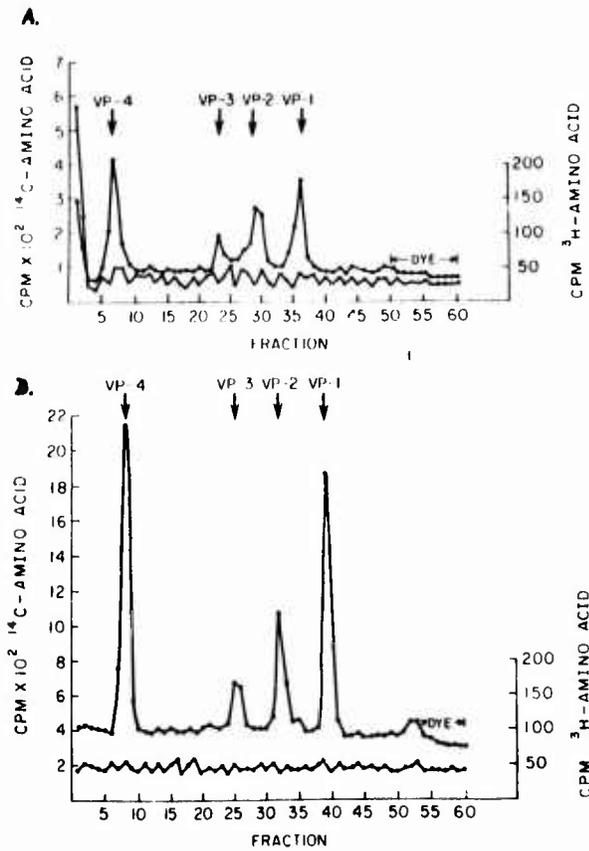


Figure 33. Electrophoretic patterns of BFS-283 CE virus proteins synthesized in BHK-21 and LLC-MK<sub>2</sub> cells. BHK-21 or LLC-MK<sub>2</sub> cells were infected with BFS-283 CE virus, labeled with <sup>14</sup>C-amino acid mixture (8  $\mu$ ci/ml), harvested and purified. BHK<sub>21</sub> and LLC-MK<sub>2</sub> cells receiving no virus served as controls and were exposed to <sup>3</sup>H-amino acid mixture (10  $\mu$ ci/ml) throughout the virus growth period. (A) <sup>14</sup>C-labeled proteins of virions grown in LLC-MK<sub>2</sub> cells plus <sup>3</sup>H-labeled proteins from uninfected LLC-MK<sub>2</sub> cells. (B) <sup>14</sup>C-labeled proteins of virions grown in BHK-21 cells plus <sup>3</sup>H-labeled proteins from uninfected BHK-21 cells. o = <sup>3</sup>H CPM; ● = <sup>14</sup>C CPM.

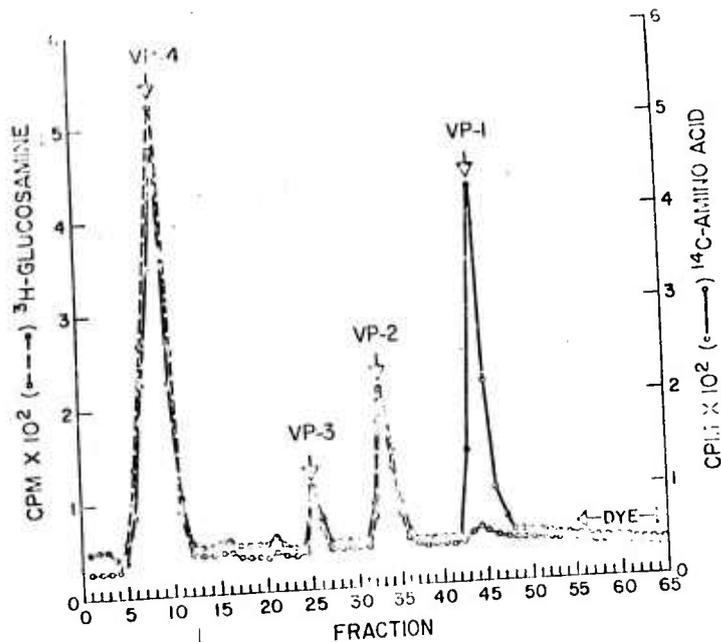


Figure 34. Electrophoresis of polypeptides of virus grown in LLC-MK<sub>2</sub> cells, doubly labeled with <sup>3</sup>H-glucosamine and <sup>14</sup>C-amino acid mixture.

**Discussion.** The findings reported here indicate that BFS-283 CE virus consists of four polypeptides when analyzed by polyacrylamide gel electrophoresis. Three large polypeptides probably represent envelope glycoproteins, only two of which were previously reported (McLerran and Arlinghaus, 1973; Rosato et al., 1974a & b). The largest viral protein (VP-4) was a glycoprotein of 82,000 daltons molecular weight, and appeared similar to VP-3 described by McLerran and Arlinghaus (1973) for La Crosse virus (McLerran and Arlinghaus, 1973), and to VP-3 of BFS-283 and Tahyna California encephalitis group viruses (Rosato et al., 1974 a & b). Viral protein (VP-2) with a molecular weight of 30,000 daltons appeared similar to the VP-2 glycoprotein described by Rosato et al (1974b). Viral protein one (VP-1), with a molecular weight of 17,500 daltons, was shown to be "core" or nucleoprotein and is probably similar to that described by Rosato et al., (1974b) for Oriboca virus. The possibility that the VP-3 was a host-cell protein seems unlikely since coelectrophoresis of purified virions and uninfected cell extracts showed that

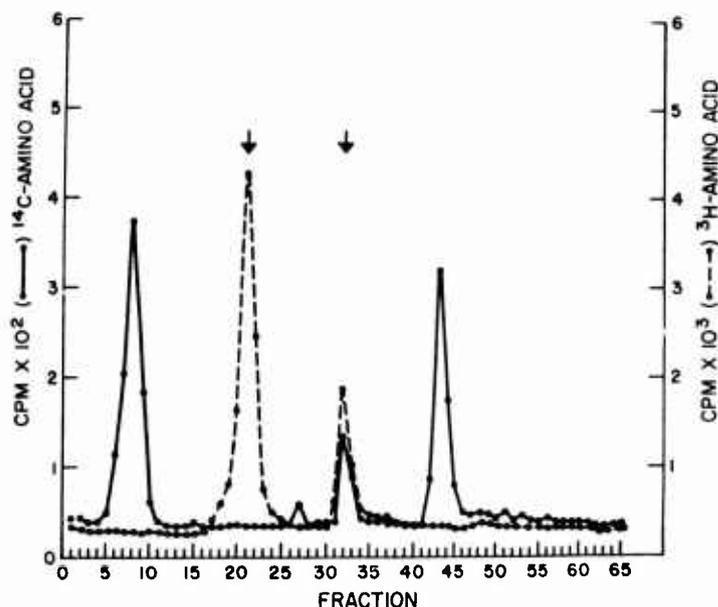


Figure 35. Coelectrophoresis of polypeptides of BFS-283 CE virus, labeled with  $^{14}\text{C}$ -amino acid mixture and Sindbis virus marker proteins labeled with  $^3\text{H}$ -amino acid mixture. Sindbis virus polypeptides (indicated by arrows) with molecular weights of 30,000 and 53,000.

uninfected cells do not contain a protein similar to VP-3.

Even though a precise experiment has not been conducted to determine the exact conditions under which VP-3 is detected, two observations were made that should be mentioned: 1) if radioactive amino acid mixtures were added to infected cells after twelve hours post-infection, and 2) if released virus was harvested after at least 50% cytopathic changes occurred in the infected cells, VP-3 was seldom if ever detected. Further, Lyons (1974) recently reported the detection of a fourth polypeptide in La Crosse virions (a closely related CE virus) which appeared to be similar in PAGE to the VP-3 reported in this paper. Lyons' experimental conditions were similar to the ones used in these experiments with regard to MOI, labeling time, and time of harvesting virus. Thus, this suggests that VP-3 may be a minor envelope protein that is synthesized during the early stages of the infectious cycle, that may be cleaved by host cell proteolytic enzymes that are released by the cell during cytopathic

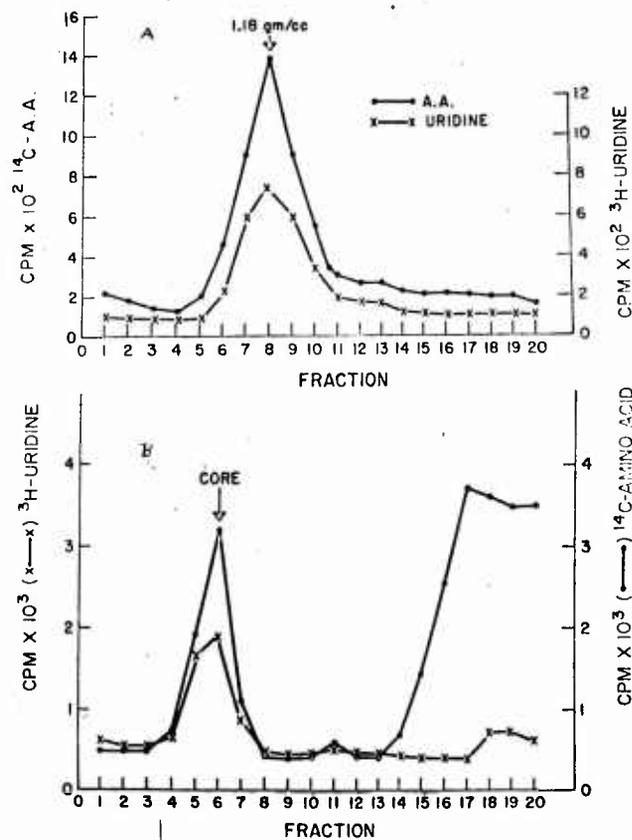


Figure 36. Isopycnic centrifugation of  $^{14}\text{C}$ -amino acid and  $^3\text{H}$ -uridine labeled (A) California encephalitis (BFS-283) virions; (B) Virions from fraction 9 in part A treated with 1% NP-40 for 15 min at  $4^\circ\text{C}$ . Samples were layered onto a 15-50% sucrose- $\text{D}_2\text{O}$  gradient in TNE, pH 8.2. Centrifugation was at  $205,000 \times g$  for 18 hrs at  $4^\circ\text{C}$ .

changes in the latter stages of infection. This reaction may be similar to the proteolytic cleavage of envelope glycoproteins in influenza virus where the HA polypeptide is cleaved to HA1 and HA2 by host-cell proteolytic enzymes late in the infectious cycle (Lazarowitz et al., 1973). However, unlike the influenza HA polypeptide, since VP-3 is a minor polypeptide, VP-3 is probably cleaved from the virion, or reduced to undetectable levels using the SDS, polyacrylamide system.

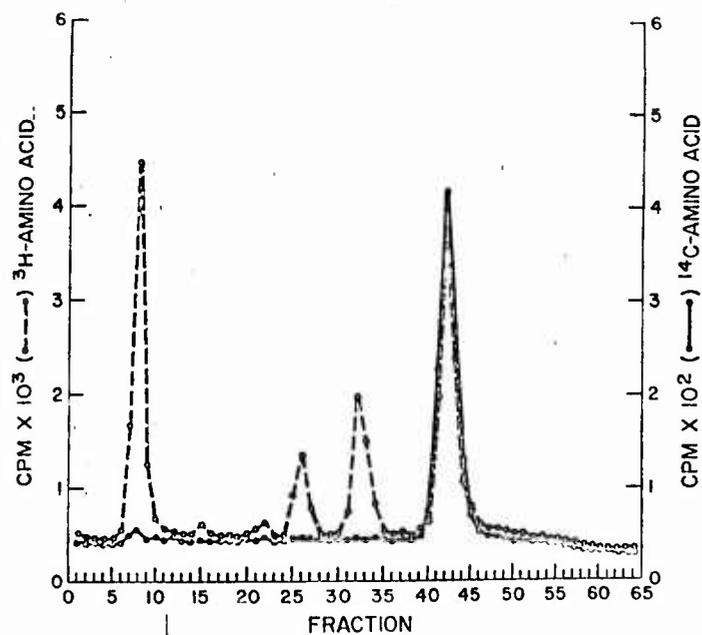


Figure 37. Coelectrophoresis of  $^{14}\text{C}$  "core" protein and  $^3\text{H}$ -BFS-283 CE virus structural proteins. NP-40-derived cores were isolated from  $^{14}\text{C}$ -amino acid labeled CE virions, as described in Fig. 36. The peak fraction was diluted 1/10 with TNE, pH 8.2, dissociated with SDS and 2-ME, mixed with  $^3\text{H}$ -amino acid labeled BFS-283 CE structural proteins and then coelectrophoresed.

A budding virus of this size and density containing one nucleoprotein, three glycoproteins, and three segments of RNA (Bouloy et al., 1974; Schlesinger et al., 1972), indicates that this is a rather complex virion. In its structural complexity it seems to fall somewhere between the simpler alphavirus which contains two glycoproteins, one nucleoprotein, and a single strand of RNA, and the more complex influenza A virus of the myxovirus group which contains four glycoproteins, three nucleoproteins, and five segments of RNA (Schlesinger et al., 1972; Compans et al., 1970).

There is at present no unanimity of opinion regarding the exact number and types of structural proteins these viruses contain. There have been no reports on the number and characteristics of Bunyavirus nonstructural proteins.

## F. Arbovirus field studies

### 1. California encephalitis virus ecology in the Pocomoke Cypress Swamp, Maryland

The continuing program of arbovirus ecology on the eastern shore of Maryland concentrated on the role of transovarial transmission in the maintenance of California encephalitis virus. This investigation was again the combined effort of the Departments of Entomology and Virus Diseases, WRAIR. The specific goals of these investigations were to confirm the observation of transovarial transmission of Keystone strain California encephalitis virus by Aedes atlanticus and to investigate the role of certain vertebrate residents of the swamp as primary hosts involved in maintenance, transmission and amplification of California encephalitis virus.

a. Mosquito collection and virus isolation. All mosquito collections (larvae and adults) were identified, pooled, and passed a single time in suckling mice by personnel in the Department of Entomology. All suspect positives were delivered to this Department where additional mouse passages were made to either confirm or rule out virus isolation. Seven of the 9 larvae isolates and a representative 10 of the 30 adult mosquito isolates were tested by plaque reduction neutralization tests (PRNT), using prototype Keystone and Jamestown Canyon strain mouse hyperimmune ascitic fluids (MHAF). All virus isolates were neutralized to high titer by Keystone MHAF, indicating Keystone virus isolates. Details of the distribution of isolates and infection rates of A. atlanticus are presented in the Department of Entomology section of this report.

b. Neutralizing antibody in deer sera. Deer sera were again examined for neutralizing antibody to both Keystone and Jamestown Canyon viruses. Blood specimens were collected from freshly killed deer at the Snow Hill, MD, deer-check station during the week of 30 Nov - 6 Dec 74. Ages of these deer were estimated by examination of lower jaw teeth by Game and Wildlife personnel. A representative 61 deer sera of the 131 collected were examined against local isolates of Keystone virus (strain 71-1736) and Jamestown Canyon virus (strain pool 4) by PRNT. These data are summarized in Table 24. It was interesting to note that all of the 17 deer lacking N antibody to either virus were less than 9 months old. The possibility that maternal antibody contributed to their resistance to infection during their first year is currently being investigated.

c. Neutralizing antibody in personnel associated with field investigations. A preliminary screen of sera from 25 field investigators versus California encephalitis virus by PRNT yielded tentative positives in 6 persons. These six sera were further examined by PRNT, using prototype Keystone, Jamestown Canyon and LaCrosse viruses. These results are presented in Table 25. Both Davis and

Table 24. Neutralizing antibody in selected deer sera from Snow Hill, Maryland

Number tested	Plaque reduction				Negative
	Keystone virus		Jamestown Canyon virus		
	(> 70%)	(50-70%)	(> 70%)	(50-70%)	
68**	19	10	15	7	17
	43%		32%		25%

\* Percent reduction of a plaque dose of approximately 50-100 pfu

\*\* Of the total tested, 18 sera were positive to both viruses but were titrated and assigned to the virus yielding the highest antibody titer

Table 25. PRNT antibody titers of swamp personnel to California encephalitis viruses

	Virus		
	Keystone	Jamestown Canyon	LaCrosse
Berger	< 10*	< 30	< 10
Bergman	< 10	< 30	< 10
Dalrymple	20	< 10	< 10
Davis	20	400	< 10
Le Duc	60	< 10	< 10
Martin	450	230	< 10

Martin had neutralizing antibody to Jamestown Canyon and Keystone virus, respectively. Sera from these investigators obtained before commencement of their field work currently being sought in an attempt to relate infection to exposure in the Pocomoke Cypress Swamp.

d. Development of viremia and N antibody to CEV strains in experimental animals. Laboratory rabbits (female New Zealand whites) have been used as field sentinels for California encephalitis viruses; however, precise susceptibility and antibody studies have not been reported. Five laboratory rabbits were injected with 0.5 ml containing 5,000 pfu of a local Keystone virus isolate (71-1736) and bled daily for one week to detect viremia. These data are presented in Table 26. Only two rabbits (#353 and #429) produced detectable viremia and both were low titered and of short duration.

Table 26. Viremia of Keystone virus in laboratory rabbits

Rabbit No.	Pre-bleed	D a y						
		1	2	3	4	5	6	7
349	0*	0	0	- - - - -	- - - - -	Died	- - - - -	- - - - -
351	0	0	0	0	0	0	0	0
353	0	0	140	170	0	0	0	0
355	0	0	0	0	0	- - -	Died	- - - -
429	0	0	29	3	0	0	0	0

\* Pfu detected in 0.2 cc of a 1:10 dilution of serum obtained on indicated day post infection

A similar group of five rabbits were injected with 500 pfu of a local Keystone isolate and their sera screened at intervals for detectable neutralizing antibody (PRNT). The appearance of Keystone neutralizing antibody is shown in Table 27. It was interesting that even though the infective inoculum was less than in the previous viremia study, all animals had detectable antibody by day 7. The early appearance of antibody to a relatively low virus dose suggests that the laboratory rabbit is a sensitive indicator of Keystone virus transmission.

Table 27. Onset of neutralizing antibody in laboratory rabbits injected with Keystone virus

Rabbit No.	Pre-bleed	D a y			
		3	7	10	15
18	-*	-	-	+	+
19	-	-	+	+	+
21	-	-	+	+	+
22	-	-	+	+	+
727	-	-	+	+	+

\* Negative (-) or positive (+) determined by the ability of a 1:10 dilution of serum to neutralize > 50% of a virus plaque dose of 50-100 pfu.

Hamsters were similarly investigated for both viremia and antibody production. Five groups of 5 hamsters were injected with a local isolate of Jamestown Canyon virus (Pool 4) at two different virus doses (900 and 90 pfu) and daily bloods examined by plaque assay for infectious virus. Viremia patterns for these hamsters are shown in Table 28. Although considerable animal variation was noted, 90 pfu appears to be near the threshold dose infecting only 2 of 5 animals with viremia on days 3 and 5, respectively. The 900 pfu dose infected 4 of 5 animals with viremia, appearing within 48-72 hrs.

Hamsters were similarly infected with a local isolate of Keystone virus and the hamsters infected with Jamestown Canyon virus were challenged with Keystone virus some 2 months following their primary infection. All animals were injected with 1000 pfu of isolate #71-1736. These data are summarized in Table 29. It would appear from these data that previous exposure to Jamestown Canyon virus does reduce the probability of the detection of viremia; however, the development of antibody after inoculation of Keystone virus indicates that infection did occur.

Various other vertebrate species were examined for both viremia and antibody production following injection of Keystone virus. These include turtles, raccoons, opossums, and squirrels, with essentially

Table 28. Jamestown Canyon viremia in hamsters

Animal	D a y				
	1	2	3	4	5
<u>900 pfu group:</u>					
900-1	0	25*	150	0	0
900-2	0	200	25	0	0
900-3	0	50	0	0	0
900-4	0	0	0	0	0
900-5	0	0	120	25	0
<u>90 pfu group:</u>					
90-1	0	0	0	0	0
90-2	0	0	0	0	0
90-3	0	0	50	0	0
90-4	0	0	0	0	375
90-5	0	0	0	0	0

\* pfu detected in 0.2 cc of a 1:20 dilution of serum drawn on the indicated day post infection.

negative results. Experimental keystone infection of grey squirrels is under current study by the Department of Entomology.

Table 29. Swamp 6: Keystone viremia in normal hamsters and animals previously infected with Jamestown Canyon virus

	D a y					Antibody (% reduction)	
	1	2	3	4	5		
<u>Animal:</u>							
Key 1	0	44 <sup>(1)</sup>	150	0	0	0 <sup>(2)</sup>	86
Key 2	0	0	1	103	0	0	98
Key 3	0	0	0	83	0	0	99
Key 4	0	7	5	0	0	0	90
Key 5	0	0	0	0	0	0	1
Key 6	0	53	10	0	0	0	92
<u>Jamestown Canyon:</u>							
900-1	0	0	0	N.T.	N.T.	77	N.T.
900-3	0	0	0	0	0	97	99
900-4	0	0	0	0	0	0	94
900-5	0	0	0	0	0	0	99
90-2	0	0	0	N.T.	N.T.	0	0
90-5	0	27	450	0	0	0	50

<sup>1</sup> pfu in 0.2 ml of 1:10 dilution

<sup>2</sup> percent reduction of 1:10 serum dilution in PRNT

N.T. = not tested

## 2. The etiology of febrile illnesses in Brazilian trans-Amazon colonists

In September 1974, USAMRU-Belem (Trans-Amazon) began a study of the etiology of fevers in colonists newly settled along the trans-Amazon highway from Maraba to Altamira in Para, Brazil. Details of this study are included elsewhere in this WRAIR annual report. In essence, a 20% sample of households along the highway is being followed by twice monthly visits for illness. Blood samples for serology from available family members of study households are being obtained at the beginning of the study and at 6-month intervals thereafter. Acute blood is obtained on colonists with fever or history of fever since the last visit; an aliquot of blood for viral isolation is frozen immediately in liquid nitrogen. Convalescent blood is obtained on the succeeding bimonthly visit. The study is planned to continue for 12 to 18 months.

Over 25 different arboviruses have been shown to infect humans in Para, Brazil, originally by the Rockefeller Foundation Laboratory and subsequently by its successor, the Instituto Evandro Chagas in Belem. Although extensive ecologic studies of several Brazilian viruses have been undertaken, relatively little is known of their potential military medical importance. Specifically, the total impact of the rich fauna of arboviruses in the etiology of human fever has not been addressed. Also a relatively large number of those arboviruses infecting humans in Para, Brazil, are members of the family Bunyaviridae, the epidemiology of which is poorly understood. The settling of lots along the Trans-Amazon highway by colonists emigrating principally from cities in northeast Brazil affords an opportunity to study the arbovirus etiology of "jungle" fevers in a population lacking previous infection with these viruses.

Although it was intended originally that virologic aspects of this study be handled by virologists at Institute Evandro Chagas, this department assumed responsibility when it was evident that the IEC could not offer full support immediately. Our current approach is a serologic study of the etiology of fever in colonists along the canal. In collaboration with Dr. Robert Shope, Yale Arbovirus Research Unit, acute and convalescent fever sera from febrile colonists will be tested for CF antibody against the following 18 arboviruses indigenous to Para, Brazil:

<u>Group A</u>	<u>Group B</u>	<u>Group C</u>
Mayaro	Yellow Fever	Marituba
Mucambo	Bussuquara	Caraparu
Eastern Equine Encephalitis	Ilheus	Murutucu
Western Equine Encephalitis	St. Louis Encephalitis	

Bunyamwera Group

Guaroa  
Maguari

Simbu Group

Oropouche

Phlebotomus Fever Group

Candiru  
Itaporanga

Guama Group

Guama

Vesicular Stomatitis Group

Piry

At this time, antigens and antisera of all 18 viruses have been received from YARU or made at the WRAIR and have been tested for antigenic potency against homologous hyperimmune antiserum. Paired sera from the 60 febrile colonists received in the first shipment from USAMRU-Belem is being tested. Although the CF test is less sensitive and recognized as less suitable for antibody prevalence studies than the hemagglutination-inhibition test, we have elected to use the former assay for etiologic diagnosis of illness because of its greater technical simplicity in assaying antibodies to the many antigens. Hemagglutination-inhibition or plaque reduction neutralization tests may be established, if appropriate, for viruses shown to cause illness in colonists.

In order to confirm the specificity of serologic tests and to include appropriate viral antigens other than the 18 selected in serologic tests, acute bloods from patients admitted to Maraba or Altamira Hospitals with febrile illnesses and from trans-Amazon colonists with fever of less than 6-days duration, are inoculated into suckling mice and Vero cell cultures at WRAIR for virus isolation. From the first shipment of specimens from USAMRU-Belem, 256 specimens for viral isolation have been tested. (Many acute bloods in this first shipment tested were drawn more than 6 days after the onset of febrile illness, a time during infection when viremia is highly unlikely!) From the information available at WRAIR, 51 represent acute bloods from febrile trans-Amazon colonists, 38 are from fever patients at Maraba Hospital, 119 are from febrile residents of a town south of Maraba, Palestina (most of whom were shown to have malaria!), and 48 from an unspecified source.

A virus, Br-H-001032, has been isolated in suckling mice from one of the Maraba hospital patients. Preliminary tests suggest that it is similar, if not identical, to Guaroa virus. A second agent, Br-H-001378, has given inconsistent morbidity and mortality in suckling and weanling mice and requires further passage before characterization is possible.

## G. Immunology of viral infections

Studies on the effects of cell mediated immunity to attenuated and virulent arboviruses was first approached by defining and working with the various populations of cells. This work is in the final stages.

### 1. Effects of carrageenan on macrophages

Carrageenan, a high molecular weight, sulfated polygalactose, resembling agar, possesses the ability to suppress the cutaneous expression of established delayed hypersensitivity (DH) (Schwartz and Leskowitz, 1968). The fact that carrageenan was selectively cytotoxic to macrophages *in vitro* (Allison et al., 1966; Catanzaro et al., 1971), but did not affect the viability of lymphocytes (Catanzaro et al., 1971) or their ability to undergo mitogen-induced blast transformation (Bice et al., 1971), suggested a possible mechanism for carrageenan's immunosuppressive effects. Therefore, it seemed important to establish whether carrageenan was indeed a macrophage cytotoxin *in vivo*. This required a demonstration that (a) carrageenan, given *intraperitoneally* (i.p.), was widely distributed throughout the reticuloendothelial system (RES) and (b) as a result of its presence, macrophage cytotoxicity occurred. Thus, the plan of the present experiment was to administer carrageenan i.p. to normal guinea pigs and to detect from 6 hr to 6 days its presence in the RES by histochemical means, and its cytotoxic effects by histopathologic criteria. Carrageenan, by virtue of its chemical composition as a sulfated polygalactose (Anderson, 1969), could be detected histochemically in tissue cells (Gangolli et al., 1973). In this report, attempts were made to detect carrageenan by: (a) the periodic acid-Schiff (PAS) stain of Mowry (1957) with prior diastase digestion of glycogen; (b) metachromatic staining with toluidine blue (Gangolli et al., 1973), and the alcian blue technique at pH 1 (Lev and Spicer, 1964).

Carrageenan (Seakem 21, kindly supplied by Marine Colloids, Springfield, NJ) was dissolved at a concentration of 10 mg/ml in warm, sterile, physiological saline. Most animals receiving carrageenan had not been immunized; i.e., they were "normal" animals. Experimental animals received either 10 or 30 ml of carrageenan i.p. (i.e., 100 or 300 mg, respectively). Control animals received either 10 or 30 ml of warm sterile physiologic saline i.p. The potency of the carrageenan preparation was verified by its ability to suppress skin reactions in tuberculin-sensitive guinea pigs (these skin test sites were biopsied). The tissues obtained above were fixed in buffered neutral formalin.

Following the i.p. administration of carrageenan, the peritoneal cavity contained a viscid gray fluid resembling carrageenan, which increased in amount with time. This fluid stained metachromatically

when toluidine blue was added, confirming the persistence of carrageenan (Di Rosa, 1972). Positive (or negative) results obtained with one histochemical procedure were usually matched by similar results with the others. These will be referred to collectively in the text as 'histochemically positive' (or 'negative'). Controls were uniformly negative.

Macrophages, but not lymphocytes, obtained from the peritoneal cavity of animals given carrageenan (100 or 300 mg i.p.) contained histochemically positive granules.

Histochemically positive material, similar to that seen in peritoneal macrophages, could be demonstrated within the Kupffer cells of the liver (Fig. 38) at, but not earlier than, 24 hrs following the i.p. injection of either 100 or 300 mg of carrageenan. The higher dose of carrageenan resulted in a greater amount of histochemically positive material demonstrable within the Kupffer cells, but had no effect on its lobular or intracellular distribution. This material increased in amount with time.



Figure 38. Alcian blue positive material in sinusoids and Kupffer cells of liver following administration of 300 mg of carrageenan 24 hrs previously. (Alcian blue without counterstain; 120X).

Unlike the liver, histochemically positive material could not be demonstrated within lymph nodes, spleen or bone marrow in the 24 hrs following the i.p. injection of 100 mg of carrageenan. However, when 300 mg of carrageenan was administered i.p., histochemically positive material, similar to that seen in peritoneal macrophages, could first be observed at 24 hrs in macrophages of the splenic red pulp, those lining the medullary sinusoids of lymph nodes (Fig. 39), and in bone marrow macrophages (Fig. 40). Although this material increased in amount with time, it was present in only occasional cells. RES cytotoxicity was restricted to peritoneal macrophages and Kupffer cells in the liver. No histopathologic alterations were observed in the regional lymph nodes or bone marrow at either dose of carrageenan. Thus carrageenan did indeed prove to be a selective macrophage cytotoxin *in vivo*. However, the expectation that carrageenan would be uniformly cytotoxic to macrophages but not lymphocytes was not fulfilled. While lymphocytes were spared from cytotoxic effects, only certain macrophages - i.e. those from the peritoneal cavity and the liver - exhibited toxic effects. Since macrophages from the peritoneal cavity or liver do not seem crucial to either afferent or efferent events which occur in the expression of DH, it is doubtful that cytotoxicity in these sites results in immunosuppression.

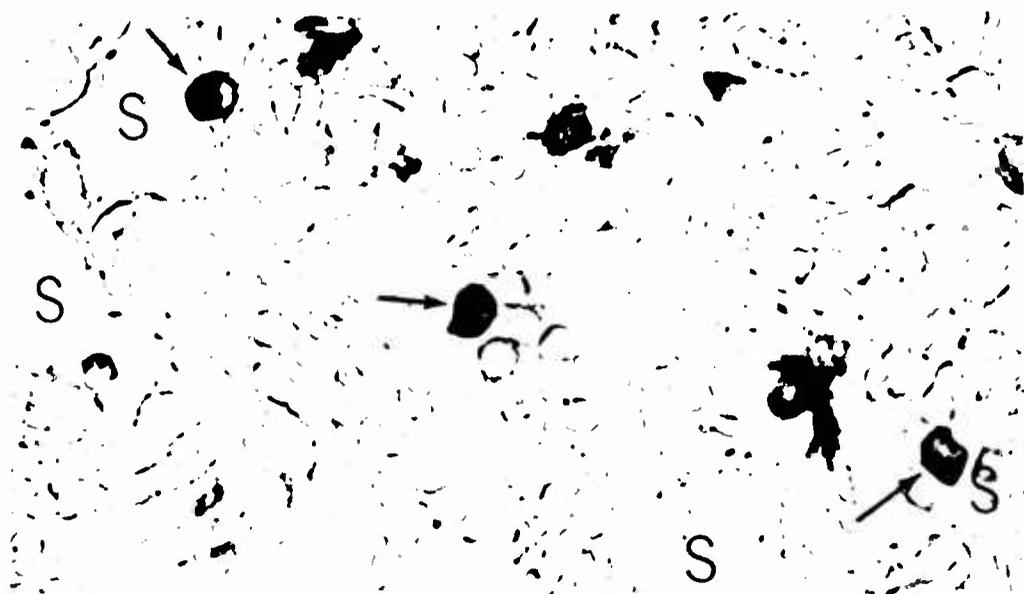


Figure 39. Lymph node from an animal 4 days after the administration of 300 mg of carrageenan i.p. The arrows indicate PAS positive material in medullary macrophages lining sinusoids S. (PAS following diastase digestion without counterstain; 500X).

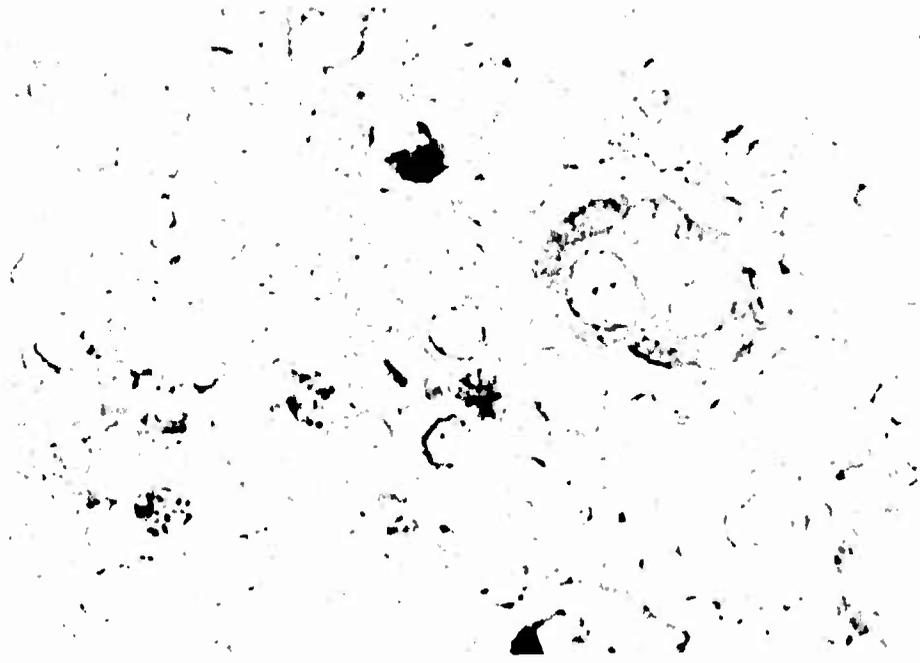


Figure 40. Bone marrow from animal given 300 mg of carrageenan i.p. 6 days previously. Alcian blue positive material is in the macrophages. M denotes megakaryocytes. (Alcian blue without counterstain; 640X).

Afferent events, following intradermal challenge of immune animals, take place in the skin test site (Dvorak et al., 1970) and the regional lymph nodes (Bloom and Bennett, 1970), as well as in the spleen and remote lymph nodes (Dvorak et al., 1970). In the expression of established DH, the majority of the cells appearing at the skin test site were newly formed cells from the marrow (Lubaroff and Waksman, 1968a, b). Thus, in terms of both afferent and efferent events, to support a mechanism of immunosuppression by in vivo macrophage cytotoxicity, there would have to be demonstrated involvement of macrophages in lymph nodes, spleen, bone marrow and/or skin test sites. Clearly, the results presented above demonstrated that 'critical' RES organs were spared from the cytotoxic effects of carrageenan and did not support the hypothesis that carrageenan exerts its immunosuppressive effects by macrophage cytotoxicity in vivo.

Because of these findings, recent experiments have focused on carrageenan's effect on the clotting system, since there exists a large body of evidence linking the clotting system to DH reactions (Nelson, 1965; Cohen et al., 1967; Colvin et al., 1973). In this regard carrageenan was shown to interfere with the intrinsic pathway

of the coagulation system; final experiments are in progress.

## 2. Suppression of induration via clotting system

Colvin et al., (1973) have shown that induration at skin test sites in delayed hypersensitivity is due to local deposition of fibrin. Tuberculin sensitive guinea pigs were given  $^{125}\text{I}$  labeled human fibrinogen and albumin at the time of skin test. Experimentals had received 100 mg of carrageenan i.p. 6 hrs previously. Twenty-four hrs later, the animals were exsanguinated and the skin test site excised. The presence of fibrinogen and albumin in the skin was expressed as a ratio (skin)/cpm (blood) so as to correct for excretion, catabolism, etc. While the levels of albumin remained the same, there was a 40% decrease in fibrin levels in the skin test sites of carrageenan treated animals. This indicated that carrageenan suppressed the induration of delayed hypersensitivity by interfering with fibrin accumulation at the site of the lesion. This work was done in conjunction with MAJ R. B. Colvin, Department of Experimental Pathology, WRAIR.

## 3. Effects of carrageenan on coagulation

In vitro carrageenan displayed a biophasic effect on coagulation. Carrageenan was added to either guinea pig or human plasma in final concentrations from 1 mg/ml to  $10^{-8}$  mg/ml. From the highest concentration to  $10^{-2}$  mg/ml, carrageenan had an anticoagulant effect while at lower concentrations a pronounced anticoagulant effect was observed. The site of action appears different in guinea pigs and humans. In humans, carrageenan activates and depletes factor XII (Hageman factor), while in guinea pig, factor XI is affected and factor XII levels normals. The data in these studies is not complete but points to a rather complex interaction of carrageenan with the clotting system, which eventuates in decreased accumulation of fibrin at the skin test site. These studies were done in conjunction with Dr. Frederick Rickles, Department of Medicine, University of Connecticut.

## 4. Kinetics of immunological sensitization

The objectives of such studies as are described below are to identify which factors affect i) the generation of effector cells, ii) the anatomic location of these effector cells, and iii) the traffic of such effector cells to and from target tissues.

Although such information has universal relevance with regard to infectious diseases, neoplasia and the homograft reaction, we have chosen one set of antigens to study; histocompatibility antigens. This was done because antigen reactive cells (ARCs) are easily detected by their blastogenesis in the mixed lymphocyte culture (MLC). Further effector capability can be evaluated by the ability of ARCs

to kill target cells bearing the histocompatibility antigens.

Peritoneal exudate lymphocytes (PELs) have long been known to be an important effector cell in cell mediated immunity (Koster et al., 1971 and Berke et al., 1972). Most studies have suggested that it constitutes a committed population. It is unknown whether antigen reactive cells (ARCs) existed in the absence of specific immunization. The clonal selection theory postulates the presence of small numbers of these cells (clones) and indeed they have been demonstrated in splenic lymphocytes (SpLs), peripheral blood, lymph nodes, etc. The presence of preformed clones in naive animals is particularly true with respect to the histocompatibility system (H-2) in which large numbers of histocompatibility ARCs (HARCs) exist in the unimmunized mouse. The presence of HARCs was detected *in vitro* in the mixed lymphocyte culture (MLC). This methodology has been described in detail elsewhere (Phillips et al., 1973). Briefly, BALB/c mice differ from C57 BL<sub>6</sub> mice at a H-2 locus and so give a vigorous MLC response. It has been shown that the response in the MLC, detected by incorporation of <sup>3</sup>H-Thymidine accurately reflects the number of HARCs responding. To make the MLC unidirectional, target cells were X-irradiated with 1200 M *in vitro*. Experimental cultures contained as targets X-irradiated C57 BL<sub>6</sub> SpLs, while control cultures contained X-irradiated BALB/c SpLs. In all cases the responding cell was from BALB/c. The MLC response was either accomplished with responding cells which had been harvested from naive or preimmunized (with target C57 BL<sub>6</sub> cells) BALB/c animals.

Results: (a) While the MLC response of SpLs from nonimmunized animals was brisk, PELs from nonimmunized animals did not support an MLC over background; (b) This implied the existence of HARCs in SpLs (which is in full agreement with all previous reports) but added the new information that PELs contained rare (if any) precommitted cells, at least with respect to histocompatibility antigens; (c) Commitment or the appearance of HARCs in PELs depends solely on preimmunization. Peak MLC responses occur 6 days after immunization; (d) A dual effect of immunization occurs in SpLs: i.) there is an increase in the response (i.e., in absolute CPM <sup>3</sup>H-Thymidine incorporated) which reaches peak value at 6 days post immunization, and ii.) peak incorporation of <sup>3</sup>H-Thymidine occurs earlier in the culture; thus the kinetics of the response *in vitro* are altered. Both these findings have been reported by Phillips et al., (1972).

With at least histocompatibility antigens, we have shown that precommitted ARCs do not exist in the PELs while such cells exist in every other lymphoid compartment (Elkins, 1971). That they appear following immunization in peritoneal exudates implies strongly that exudates represent a concentrating mechanism for effector lymphocytes directed against antigens to which the animal has been recently exposed.

### 5. Traffic into the peritoneal cavity

Since PBLs appear such an important source of effector cells, their origin and traffic were studied. Peripheral blood lymphocytes (PBLs) from normal animals and animals which had an exudate were harvested. These were lightly labeled with  $^{51}\text{Cr}$  and injected intravenously and harvested from the peritoneal cavity. Fig. 41 summarized the results:

- a. There appears to be little if any traffic of normal PBLs into the quiescent peritoneal cavity.
- b. A slight but significant increase in traffic occurred if normal PBLs were injected IV into animals with an exudate.
- c. The greatest traffic into the peritoneal cavity occurred if PBLs harvested from animals with an exudate were injected into animals with peritoneal exudate.

These studies suggested that two factors were needed for emigration into exudates: i) an inflamed endothelial cell through which emigration takes place, and ii) a lymphocyte, not normally present in

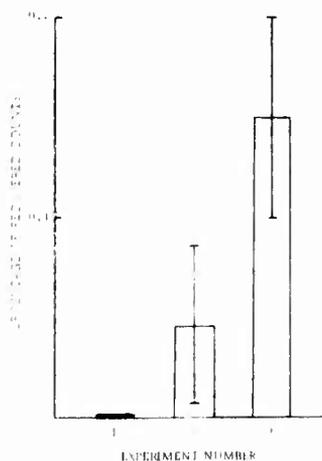


Figure 41. Traffic to the peritoneal cavity of  $^{51}\text{Cr}$ -labeled peripheral blood lymphocytes infused intravenously into recipients with normal or inflamed peritoneal cavities. 1 = PBLs into normals; 2 = PBLs into inflamed; 3 = PBLs from inflamed mice into inflamed cavities.

the blood, called forth by an inflammatory stimulus in the body. It is thought that these cells added to the blood with the capacity of emigrating into exudates are the effector cells described above.

#### 6. Traffic of PELs out of the peritoneal cavity

Ninety % or more of the recovered counts after  $^{51}\text{Cr}$  labeled PELs were injected intraperitoneally, were found at various times in the peritoneal cavity. This suggested that traffic of PELs into the inflamed peritoneal cavity is one way and that immune effector cells are not part of the recirculating pool. In contrast to PELs, when PBLs are injected into the peritoneal cavity, the vast majority of these cells leaves the peritoneal cavity in as little as 8 hrs.

## II. Respiratory viruses

### A. Influenza virus

#### 1. Immune cytolysis as a tool for studying viral entry

For some years, a controversy has existed between groups who believe myxoviruses enter cells by pinocytosis and those who believe cell entry occurs by fusion of viral and cellular envelopes. Immune cytolysis may provide a definitive answer to this question. If virus enters by fusion with the cellular membrane, the envelope of the virus should be incorporated into the cellular membrane shortly after viral penetration. This might make the cell susceptible to immune cytolysis by anti-viral antibody and complement within one hour after infection. However, if virus enters by pinocytosis, the cells should not be susceptible to immune cytolysis shortly after infection.

BHK-21 cells were seeded into the 24 wells of a Linbro plate. Cells were labeled by adding 0.1 ml of a solution of  $^{51}\text{Cr}$  containing roughly 200,000 cpm to 1 ml of media 199 with 10% fetal calf serum (FCS) in each well. After 18 hrs at  $37^{\circ}\text{C}$ , the media was removed from the confluent cells. Alternatively, the cells were labeled for 3 hrs prior to use by removing the media from the cells and adding 0.1 ml of chromium solution. The cells were inoculated with 0.2 ml WSN influenza virus at various multiplicities and covered with maintenance media 2 hrs later. Seven hrs after infection, the cells were washed three times with phosphate buffered saline (PBS) and once with triethanolamine-buffered saline (TBS) containing  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  (Rife and Muschel, 1961). Then 0.1 ml of a 1:100 dilution of heat inactivated rabbit anti-WSN-antibody (HAI titer 1:4096) in TBS was added along with 0.1 ml of a 1:2 dilution of fresh guinea pig complement (C). When necessary, serum was absorbed twice with 0.1 volume packed BHK cells prior to use. The following controls were included: infected cells incubated with C alone, a mixture of C and preimmune rabbit serum, and a mixture of

heat-inactivated C and immune serum. Uninfected cells were incubated with immune serum and complement. After one hr at 37°C, the supernatant was removed and the cell monolayers dissolved in 1 ml of 2 N sodium hydroxide. Both supernatant and dissolved cells were assayed for radioactivity. The percent lysis equals cpm supernatant/cpm (supernatant and cells) X 100.

We found that seven hrs after infection with WSN virus, the cells were positive for hemadsorption and approximately 70% lysis occurred, compared to 13% for controls. A large amount of lysis could be achieved even with only a 10 min lysis time. At eighteen hrs after infection, backgrounds were much higher. These results were repeated, using Sendai virus-infected cells 7 hrs after infection. A rabbit anti-Sendai virus-serum (HAI titer 1:32) was used undiluted. Maximum lysis was 40-50% versus a background of 10 to 16%. Attempts were then made to look for cytolysis one hr after infection. Virus was added to the cells at a multiplicity of infection of 50 to 500 and allowed to adsorb at 4°C for one hr. The cells were warmed to 37°C and one hr later serum and complement were added. No cytolysis was obtained with guinea pig C, so rabbit C was tested. High backgrounds were often obtained but occasionally specific cytolysis appeared. Numerous attempts could not reduce the variability in these results. A different procedure was developed where the antiserum is first allowed to react with virus-infected cells for 30 min and removed. Then C is added for 1 hr. Using this procedure, results show guinea pig C can cause specific lysis of Sendai-infected cells one hr after infection (Table 30). No lysis at all is caused by Sendai virus which has adsorbed to cells at 4°C and been treated with antibody in the cold (sample 2, Experiment II, Table 30). In accord with the results of others showing rapid penetration of Sendai virus by electron microscopy, we found 23% lysis only 10 min after the cells were warmed. Thus, Sendai virus, a parainfluenza virus which appears to enter cells by fusion, could serve as a positive control for detecting viral fusion with the cell membrane. Influenza virus, in contrast, does not cause lysis of cells one hr after warming. Attempts are being made to confirm these preliminary experiments which suggest that influenza virus does not enter cells by fusion with the external membrane.

## 2. Effect of different plastics on WSN viral growth

During the course of our work, WSN virus hemagglutinin yields from MDBK cells dropped from a high of 1:1280 to 1:8 or 1:32. As this occurred, HA/pfu ratios dropped from around 105.0 to around 10<sup>4</sup>. The growth rate of the virus and the growth rate of the cells also slowed considerably. The virus and the cells (at passage 45) were obtained from Dr. R. Compans at the Rockefeller University. He had noticed a large increase in virus yields after passing cells on plastic flasks instead of glass bottles (Choppin, 1969). We used the same media and culture conditions as Compans, but in the course of our work we changed from Falcon brand plastic flasks to CoStar brand plastic

Table 30. Early immune cytolysis of virus infected cells

Sample	Virus	EID <sub>50</sub> /cell	Time virus at 37°C	Serum	C	% lysis <sup>a</sup>
<u>Exp. I</u>						
1	Sendai	500	1 hr	anti-Sendai	1:4	46
2	"	100	"	"	"	34
3	"	50	"	"	"	26
4	"	25	"	"	"	13
5	"	12.5	"	"	"	7
6	"	6.25	"	"	"	6
7	"	500	10 min	"	"	23
8 <sup>b</sup>	"	"	"	"	"	24
9	"	"	1 hr	normal	"	5
10	"	"	"	anti-Sendai	heated 1:4	4
11	None	-	"	"	1:4	7
12	WSN	500	"	anti-WSN	"	7
13	"	"	"	normal	"	6
14	None	-	"	anti-WSN	"	
-----						
<u>Exp. II</u>						
1	Sendai	500	1 hr	anti-Sendai	1:4	55
2	"	"	0 hr	"	"	10
3	"	"	1 hr	normal	"	19
4	"	"	"	anti-Sendai	heated 1:4	4
5	None	"	"	"	1:4	14

<sup>a</sup> Average of 3 tests

<sup>b</sup> Serum incubated only 15 min, C only 1/2 hr

flasks. In order to reduce the variability in cells, we cloned the MDBK line and selected a clone yielding the greatest amount of virus. At the same time, we undertook a brief investigation to determine if the change in flask brand could account for our reduced yields.

Cells derived from the same flask at passage 68 were passaged twice on CoStar flasks, then divided into a Falcon subline (1F) and a CoStar subline (1C). For a second experiment, two sublines were also started from one vial of cells frozen at passage 52. These cells had been passed on Falcon flasks until passage 52, passed once on CoStar flasks after thawing, then split into a CoStar (2C) and Falcon (2F) subline. The four cell sublines were passed a number of times and each plaque flask which contained  $4 \times 10^6$  cells was infected on the same date with similar volumes of aliquots of serial dilutions of WSN virus (egg grown,  $10^{-7}$  EID<sub>50</sub>/0.1 ml). The results are presented in Table 31. The most dramatic difference between the CoStar and Falcon flasks appeared at the  $10^{-5}$  and  $10^{-6}$  dilutions of experiment 2. Here, the CoStar flasks appeared uninfected as opposed to the infected Falcon flasks. Falcon flasks always gave a higher yield of virus. These studies indicated to us that a) the growth of WSN in MDBK cells varies with cell passage, and b) the surface of culture flasks exert a significant influence on the efficiency of replication of influenza virus in MDBK cells.

### 3. Attempts to produce a model for a vaccine strain grown in diploid human fibroblasts

Influenza virus vaccines are presently produced in embryonated hens' eggs. A disadvantage of this method is that eggs are not uniformly sterile and therefore a certain amount of bacterial endotoxin is found in vaccine lots. There are also problems in obtaining enough certified eggs when rapid production of vaccine is necessary. To alleviate these problems, we are trying to produce a strain of influenza virus which will grow in WI-38 diploid human fibroblasts. Cells of this type have already been used for production of adenovirus vaccine. However, Kilbourne et al., (1964) showed that this cell line grew influenza virus poorly, if at all. We planned to adapt the WSN strain of influenza virus, which is well known for its wide tissue tropism, to WI-38 cells, and transfer the genetic elements responsible for its increased growth to current strains of virus which can be used for vaccine production.

WSN virus was grown in screw-capped tubes of WI-38 cells obtained from HEM Laboratories and maintained with one ml of Medium 199, with 2% noninactivated fetal calf serum. These concentrations of calf serum do not inhibit WSN virus hemagglutination (HA). For the first passage approximately  $1 \times 10^4$  pfu (titered on chick embryo cells) of MDBK grown WSN virus was added to each tube of WI-38 cells in serial 10 fold dilutions. No HA was produced, but in 10 days some viral cytopathic effect appeared in the tube inoculated with undiluted virus. Passage of the fluids from the undiluted culture in serial 10-

Table 31. Influence of culture vessel on yield of WSN virus from MDBK cells

Cell line	Passage No.	Dilution of virus inoculum	Maximum HA yield	Day of maximum HA yield
<u>Exp. 1 (line 2)</u>				
C*	57	10 <sup>-3</sup>	1:16	5
F**	58	10 <sup>-3</sup>	1:256	5
C	57	10 <sup>-4</sup>	1:16 <sup>±</sup>	6
F	57	10 <sup>-4</sup>	1:64	4
C	57	10 <sup>-5</sup>	1:32	6
F	58	10 <sup>-5</sup>	1:64	4
C	57	10 <sup>-6</sup>	1:16	7
F	58	10 <sup>-6</sup>	1:64	4
-----				
<u>Exp. 2 (line 1)</u>				
C	77	10 <sup>-3</sup>	1:32	4
F	75	10 <sup>-3</sup>	1:256	5
C	77	10 <sup>-4</sup>	1:64 <sup>±</sup>	6
F	75	10 <sup>-4</sup>	1:128 <sup>±</sup>	6
C	77	10 <sup>-5</sup>	0	-
F	75	10 <sup>-5</sup>	1:64	4
C	77	10 <sup>-6</sup>	0	-
F	75	10 <sup>-6</sup>	1:32 <sup>±</sup>	6

\* CoStar flasks  
 \*\* Falcon flasks

fold dilutions led to the production of an HA titer of 1:4 in 15 days only at the  $10^{-3}$  dilution. The virus has been passed for a total of 10 passages at limit dilution. The adapted virus routinely produces HA on day 2 at the stronger virus dilutions and HA yields will reach 1:128 to 1:256 by day 8. Virus in the later passages was usually harvested and passed when it had reached an HA titer of 1:16 and produced virus when diluted to  $10^{-5}$ . Adapted virus has been plaqued on WI-38 cells, using an overlay consisting of 10 ml of 10X medium 199, 1.2 ml sodium bicarbonate, 2 ml fetal calf serum, 5.33 ml of 7.5% bovine serum albumin, 1.5 ml of 100X amino acids, 1.5 ml of 100X vitamins, 0.5 ml of DEAE dextran (2%), 32 ml of 2% Ionagar #2, penicillin and streptomycin and 52 ml double distilled water. Plaques appeared on day 7 when stained with neutral red. Attempts to recover virus from plaques have not yet been successful. When the WI-38-adapted WSN virus is plaque purified, attempts will be made to use it in recombination experiments to produce a virus with A/Port/Chalmers/1/73 surface antigens and the ability to grow to high titer in WI-38 cells. The virus produced should serve as a model for production of inactivated influenza vaccines in WI-38 cells.

#### 4. Role of the viral carbohydrate

Studies are proceeding to investigate the role of the carbohydrate moiety which is covalently bound to the surface proteins of influenza virus. Schloemer and Wagner (1975) provided a model for this study when they showed that enzymatic removal of the sialic acid from the glycoproteins of VSV resulted in a 100-fold drop in HA ability and infectivity. Treatment with an enzyme which transferred sialic acid to the virus restored much of the activities. Our studies involved treatment of virus infected cells with glucosamine which prevents glycosylation and thus produces virus with less carbohydrate (defective virus). Attempts were then made to replace the carbohydrate using the system of Spiro and Spiro (1966). Studies were initially directed toward further characterizing the defective virus, the production of which was described in the previous WRAIR annual report. Virus infected cells were treated 30 min to 1 hr after infection with 0 mg/ml, 5.0 mg/ml, 10 mg/ml, 20 mg/ml, or 30 mg/ml glucosamine and 20  $\mu$ Ci/ml  $H^3$ -amino acids. Twenty four hrs later, the flasks were harvested. SDS gels were run with extracts of infected cells and supernatants from which virus was concentrated by precipitation with anti-WSN antibody. No significant difference was seen between the migration patterns of the labeled defective virus produced with various concentrations of glucosamine and a  $C^{14}$ -labeled normal virus standard. The same was true for defective virus which was produced using 20 mg/ml glucosamine and purified by tartrate gradient centrifugation as described previously.

Studies directed toward glycosylation of defective virus have been carried out as described by Spiro and Spiro (1966) with the

following modifications:  $MnCl_2$  at  $6 \times 10^{-6}$  was added to the incubation mixture, phosphoenol pyruvate and leucine were omitted; the amount of cellular protein used was in the range of 0.5 to 2 mg/ml; the cells used were uninfected BHK-21 or MDBK cells, and defective virus, partially purified by differential centrifugation, was added. Occasionally Triton X and/or B-mercaptoethanol ( $6 \mu M/ml$ ) was added to the mixture. Incorporation of carbohydrate (.02-.1%) into defective virus was monitored by addition to the reaction mixture of  $C^{14}$ -glucosamine (in amounts far below inhibitory concentrations) and/or  $C^{14}$ -galactose at between  $2 \mu Ci/ml$  and  $13 \mu Ci/ml$ . After 1-3 hrs at  $37^\circ C$  the reaction mixture was assayed for the presence of HA and then frozen. Later, a portion of the mixture was run on an SDS gel with a  $H^3$ -amino acid-labeled virus standard. Some samples were dialyzed overnight against 0.25 N saline before electrophoresis. Controls included no virus in the reaction mixture, normal virus (HA titer 1:64), no nucleotides, and no cells.

An increase in HA titer from 1:2 to 1:32 after incubation was noted in several experiments. However, since this HA could not be neutralized by anti-viral antibody, it was probably caused by cellular hemagglutinins. The cell mixtures themselves were found to inhibit virus HA. Viral-specific HA could be recovered by treatment of the Mixture with *V. cholera* neuraminidase and EDTA (0.01 M). A viral specific increase in HA activity has not yet been demonstrated. Neuraminidase activity of the virus has not yet been assayed after incubation of defective virus in the glycosylation system because the sucrose present in the system inhibits the assay. When larger samples are used, sucrose will be removed by dialysis. Preliminary results of SDS gels of the reaction mixture show small amounts of  $C^{14}$ -galactose labeled proteins from the reaction mixture migrating with the viral glycoproteins of the  $H^3$  standard.

Experiments are proceeding to increase the amount of radiolabel incorporated in the reaction mixture. Experiments will also be initiated to specifically cleave the terminal N-acetylhexosamine of normal virus as described by Bikel and Knight (1972). The resulting virus might serve as a receptor for the enzyme mixture described above or for a specific enzyme reaction using labeled UDP-N-acetylhexosamines.

#### B. Adenovirus ARD in basic combat trainees

During fiscal year 1975, acute respiratory disease (ARD) rates in basic combat trainees (BCTs) continued to be low as was the experience in FY 1974. The low rates were a reflection of the potent and stable adenovirus vaccines used. Initial titers of the vaccine lots used were as follows: Type 4, Lot 05601 -  $5.5 \log_{10} TCID_{50}$ , and Lot 6501 -  $5.4 \log_{10} TCID_{50}$ ; Type 7, Lot 04701 -  $6.5 \log_{10} TCID_{50}$ , and Lot 6601 -  $6.6 \log_{10} TCID_{50}$ . The former lot of each type was used from commencement of immunization at BCT posts through January 75, and the

latter lots of each type thereafter. The initial titers, listed above, were verified in human embryonic kidney cells at WRAIR and all 4 lots were shown to maintain stable titers over the 6 months of their use.

Following is a summary of data from the adenovirus surveillance program from 1 July 74 - 1 May 75.

Fort Dix. Adenovirus vaccines were used from 1 September 74 through 1 May 74. The median weekly ARD rate by month was highest (1.6/100 BCTs/week) in September, because of adenovirus type 4 disease first evident in mid-August. From 24 August - 21 September 74, 44% of all ARD patients sampled yielded Adv-4. Thereafter ARD rates were below 1.5/100 men/week and isolation of adenoviruses from ARD patients uncommon. Indeed, an adenovirus was not isolated from the 194 patients sampled from mid-November through 1 May 75. Numerous isolates of coxsackie virus A-21 were made from ARD patients from mid-October to mid-December 74 and in March 75, but ARD rates did not exceed 1.3/100/week in this period. Scattered isolates of influenza A virus, similar to A/Port Chalmers/1973 were made in January, February, and March 1975 when the median weekly ARD rate was 1:0, 1:4, and 1:1, respectively.

Fort Jackson. Adenovirus vaccines were used from 1 October 74 - 1 May 75. The highest ARD rate was 1.3/100/week in December and January. Between 1 October 74 and 2 May 75, only 10 of 327 ARD patients sampled (3%) had isolates of adenovirus, all type 4.

Fort Knox. This post had an earlier outbreak of ARD than in previous years. Adenovirus type 4 was isolated in more than 10% of ill trainees first at the end of August. Table 32 lists ARD rates and proportion of ill trainees with Adv-4 isolates at Fort Knox from August through December 1974.

Newly arriving BCTs were immunized with adenovirus vaccines beginning 1 October 1974. ARD rates declined 3 weeks after immunization was begun and further strains of adenovirus were not isolated from ill patients 6 weeks after immunization was begun.

A small increase in weekly ARD rate to 1.5 was noted in early February, associated with isolation of influenza A viruses. From January through April only 2 of 405 patients sampled (0.5%) had adenovirus isolates (both Adv-4).

Fort Polk. Adenovirus vaccines were used at Fort Polk from 2 November through 1 May. An adenovirus type 4 outbreak, with weekly ARD rates between 2.0 - 3.7/100/week, beginning in mid-October, prompted earlier vaccine use than projected (for January - May 75). A significant decrease in ARD rate was not apparent until mid-December, 6 weeks after immunization of incoming BCTs began. From 6 October through 14 December, 92 of 219 (42%) ARD hospitalizations sampled yielded adenovirus type 4. During December, nearly all Adv-4 isolates

Table 32. Adenovirus surveillance, Fort Knox, KY, Fall of 1974

Week ending	ARD rate/100/week	ARD patients		
		Sampled (No.)	With pharyngeal isolates of Adv-4	
			(No.)	(%)
Aug 24	0.4	9	0	0
31	0.6	18	2	11
Sep 7	0.7	22	2	9
14	0.9	23	4	17
21	1.5	17	10	59
28	2.2	27	12	44
Oct 5	2.9	42	23	55
12	3.4	45	26	62
19	3.3	47	28	60
26	1.2	48	22	46
Nov 2	0.6	48	11	23
9	0.9	41	3	7
16	0.8	48	0	0
23	1.1	27	0	0
30	0.9	39	0	0
Dec 7	1.2	40	0	0
14	1.4	45	0	0
21	0.8	26	0	0

were obtained from trainees beyond the 5th week of BCT; i.e., trainees who did not receive adenovirus vaccines.

From January through April, the ARD rate remained below 1.6/100/week; only 3 adenovirus isolates (2 type 7 and one type 16) were made from the 247 ARD patients sampled (1.2%). Two strains of influenza A were isolated in February and five strains of coxsackie virus A-21 were isolated in March from BCTs at Fort Polk.

Fort Wood. Adenovirus vaccines were used from 1 October through 1 May. ARD rates remained below 1.6/100/week through December when adenoviruses were isolated from only 10 (8 Adv-4 and 2 Adv-7) of 214 ill BCTs (4.6%). The ARD rate increased in January to peak at 5.2 the week ending the 18th. Disease was associated with influenza A, which continued to be isolated from ill trainees until March. Despite median weekly ARD rates of 2.9, 1.9, and 2.1 in January, February, and March 75, respectively, only 8 adenoviruses (6 Adv-4 and 2 Adv-7) were isolated from 402 hospital admissions sampled (2%) from January through April.

In summary, potent and stable adenovirus vaccines effectively controlled adenovirus ARD in the 6 BCT posts, as they did in FY 1974. ARD morbidity at Fort Knox and Fort Polk might be further decreased by commencing adenovirus immunization in October rather than the January starting dates used in the past years. Adenovirus-associated ARD rates were so low that for the first time in the history of the adenovirus surveillance, influenza outbreaks could be recognized at individual posts by increases in ARD rates. Over 90% of ARD hospitalizations associated with influenza occurred in BCTs in the first 3 weeks of training. This suggests the current military influenza vaccines were highly effective within 3 weeks after immunization.

### III. Clinical virology

#### A. Herpes virus infections

##### 1. The spectrum of herpes simplex virus disease at Walter Reed General Hospital

Herpes simplex viruses (HSV) may be separated into two types based upon antigenic and other biologic characteristics. The most consistent method of typing is by neutralization test (Stalder et al., 1975). The simplified micro neutralization test of Herrman was adapted using mouse hyperimmune ascitic fluid (HMAF) prepared by the method of Chewsilp and McCown (1972). This test was applied to clinical isolates of Herpes simplex obtained in 1972 through 1974. A dose of previously titered virus was tested simultaneously against dilutions of HMAF prepared against type 1 HSV and of HMAF prepared against type 2 HSV. Fig. 42 depicts dilutions of type 1 and type 2 HMAF which neutralized individual strains of virus by Herrman's

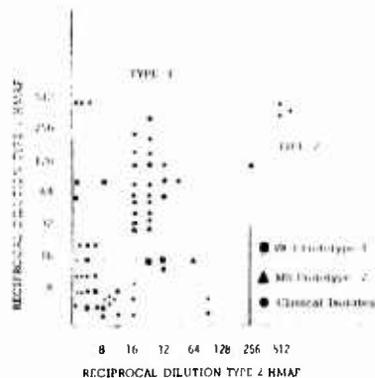


Figure 42. Herpesvirus neutralization typing test. Each data point represents one test, with some strains tested more than once, against both type 1 and type 2 herpesvirus hyperimmune mouse ascitic fluid (HMAF).

technique. Typing results of 54 Herpes simplex strains isolated from 1972-1974 are shown in Table 33.

Table 33. Herpes simplex virus isolates (54 isolates)

Site from which isolate obtained	Type 1	Type 2
Respiratory mucous membranes*	25	2
Genitals or buttocks	2	9
CNS	4	1
Hand vesicles	1	2
Face or scalp vesicles	3	1

\*Including conjunctivae

All but three isolates were obtained from adults. One isolate of type 1 was obtained from the scalp of a 5-month-old infant whose father had a lip lesion 1 week previously and had kissed the infant on the scalp. One isolate of type 2 was obtained from a lesion on the mouth of a 6-year-old renal transplant recipient and another isolate of type 1 was obtained from an 8-year-old child with primary Herpes simplex gingivostomatitis. Types of herpesvirus recovered from patients with neurological disease are shown in Table 34.

Table 34. Neurologic disease associated with Herpes simplex isolates

Disease	Herpes simplex type isolated		Source of isolate
	Type 1	Type 2	
Encephalitis	2	0	1 brain 1 brain and CSF
Aseptic meningitis	0	2	1 CSF, 1 penile
Radiculitis:			
cervical	1	0	1 CSF isolate;
sciatic	1	1	type 1 CSF isolate; type 2 from buttocks lesion
Transverse myelitis	1	1	type 1 isolated from diffuse oral lesions; type 2 isolate from diffuse vesicles on trunk*
Hemiplegia	1	0	Contralateral scalp lesion

\* Patient also had acute onset of monocular blindness and was diagnosed to have multiple sclerosis

Encephalitis in adults has not been reported to be caused by type 2 virus. Both cases of aseptic meningitis in this series were associated with type 2 virus, one with virus recovered from CSF and

another with 3 separate episodes of aseptic meningitis associated with penile lesions from which type 2 was isolated between episodes of meningitis.

Two cases of transverse myelitis were studied with one isolate of each type. Transverse myelitis has been associated with type 1 virus previously (Klastersky et al., 1972). An association between HsV and radiculitis has been reported rarely (Layzer et al., 1974 and Hunt et al., 1955). One CSF isolate in this series was obtained in high titer ( $10^5$  TCID<sub>50</sub>) from a 38-year-old man with a one month illness which began with cervical radiculopathy; next he developed vesicles over his left upper extremity, and following their clearing, he developed lumbosacral radiculopathy for which he underwent 2 separate laminectomy procedures to no avail. Virus was isolated from the CSF at the time of a myelogram in preparation for a third laminectomy. He was treated with bed rest and observation and symptoms gradually cleared. Another man developed lumbo sacral radicular symptoms coincident with recurrent Herpes lesion on his ipsilateral buttock.

The patterns of involvement in this predominately adult population are typical of published reports (Juel-Jensen et al., 1972) with 92% of the isolates from respiratory mucous membranes being type 1, and 88% of the genital area isolates being type 2. One transplant patient had a type 1 strain recovered from a lip lesion and a type 2 strain recovered from a penile lesion. Strains isolated from more distant sites are more diverse. Type 2 was isolated twice from the hands of adult men; one with concomitant primary type 2 oral disease and another with a 6-year history of recurrent lesions on the 3rd finger of his right hand. Type 1 was isolated from zoster-form facial lesions of 3 adults, 2 of whom also had eye involvement. Diffuse type 1 skin vesicles from 2 patients were seen at sites of apparent auto-inoculation, during scratching. A type 2 strain was isolated from a lesion on the cheek of an adult woman.

The types of neurologic disease associated with Herpes simplex isolates are varied. Both cases of adult encephalitis were caused by type 1, with virus being isolated from the brain alone of one case and from several sites in brain and from the CSF in one fatal case.

The man with hemiplegia was a 62-year-old man with diffuse zoster-form Herpes simplex lesion involving his contralateral eye and scalp. This syndrome of hemiplegia has been reported previously with contralateral "zoster form" skin lesions and has been attributed to varicella zoster virus, but without appropriate virologic studies. A granulomatous angitis has been observed in the cerebral lesions of the involved side (Gilbert, 1974). It is known the Herpes simplex may mimic the skin lesions of Herpes zoster.

The young lady with De Vics syndrome (monocular blindness and

diplegia) had diffuse type 2 Herpes simplex skin lesions and was clinically felt to have multiple sclerosis. Some patients with multiple sclerosis have been found to have elevated anti-Herpes simplex antibody in the CSF and type 2 virus has, on occasion, been isolated from the spinal cord of patients with this disease (Norby et al., 1974).

Both type 1 and type 2 virus have been shown to be latent in the ganglion cells of a high percentage of unselected cadavers. Further studies are necessary to understand the pathogenesis of these heterogeneous CNS disorders associated with Herpes simplex virus.

## 2. Herpes virus replication in gingival cell cultures

Herpes simplex virus type 2 (HSV-2) has been shown to be associated with select carcinomas in humans, and recently has been recovered with greater frequency from the oral cavity. This study was conducted to determine if a HSV-2, isolated from the lip (mucocutaneous junction) of a 6-year-old female, and several other HSV-2 viruses could be propagated in gingival cell culture. These investigations showed that HSV-2 replicate in gingival cell monolayer cultures at 35°C, producing a characteristic cytopathic effect (CPE) with giant cell formation, cell rounding and finally, cell destruction. Equal multiplicity of infection of type 1 and type 2 HSV in gingival cells showed that they replicated to approximately the same viral titer, but the CPE produced by HSV-2 appeared more pronounced. An additional indication of HSV-2 replication in gingival cells was illustrated by electron micrographs of thin sectioned preparations showing HSV-2 virions within the gingival cell nucleus (Fig. 43). The inner bodies contain central

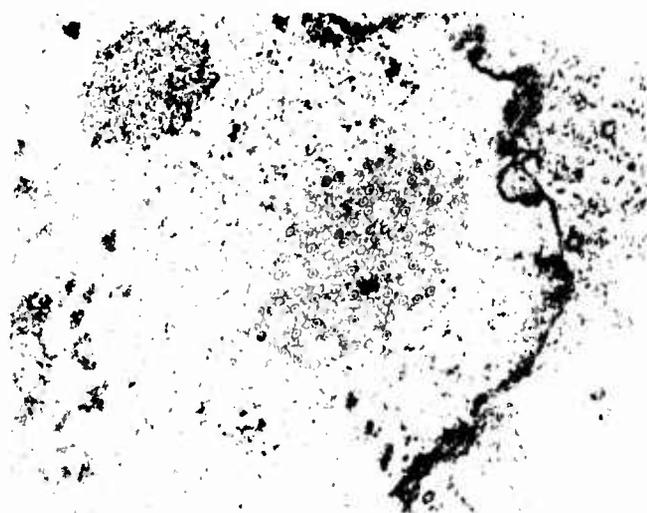


Figure 43. Electron micrograph of a gingival cell infected with type 2 Herpesvirus.

zones of less density (hollow core forms of naked particles), but in general there appeared to be a majority of dense core naked particles. This gingival cell line appears to provide an ideal environment for isolation and identification of Herpes simplex virus, type 2.

#### B. Hepatitis after renal transplant

Hepatitis is common after renal transplantation (Penn et al., 1969 and Luby et al., 1974). Drug toxicity, hepatitis B virus, and cytomegalovirus infection have been associated with the majority of cases of post transplant hepatitis. Seventy-one consecutive patients undergoing renal transplantation at the Walter Reed Army Medical Center, Washington, DC, were studied in a two and one-half year period to ascertain the incidence and the etiology of hepatitis in this patient population. Hepatitis is arbitrarily defined as an elevation of the serum SGOT to greater than 50 units, often with concomitant rises in LDH and serum bilirubin. HB<sub>s</sub>Ag associated hepatitis is an episode of hepatitis with a test positive for HB<sub>s</sub>Ag or with a fourfold or greater rise in anti-HB<sub>s</sub> within 2 months of an episode of hepatitis. One patient had repeatedly negative serology for these antigens but had hepatitis antigen demonstrated by immunofluorescence of renal biopsy specimen and was included in this group.

Seventy-one consecutive patients undergoing renal transplantation between January 1971 and September 1974 were studied. Sera were collected at intervals and stored at -20°C until the time of testing. The average number of sera studied after each renal transplant was 5. Titers of CF antibody to the broadly cross reacting AD 169 strain of CMV were determined. The Ausria 2 solid phase radioimmunoassay was used to detect hepatitis B surface (HB<sub>s</sub>Ag) antigen. HB<sub>s</sub>Ag negative sera were studied by passive hemagglutination for antibodies to hepatitis surface antigen (anti-HB<sub>s</sub>).

Results: Twenty-eight of seventy-one consecutive renal transplant recipients developed hepatitis within 3 months after transplantation (39%). Only four of the twenty-eight cases of hepatitis (14%) were associated with HB<sub>s</sub>Ag. Four-fold titer rises to CMV CF antigen occurred in fifteen of the twenty-nine cases of hepatitis (54%). Four-fold CMV titer rises also occurred in twenty of the forty-three patients who did not develop hepatitis (63%). Diagnostic cytomegalovirus titer rises occurred in forty-two of the seventy-one patients (59%). Thus, etiologic role of CMV in post transplantation hepatitis remains unproven. Depending upon the significance of cytomegalovirus in the etiology of PTH, a minimum of forty-seven and a maximum of eighty-seven % of the hepatitis occurring in patients with 3 months of renal transplant remains without determined etiology. (See Table 35).

Hepatitis is extremely common in the period post renal transplantation. The situation is analogous to the events occurring after multiple whole blood transfusions. Knodell and associates have

Table 35. Seventy-one renal transplant recipients

Patients with hepatitis	Patients without hepatitis	
28/71 (39%)	43/71 (61%)	
-----		
CMV fourfold titer rises:	42/71	(59%)
15/28 with hepatitis		(57%)
15/18 without hepatitis		(61%)
HB <sub>s</sub> Ag associated hepatitis	4/28	(14%)*

\* Does not include 3 asymptomatic carriers for a total of 7 of 71 renal transplant recipients (10%) positive for hepatitis antigen

described hepatitis occurring in 21% of patients receiving multiple transfusions with 7% associated with CMV seroconversion and another 12% due to hepatitis B virus (Knodell et al., 1975). Neither EB virus nor hepatitis A viruses could be implicated as a cause of hepatitis in these multiply-transfused patients (Prince et al., 1974, Feinstone et al., 1975) and others have postulated yet another infectious agent hepatitis C, as the cause of the majority of hepatitis in a multiply-transfused patient population.

If neither EB virus nor hepatitis A is the cause of the hepatitis in renal transplant recipients, hepatitis C may be implicated. Oro-pharyngeal excretion of EB virus occurs in up to 47% of renal homograft recipients, many of whom were seropositive for EB virus. Immunosuppressive therapy reactivates latent EB virus infection. Hepatitis has not been observed in relationship to this EB virus shedding (Strauch et al., 1974). Hepatitis A has not been studied in these patients.

Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable Disease and Immunology

Work Unit 166, Viral Infections of Man

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL DD-DNAF (AK) 16	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8A. DISPN INSTN <sup>6</sup>	8B. SPECIFIC DATA CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		9. LEVEL OF SUM A. WORK UNIT
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10. NO./CODES <sup>7</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER				
8. PRIMARY	61102A	3A101102B71Q	00	168				
9. CONTRIBUTING								
9. SUPPORTING	CARDS 114F							
11. TITLE (Precede with Security Classification Code)								
(U) Bacterial Diseases of Military Importance								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>8</sup>								
010100 Microbiology								
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
58 05		CONT		DA		C. In-house		
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS		B. FUNDS (in thousands)
A. DATES/EFFECTIVE:		EXPIRATION:		PREVIOUS				
NA				75		5		100
B. NUMBER <sup>9</sup>		C. TYPE:		FISCAL YEAR				
		D. AMOUNT:		CURRENT				
		E. CUM. AMT.		76		6		281
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research				
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide MAN if U.S. Academic Institution)				
NAME: Buescher, COL E. L.				NAME: Artenstein, M. S.				
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER				
Foreign intelligence not considered				[REDACTED]				
22. KEYWORDS (Precede Each with Security Classification Code)				ASSOCIATE INVESTIGATORS				
(U) Pseudomonas aeruginosa; (U) Mycoplasmas; (U) Neisseria gonorrhoeae; (U) Immunology; (U) Antibiotics; (U) Infectious Diseases				NAME: DA				
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23 (U) Studies on the etiology, ecology, epidemiology, pathogenesis, physiological, immunological and diagnostic aspects of diseases of microbial origin which are current or potential problems to military forces. Current emphasis on meningococcal, gonococcal, pseudomonas, mycoplasma and staphylococcal infections in military forces.								
24 (U) Development of bacteriologic techniques for study of various infectious diseases. Field studies on prophylactic regimens, spread and persistence of organisms in various military populations.								
25 (U) 74 07 - 75 06 The outer cell wall membrane antigens of Neisseria gonorrhoeae have been isolated and characterized. The lipopolysaccharide is serologically related to a common antigenic determinant of N. meningitidis as demonstrated by hemagglutination inhibition assays. The protein forms the basis of an immunologic typing scheme developed for N. gonorrhoeae based on inhibition of a solid phase radioimmunoassay. Clinical strains of gonorrhea have also been typed using meningococcal antisera in a bactericidal system. A previously undetected capsule has been identified in N. gonorrhoeae. Lipids extracted from pathogenic Neisseria and other gram negative organisms were toxic for N. gonorrhoeae. Gonococci invading tissue cultures were demonstrated to have intracellular viability. Clinical laboratory support of infectious diseases problems has included studies on the epidemiology and antibiotic sensitivity patterns of Pseudomonas aeruginosa, a study demonstrating increased susceptibility of dacron grafts to bacterial infection as compared to autogenous vein or bovine heterographs, and demonstration that the Wright's stain and culture of amniotic fluid is of limited use in predicting infection secondary to intrauterine monitoring. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.								

PII Redacted

DD FORM 1498  
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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable Disease and Immunology

Work Unit 168 Bacterial diseases of military importance

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I. Investigations on the control of *Neisseria gonorrhoeae*.

The overall goal of the gonococcal research projects is to develop effective control of *Neisseria gonorrhoeae*. The development of immunoprophylactic control has been one approach currently under investigation. To accomplish this objective the immunochemistry of gonococcal surface structures has been studied with the view that suitable vaccine candidates could be discovered and that common and individual strain specific antigenic structure could be unraveled. Other approaches to typing of the gonococcus such as individual sensitivity in a complement mediated bactericidal antibody system and differential sensitivity to lipids derived from *Neisseria* have also been investigated. Likewise, in vitro models of gonococcal infection such as invasion of tissue culture and attachment to buccal epithelial cells have been studied with the view of developing in vitro correlates of immunity.

A. Immunochemical investigations of the cell surface.

An outer membrane complex similar to that isolated from the meningococcus [1] has been isolated from *N. gonorrhoeae* as described in previous annual reports [2]. Electron microscopy of gonococci grown on plates or liquid culture demonstrate budding and overlapping of outer membranes. These buds of outer membrane can be isolated from filtered culture supernatant by differential ultracentrifugation. The isolated buds demonstrate the same protein and lipopolysaccharide band patterns on 7.5 percent sodium dodecyl sulfate polyacrylamide gels (PAGE) as outer membranes isolated from the whole organism by the EDTA-shear method. This outer membrane blebbing may be playing a role in the infective process as this blebbing phenomena can be seen in electron micrographs of urethral scrapings from infected patients [3].

Over 100 outer membrane preparations have been prepared from clinical specimens to date. These outer membrane complexes consist of three major components: lipid, lipopolysaccharide, and protein. Each of these three components has been analyzed by chemical and immunochemical means.

The lipid was extracted from outer membrane preparations by means of the Bligh-Dyer procedure [4]. The chloroform soluble lipid constituted 25-30 percent of the dry weight of the complex. The lipid was fractionated on silicic acid columns by the method of Vance and Sweely [5] into a F-1 free fatty acid fraction, F-2 glycolipid and F-3 phospholipid fraction (recovery on the column was greater than 95 percent for the phospholipids). Each of the fractions was analyzed by thin layer chromatography (Silical Gel 60, Applied Science Laboratories, Inc., State College, Pa.) with appropriate solvents (Chloroform:Methanol:H<sub>2</sub>O:Acetic acid 50:30:4:2). Fatty acid methyl esters prepared by the Boron-fluoride methanol technique of Morrison and Smith were analyzed by gas liquid chromatography (F&M, 3% ECX X on a gas-chrome Q column, 16:1 and 18:1 conformations confirmed by hydrogenation). The results of the lipid analysis are shown in Table 1.

The composition is similar to that found in Escherichia coli outer membrane [6]. Growth inhibiting activity for other gonococcal strains was found in the F-1 and F-3 fractions confirming Sparlings' work that phospholipids and free fatty acids from GC have inhibitory activity.

Solubilization of the native outer membrane with deoxycholate and chromatography over a Sephadex G-100 deoxycholate column after the method of Zollinger [7] has yielded separation of the protein and lipopolysaccharide (LPS) components. Each of these components has been investigated using chemical and immunologic techniques.

The gonococcus and meningococcus cross react serologically by a variety of assays. It was felt that similarities in LPS between the two species could account for some of this cross reaction. Dr. W. D. Zollinger has identified eight different LPS types for the meningococcus. Using meningococcal outer membrane complexes, which contain LPS, to inhibit homologous LPS hemagglutination reactions, he has been able to classify most meningococci by their LPS type [8]. When 50 different outer membrane complexes from GC were used in an attempt to inhibit homologous meningococcal reactions no clear cut inhibition occurred (Table 2).

Membrane complexes were standardized to contain 250 µg LPS as determined by KDO analysis. These results indicated that laboratory strains of N. gonorrhoeae did not share to any large extent specific LPS serotypes with the meningococcus. Heterologous reactions such as that between meningococcal antisera 136 and sheep RBC's coated with meningococcal LPS 6275 can be inhibited by almost all meningococcal OMC's, indicating that the reaction involves a broadly cross reactive

Table 1. Lipids in Neisseria gonorrhoeae native complex.

F-1 - 1.	Free Fatty Acids:	Lauric (12:0)	0.8%	Myristic (14:0)	3.8%	Palmitic (16:0)	44%	
		Palmitoleic (16:1)	26%	Stearic (18:0)	2.6%	Oleic (18:1)	18%	
		Unidentified (?) Beta-hydroxy fatty acid						
2.	Monoglycerides, Diglycerides							
3.	No Sterol							
F-2 -	Glycolipid (small amount)							
F-3 - 1.	Phospholipids:	Phosphatidylethanolamine, Diphosphatidylglycerol						78.7%
		Lysophosphatidylethanolamine						3.3%
		Lysophosphatidylcholine						17.4%
2.	Fatty acids of phospholipids:	Lauric (12:0)	3.0%	Stearic (18:0)	1.3%			
		Palmitoleic (16:1)	30.6%	Palmitic (16:0)	33%			
		Myristic (14:0)	11.8%	Oleic (18:1)	18.7%			

Table 2. Cross reactions between meningococcal and gonococcal lipopolysaccharides (LPS).

Meningo- coccal Antisera	Meningo- coccal LPS	Gonococcal LPS inhibitor (250 µg/ml)														
		484	162	154	199	187	129	134	527	342	128	209	357	198	120	533
136	6275	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
126	126	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35E	35E	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6275	6275	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6155	6155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
978	978	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
992	992	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
89I	89I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
98I	98I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Hemagglutination

- = Hemagglutination inhibition

determinant on the LPS. Gonococcal outer membrane complexes were in most cases able to inhibit this reaction (Table 2) indicating that gonococci and meningococci probably share some broadly cross reactive determinant in their LPS structure, and this may account for some of the serological cross reactivity between the organisms.

The protein band patterns on SDS-PAGE gels of outer membrane complexes are strain specific and constant if the organisms are grown in the same media under similar conditions of temperature and time. Most but not all strains have outer membrane proteins with molecular weights of between 47,000-50,000 Daltons; between 26,000-28,000 Daltons; and between 21,000 and 24,000 Daltons (Tables 3 and 6 for examples).

The proteins on the M.W. range between 21,000-24,000 Daltons have interesting properties in that they are resistant to proteolytic digestion by pronase, trypsin and subtilism in contrast to the other membrane proteins as demonstrated by SDS-PAGE of intrinsically radio-labeled enzyme treated membrane preparations. Their molecular size and resistance to enzymes is similar to that of pili although these proteins can be found in the membranes of all colonial types of GC while pili have been seen by electron microscopy only on colonial type T<sub>1</sub> and T<sub>2</sub> organisms.

A large but variable percentage of the total protein in the outer membrane, however, resides in proteins that vary in their molecular weight from 29,000-42,000. These may be the strain specific proteins. In order to further investigate common and strain specific antigenic determinants a solid phase radioimmunoassay as described by Zollinger [9] was employed. The test consists of sensitizing the wells of micro-titer plates with these relatively insoluble membrane antigens or their components, incubating with antibody, and then incubating with I<sup>125</sup> labeled antioglobulin. Using this technique the cross reactivity between meningococcal and gonococcal protein antigens could be demonstrated. When a protein vaccine prepared from meningococci 138I was inoculated into rabbits and pre- and post-vaccination sera were tested against gonococcal outer membrane complexes from GC108 a significant rise in titer could be demonstrated.

Using the solid phase radioimmunoassay system a great deal of cross reaction or common antigenic structure could be demonstrated between almost all strains of GC. For example, antisera prepared against GC108, GC104, GC120 and GC134 all reacted with outer membrane complex from GC108 in a titer greater than 1:10,000 (binding >3,000 cpm at that dilution with a background of <400 cpm at infinite dilution). Likewise antibody from a single antiserum prepared against GC108 bound to outer membranes prepared from GC108, GC120, GC104 and GC134 in a dilution >1:10,000 (2,000-6,000 cpm at dilution 1:10,000 with a background <400 at infinite dilution). In each case, however, the homologous antigen-antibody reaction (eg. 108 antisera vs. 108 membrane complex) yielded

Table 3. Molecular weights of membrane proteins from heterologous gonococci.

Strain -	9	105	108	112	119	220	302
	50,500				50,000		
	47,000	47,500	47,000	48,000			
	29,500	31,000		30,000	30,500	30,500	30,000
				28,000		29,000	
	27,500	27,500	27,000	26,500	27,000	27,500	27,500
	21,000	23,000	23,500	22,000	23,500	23,000	

significant binding at dilutions of antisera >100,000 (2,000 cpm at serum dilution 1:100,000), suggesting that antigen abs reactions could be investigated at dilutions of antisera where the majority of the remaining antibody was directed at immunodominant and, hopefully, immunospecific antigenic determinants.

Rabbit antisera against 20 gonococcal strains were tested against their homologous outer membranes. Points on the titer curve representing one-half maximum binding were picked (dilutions ranged 1:20,000-1:110,000) for each reaction and homologous and heterologous inhibitions were carried out at this dilution, using antigens for inhibition at a final concentration of 50 µg protein/ml. When percent inhibitions are calculated a great deal of strain and antibody specificity could be demonstrated (examples in Table 4). Thus, OMC from GC9 was able to inhibit 97.8 percent of the reaction between GC9 antisera and GC9 OMC. It was unable to inhibit any other homologous reactions to a significant degree. Likewise, none of the other heterologous antigens, 33, 105, etc., were able to inhibit the homologous GC9 reaction. As can be seen the other antigens and antisera show similar specificities. When viewed this way, although there is considerable cross reactivity among all strains, individual strains also appear to be almost unique in their antigenic structure.

In an attempt to demonstrate the usefulness of solid phase inhibition as an epidemiologic tool, membrane antigens were prepared from case strains where reliable information was available. These antigens were then used to inhibit standard homologous antigen-antibody reactions. As seen in Table 5 epidemiologically similar strains appear very similar in their ability to inhibit solid phase reactions. For example, OMC prepared from strains 161 and 162 (cervix and pharynx from the same patient) share a major determinant with antigen 120 and also demonstrate relatively the same degree of inhibition of the other reactions. Likewise, if protein band patterns (Table 6) from these case strains are looked at they appear almost identical for epidemiologically similar strains. Thus, with expansion and refinement of the solid phase inhibition system it should be possible to immunotype most strains of GC according to their protein type. If protective immunity is directed against these strain specific antigenic determinants, rather than broadly cross reactive ones, then the apparent lack of immunity in humans may be explained on the basis of a very large number of specific gonococcal strains.

Under certain conditions it has been noted in this laboratory that the gonococcus seems capable of producing a capsule. This capsule can be demonstrated by the wet India ink method (Higgins No. 4415 India Ink:Loeffler's methylene blue 4:6).

Freshly isolated strains grown on GC chocolate agar supplemented with 5 % glucose and 7 % casamino acids may demonstrate capsules. Organisms grown in Medium 199 + 10 percent fetal calf serum may elaborate capsules. Organisms grown in hyperimmune rabbit sera and

Table 4. Specificity of gonococcal antisera.

Homologous Ag-Ab Reaction	Inhibiting Antigen									
	9	33	105	108	112	119	220	302		
9	97.8	0	0	0	22.6	0	27.7	0		
33	0	95.6	1.9	6.0	7.0	16.1	23.9	16.7		
105	17.5	39.1	94.8	63.7	61.6	7.0	17.5	43.2		
108	0	9.5	23.8	90.7	9.3	0	0	18.6		
112	0	0	0	0	93.2	12.7	27.7	0		
119	0	0	0	0	19.3	91.7	16.0	13.7		
220	26.3	23.4	0	59.3	9.2	9.7	94.2	11.7		
302	0	17.7	41.2	34.4	25.3	29.9	59.9	97.9		

Table 5. Inhibition by case strain antigens.

Inhibitory Antigen	Patient	Site	Homologous Ag-Ab Reaction					
			9	117	119	120	302	
288	D.D.	Rectum	31.6	40.8	14	48	0	
302	D.D.	Vagina	31.6	49.3	15	51	0	
161	B.R.	Cervix	39	55	41	83	4	
162	B.R.	Pharynx	32	55	45	83	7	
316	G.S.	Cervix	39	53	63	59	4	
317	G.S.	Joint	36	58	59	53	14	
342	B.L.	Rectum	57	44	22	63	18	
343	B.L.	Cervix	50	47	35	69	22	
152	Consorts		42	85	29	56	34	
154			44	84	22	64	30	
198	Consorts		25	61	51	67	63	
199			48	67	51	71	63	
212	R.Mu		49	92	46	73	45	
215	R.Mu		47	86	31	67	23	
216	B.Maj.		52	95	17	52	20	

Table 6. Molecular weight of membrane protein from case strains.

Strain No. -	288	302	161	162	316	317	342	343
M.W.	30,000	30,000	30,500	31,000	31,000	32,000	48,000	48,000
Daltons	28,000	27,000	27,500	28,500	27,500	27,500	30,500	39,500
			23,000	24,500			28,500	28,000
			20,000	21,500			27,000	26,500

Strain No. -	152	154	198	199	212	215	216
M.W.	50,000	41,000		30,500	32,000	31,500	31,000
Daltons	31,500	31,000	30,500	29,000	29,500	29,000	28,000
	29,000	28,500	28,000	27,000	28,000	27,500	
	25,000	25,000	24,000	25,000			
	21,000	22,000					

passed through tissue culture may elaborate capsules. None of these methods, however, have proven to be consistent and reliable means for insuring good capsule production. Attempts at demonstrating the capsule by electron microscopy have not as yet been successful nor has successful isolation, purification and identification of the capsular substance been accomplished to date.

B. Inhibition of *Neisseria gonorrhoeae* and *Neisseria meningitidis* by lipids derived from homologous and heterologous species of *Neisseria*.

While searching for the presence of bacteriophages or bacteriocins in *Neisseria gonorrhoeae* we observed that some strains inhibited the growth of heterologous strains in mixed culture. Further study showed that inhibition by live organisms was not a reproducible phenomenon and was in some measure influenced by cultural conditions, e.g., glucose and peptone concentration and pH. Inhibitory activity could be detected in mass agar cultures of live producer strains but not in cultures of dispersed single colonies of these strains. However, when chloroform vapor was used to kill both mass cultures and dispersed single colony cultures of producer strains prior to the application of live indicator strains, we observed inhibitory activity against the homologous strain and most heterologous strains (Fig. 1).

The effect of chloroform treatment upon bacterial cells, lysis and probable solubilization and release of membrane and cytoplasmic lipids, suggested that the inhibitory activity we observed was not the result of bacteriocins but due to the release of bacterial lipids into the surrounding medium and in local concentrations high enough to inhibit the growth of indicator strains.

Inhibition of *N. gonorrhoeae* by fatty acids was described some 30 years ago by Ley and Mueller [10], who implicated naturally occurring fatty acids, 6 to 10 carbons in chain length, present in the animal protein components of bacteriologic media. They found that soluble starch, which adsorbs fatty acids, when added to media relieved growth inhibition.

To study the effect of lipids upon strains of *N. gonorrhoeae* small lipid extracts were first prepared from different strains of *N. gonorrhoeae* according to the method of Folch, et al. These lipid extracts were then screened for activity by methods similar to that used for antibiotic screening. Extracts were applied to the surface of agar plates, either directly or in saturated filter paper discs, overlaid with a semi-solid medium suspension of indicator organisms and then examined for zones of inhibition after overnight incubation. These extracts appeared to have different activity spectra against a variety of *N. gonorrhoeae* indicator strains, suggesting that lipid sensitivity could be used to clarify strains of *N. gonorrhoeae*.



Fig. 1. Effect of chloroform treatment upon the detection of bacteriocin-like activity in N. gonorrhoeae. Upper plate received no treatment and lower plate was exposed to chloroform vapor for 30 min.; both plates were then overlaid with a suspension of the same indicator strains of N. gonorrhoeae. Zones of inhibition are apparent only on chloroform treated plate. Note that chloroform treatment markedly decreases the density of the producer strain colonies.

The reasons for variation in activity among the different extracts are unclear but could be explained by qualitative or quantitative differences in the lipids present in producer strains or similar differences in the sensitivity of indicator strains. It was also remotely possible that variation was an artefact, the result of uneven distribution of the starch present in the agar media and inadvertent detoxification of the lipids being tested.

Lipid extracts of more than 20 strains of *N. gonorrhoeae* and of 15 strains of *N. meningitidis* have been prepared according to the method of Folch, et al. In addition, extracts of a variety of strains including enteric organisms, staphylococci, streptococci, gram positive bacilli and five different nonpathogenic species of *Neisseria* have also been prepared. We have used thin layer chromatography (TLC) on mylar backed silica gel layers for qualitative comparisons between these various extracts.

The lipid extract of a typical strain of *N. gonorrhoeae* when chromatographed in a polar solvent shows eight or nine distinct components which react with iodine vapor (an indicator of organic material) and with a lipid reagent phosphomolybdic acid (Fig. 2). Five components, including two minor ones, react with molybdenum blue, a reagent specific for phospholipids. When extracts are chromatographed with a mixture of authentic phospholipids they show components comparable to phosphatidyl ethanolamine and phosphatidyl choline (PE and PC), and a third component with the same chromatographic properties as lysophosphatidyl choline (LPC) but which does not react with the phospholipid reagent. There are in addition one major and two minor phospholipid reacting components which may be monoacyl (lyso) derivatives of PE or phospholipids other than PE or PC. Chromatograms also reveal a component(s) near the solvent front, the area to where free fatty acids (FFA), neutral lipids and sterols could be expected to migrate in a polar solvent system.

When examined by TLC the meningococcal lipid extracts are, for the most part, similar to the gonococcal extracts (Fig. 3). However, three strains did not show lipids comparable to the FFA-like component and three other strains did not show lipids comparable to the PC present in the gonococcal extracts. Extracts of the saprophytic *Neisseria* and of the gram-negative enteric organisms, while generally similar to the gonococcal extracts when tested by TLC, also showed diversity comparable to that seen among the strains of meningococci. Extracts of a few gram-positive organisms, *Staphylococcus aureus* and *S. epidermidis* and a  $\beta$  hemolytic streptococcus are dissimilar to those of the gram-negative organisms by TLC.

To determine which components are responsible for the inhibitory activity of the complete extract we have devised a bioautographic procedure which permits detection of zones of inhibition directly over specific components on developed chromatograms. Briefly, lipid

FIG. 2

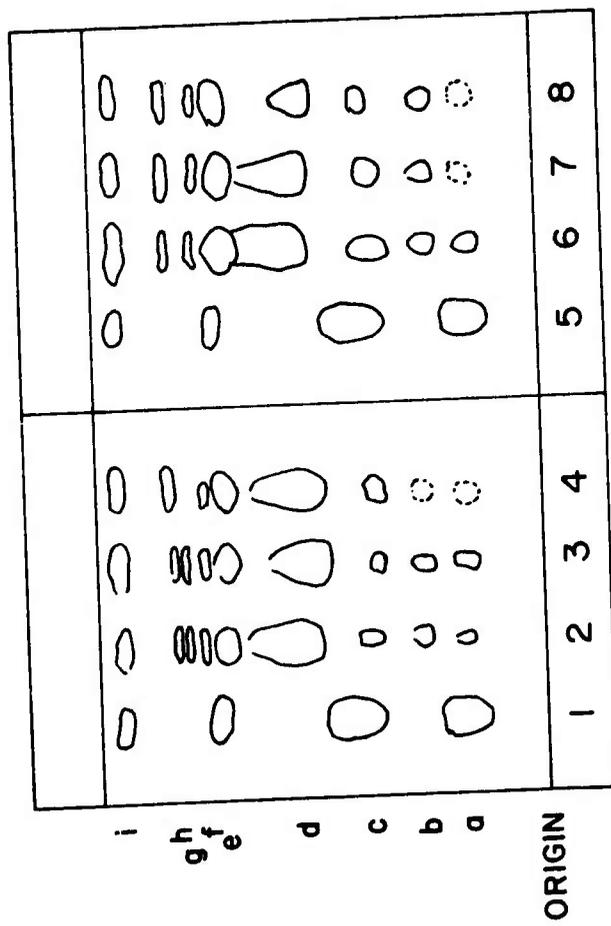


Fig. 2. Thin layer chromatography of lipid extracts of six different strains of *N. gonorrhoeae* on Baker 1B2F silica gel layers. Row 1 and 5 is a mixture of authentic phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and cholesterol; remaining rows are *N. gonorrhoeae* extracts, all at 10 mg/ml. All preparations spotted in 5  $\mu$ l volumes, chromatographed over 6 cm in  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}(65:25:4)$  overdeveloped for 5 min. and components visualized with  $\text{I}_2$  vapor and molybdenum blue reagent. Component a is LPS, c is PC and e is PE. Component i in rows 1 and 5 is cholesterol and in the *N. gonorrhoeae* extracts represents the free fatty acid (FFA) fractions. Components d, f, g and h which react with the phospholipid reagent. molybdenum blue, have not been identified. Component a in the *N. gonorrhoeae* extract, which is chromatographically similar to authentic LPC, does not react with molybdenum blue and most likely is not LPC.



Fig. 3. Thin layer chromatography of lipid extracts of eight strains of N. meningitidis. Conditions of chromatography are the same as in Fig. 2. Row 1 is a mixture of authentic lipids; LPC (a), PC (c), PE (e) and cholesterol (i). Row 2 is an extract of N. gonorrhoeae strain 1 and rows 3 through 10 are extracts of eight strains of N. meningitidis. Of particular interest are absences of specific N. gonorrhoeae lipid components in the N. meningitidis extracts; component c in rows 3 and 9, and components b, c and i in row 4.

extracts are chromatographed in a polar solvent over a distance of six cm on mylar-backed silica gel layers. This distance is sufficient to achieve adequate separation of all lipid components. The chromatograms are trimmed, applied to the surface of a basal agar layer in a standard petri dish and then overlaid with a suspension of indicator organisms in semi-solid growth medium without starch. Visualization of zones of inhibition over the opaque silica gel chromatogram is done by in vivo staining of live colonies of the indicator organisms with the redox indicator triphenyltetrazolium chloride (TTC). Clear areas of inhibition are seen surrounded by live colonies that have turned red as they take up and oxidize the dye. We have also found this procedure useful in testing a variety of authentic lipids such as saturated and unsaturated fatty acids, phospholipids and sterols for inhibitory activity.

Using this bioautographic procedure we tested chromatograms of our extracts for inhibitory activity against selected gonococcal and meningococcal strains. Three zones of inhibition are usually seen over chromatograms of gonococcal extracts when tested against gonococcal indicator strains (Fig. 4). The first of these zones of inhibition corresponds to the fast moving (high Rf) FFA-like component, the second to the PC component and the last to a component directly below PC which has been only poorly visualized with iodine vapor and phosphomolybdic acid reagent. The nature of this last component is not certain, but because it does not react with molybdenum blue or ninhydrin it is unlikely that this component is LPC. There is little variation in the response of gonococcal indicator strains to a given gonococcal or meningococcal extract component. If all three components are present in an extract there will be three zones of inhibition. When one or more of these three components are not present as occurs with some meningococcal strains and other gram negative diplococci or rods, the corresponding zone of inhibition will also be absent.

In contrast with the gonococcal strains which respond uniformly to the three toxic components, the meningococci show variation in their response (Fig. 4). This variation which is expressed as absence of an expected zone of inhibition or a diminished zone of inhibition is probably due to strain variation in their sensitivity to specific lipid components. This variation may also reflect quantitative differences in the levels of specific lipid components present in the strains used to prepare lipid extracts.

Summarizing our TLC and bioautographic data on the lipid extracts of N. gonorrhoeae and N. meningitidis we find that the extracts are similar qualitatively. Chromatographically they contain nine components three of which, FFA, PC and a third unidentified component, inhibit the growth of both N. gonorrhoeae and N. meningitidis. This third lipid component reacts with ninhydrin but does not react with phospholipid reagent and may be a glycolipid containing a hexoseamine. We have also found that authentic LPC is inhibitory. While our extracts contain a component chromatographically similar to LPC, it is neither inhibitory

FIG. 4a

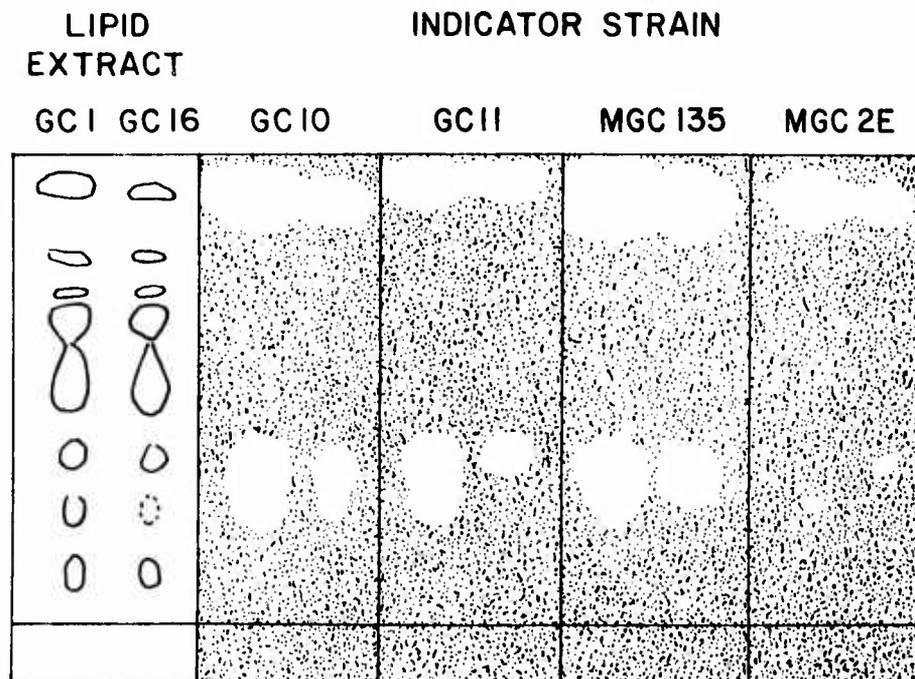


FIG. 4b

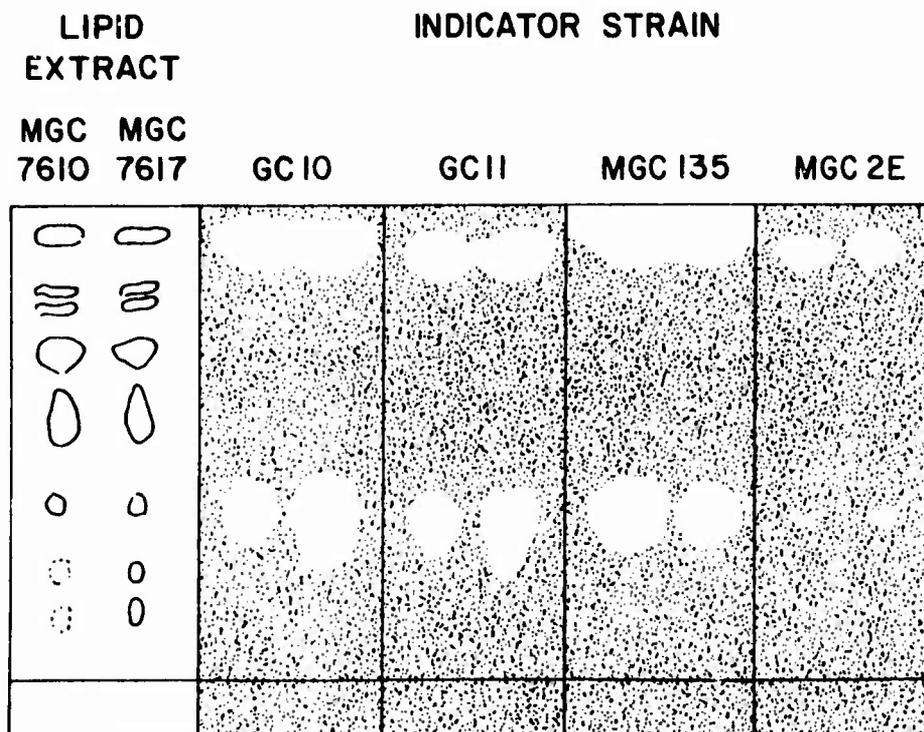


Fig. 4. Detection of inhibitory activity of components in lipid extracts of N. gonorrhoeae and N. meningitidis by bioautography. TLC of lipid extracts done as described in Fig. 2. Replicate developed chromatograms overlaid with semi-solid agar suspensions of N. gonorrhoeae indicator strains 10 or 11 and N. meningitidis indicator strains 7610 and 7617. After overnight incubation, bioautographs treated with 0.1% triphenyltetrazolium chloride to visualize zones of inhibition.

4a. Extracts of N. gonorrhoeae strains 1 and 16.

4b. Extracts of N. meningitidis strains 7610 and 7617.

nor does it react with a phospholipid specific reagent. Walstad, et al. [11], pursuing a course directly parallel to ours, recently reported that their lipid extracts of N. gonorrhoeae contain two inhibitory components, FFA and LPE, and that an extract of one strain of N. meningitidis contains FFA but lacks LPS. Differences between our data and those of Walstad, et al. could be ascribed to differences in the methods of lipid extraction, chromatography and finally in the biologic assay system for detection of inhibitory activity.

We have tested a large number of saturated and unsaturated fatty acids and some neutral lipids were tested for toxicity against pathogenic Neisseria. Saturated fatty acids from 8 to 24 carbon atoms and unsaturated fatty acids from 14 to 24 carbon atoms in length were tested in varying concentrations. With the exception of the C9 FA, all saturated FA from C8 to C15 were toxic to two strains of N. gonorrhoeae; toxicity increasing with increasing chain length. Unsaturated fatty acids of 14, 16 and 18C in length were toxic for N. gonorrhoeae. While the pattern of sensitivity of one strain of N. meningitidis was the same as N. gonorrhoeae, this strain was in each case less sensitive.

We have also tested some readily available sterols for activity against N. gonorrhoeae and N. meningitidis, using the method just described. We found that at high concentration deoxycholate and hyodeoxycholate are markedly inhibitory, that cholesterol and lanosterol show low level variable inhibition and ergosterol is non-inhibitory.

Differential sensitivity of gonococci to toxic lipids does not appear feasible as a typing scheme for gonococci. The use of these lipid compounds, however, as potential prophylactic agents is currently under investigation, as are a variety of other lipids.

#### C. Clinical correlates of bactericidal strain differentiation of Neisseria gonorrhoeae.

In the previous Annual Report a system utilizing the serum bactericidal assay was described in which strains of gonococci could be distinguished by patterns of killing [12]. We have used this test to study certain circumscribed aspects of gonococcal epidemiology concerning the transmission between consorts, reinfection and treatment failures.

#### Methods.

Gonococcal strains. Strains of N. gonorrhoeae used were freshly isolated from patients at Walter Reed Army Medical Center, Indianapolis-Marian County VD Clinic, or kindly supplied by Dr. Douglas Kellogg, Center for Disease Control, Atlanta, Ga. All strains of N. meningitidis were from the collection of the Department of Bacterial Diseases, Walter

Reed Army Institute of Research. Gonococcal colonial types T3 and T4 were used since previous studies had shown that colonial type had no effect on bactericidal activity of rabbit antisera [13].

Antisera. The antisera used were previously described [12]. For simplicity the meningococcal antisera were designated as shown in Table 7.

Bactericidal test. The microbactericidal test used was as previously described [13].

### Results.

Gonococcal strains were examined from three pairs of consorts on whom detailed case histories were available. (Initials used to identify patients are fictitious and are used only to facilitate the description of results.) Organisms isolated from two pairs were found to be the same (Table 8) (strains GC152 and GC154, strains GC198 and GC199) and those from one pair (strains GC129 and GC130) appeared to be different. Strain GC129 was recovered from a male patient (FG) who had been away for one year. However, his sex partner (LG), who denied other contacts, revealed that her symptoms (pelvic pain and fever) actually had begun on the very day that they had renewed relations.

Another set of consort strains on which epidemiological and clinical histories were not as complete were also studied (Table 9). Patient ST was infected with two different strains five months apart. His consorts, CM and JT, were infected with antigenically similar strains during his second and third episodes. Although all three patients were treated appropriately with penicillin and spectinomycin at the time of each positive culture and ST had a negative follow-up culture on 2021074, CM on 2-26-74 and JT on 4-5-74, detailed contact histories and culture results on another unlisted female consort were not available and we can only speculate as to whether these results represent a relapse or reinfection with the same strain. The organisms were all sensitive to 0.12 units/ml of penicillin.

Of the remaining three groups of consorts tested, two groups of strains showed identity (SN, WN, CK; XM, CS). Patients KL and CT were infected with different strains in June but CT became infected with KL's strain in October. Of interest is the fact that KL's strain (Ig210) was isolated from the nasopharynx in June.

Gonococci isolated at different times were available from two patients with detailed treatment records (Table 9). One patient had been treated with 4.8 million units of aqueous procaine penicillin at the time of the first culture and was negative for N. gonorrhoeae on follow-up cultures of the cervix and rectum. She returned six weeks later with recurrent symptoms and N. gonorrhoeae was again isolated.

Table 7. Meningococcal antisera used to differentiate gonococci.

Antisera	Designation
32/60-118 <sup>(1)</sup>	A
32	B
60/35	C
60/138	D
60	E
118/60	F
118/89	G
118/126-138	H
118	I
6557/6586	J
6557	K
126/60	L
126/118	M
126	N
138/60	O
138	P
35/60	Q
35	R
89/118	S
89	T

(1) 32/60-118 = antiserum raised to strain No. 32 absorbed with organisms 60 and 118.

Table 8. Bactericidal typing of consort strains.

Gonococcal strains	Patient/Sex	Date isolated	Antisera (Set IV-1)																			
			A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
GC152	CH M	7-31-72	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GC154	VH F	8- 2-72	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GC198	RJ M	12-11-72	-	-	±	+	+	+	+	ND*	-	-	-	-	-	-	-	-	-	-	-	+
GC199	VJ F	12-11-72	-	-	±	+	+	+	+	ND	-	-	-	-	-	-	-	-	-	-	-	+
GC129	LG M	5-15-72	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	±	+	+	+
GC130	FG F	5-16-72	-	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Ig349	AR M	9-17-73	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig343	CM F	9-18-73	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig357	JT F	9-19-73	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±
Ig484	ST	2-12-74	-	-	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig492	CM	2-19-74	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig527	ST	3-25-74	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig530	CM	3-26-74	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig533	JT	3-27-74	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig545	ST	4-19-74	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig550	CM	4-23-74	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig553	JT	4-25-74	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig212	SN M	7- 2-73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ig215	WM F	7- 2-73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ig216	CK F	7- 2-73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ig158	XM M	5- 1-73	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig161	CS F	5- 1-73	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig200	CT F	6-26-73	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ig210	KL M	6-29-73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ig368	CT	10-17-73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

\* ND = Not Done

Table 9. Bactericidal typing of strains from the same patient isolated at different times.

Gonococcal strains	Date	Reactions with Antisera (Set IV-1)																			
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
GC187	11- 3-72	-	-	±	+	+	-	+	-	-	+	+	-	+	+	+	+	+	+	-	+
GC197	12- 7-72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±
GC170	8-23-72	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
GC180	10- 2-72	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

When these strains (GC187 and GC197) were examined in the bactericidal system they were found to be different (Table 9). The patient had had several sexual contacts with a different partner during the interval between the two illnesses.

The second patient (GC170) was treated similarly but an intra-uterine device (IUD) was left in place. She did not return for follow-up examinations. Although she became afebrile and felt well she continued to have a slight vaginal discharge. She returned five weeks later when her husband developed acute gonococcal urethritis. At this time she was culture positive (GC180). Both strains (GC170 and GC180) were identical by the bactericidal assay (Table 9). The clinical histories of JT and CT were already discussed.

In order to determine whether gonococci recovered from different parts of the body might be dissimilar, isolates from various sites obtained during a single illness in five patients were examined. Matching patterns were observed in each case.

#### Discussion.

In the present study the ability of meningococcal antisera to distinguish antigenic differences among gonococci [12] was used to examine relatedness of gonococcal isolates. By using the standard epidemiologic method of obtaining contact histories and cultures, strains within sets met criteria which made them likely to be identical. Bactericidal patterns of individual strains of seven sets were the same and in three sets were not (Tables 8 and 9, GC129 and GC130, GC187 and GC197; Ig200 and Ig210). The differences were also shown by antibiotic sensitivity testing. Furthermore, clinical and epidemiologic findings in two of these three sets of patients corroborated the differences in strains which were determined by laboratory differentiation.

Patients ST, JT, CM, CT (Table 8) and GC187 (Table 9) were each infected with different gonococcal organisms at different times. These findings support the clinical experience that recurrent infections with different gonococcal strains occur. In contrast, the patient represented by GC170 and GC180 (Table 9) appears to be a treatment failure (perhaps due to the fact that an IUD was not removed).

Unfortunately, complete information concerning ST's second episode (Table 9, strains Ig484-553) was not available for us to be entirely confident of what took place. The fact that each patient had at least one negative follow-up culture between episodes suggests that re-infection with the same strain occurred. Nevertheless, certain questions concerning immunity to gonorrhea are raised. Three possibilities exist: 1) immunity does not develop; 2) immunity is short lived and 3) immunity is directed against a narrow set of antigens not shared by many organisms. It would seem unlikely that culturing of all potential sites of all three

patients would have been inadequate to pick up persistent infection. Although possible, it would also seem unlikely that many antigenic differences might be missed by the bactericidal assay. Thus, these results suggest that either immunity did not develop or was extremely short lived.

Isolates from different parts of the body taken during the same illness all had the same bactericidal pattern. Further studies must be done to determine whether simultaneous infections with multiple strains occur.

D. A tissue culture model of *Neisseria gonorrhoeae* infection.

Work has continued on the use of infected tissue cultures in the study of the pathogenesis of gonococcal infections. In the previous Annual Report it was reported that the gonococci enter tissue culture cells and that this is an active phagocytic process which can be blocked with a microfilament binding substance, Cytochalasin-B. This may be of importance because histologic studies by others [3] have demonstrated that gonococci in human infection are taken up by epithelial cells, pass through the epithelium and then set up micro abscesses in sub-epithelial areas. Thus the uptake of gonococci by tissue culture cells may reflect the means by which these pathogens invade a host.

During the present reporting period it has been determined that the gonococci phagocytized by baby hamster kidney (BHK) cells remain viable and are protected while intracellular from the bactericidal effects of immune serum. It has also been determined that the bacterial factor responsible for phagocytosis in this model can be stabilized and possibly isolated for further study.

Methods.

The tissue culture cells used, BHK-21 cells, were passed in continuous culture in Medium 199 plus 10 percent fetal calf serum. Gonococci were grown on Difco GC agar.

In the assay of the viability of intracellular bacteria, BHK cells were first infected with gonococci for six hours and then treated with three changes of rabbit antisera, plus a source of fresh rabbit complement, in order to lyse bacteria on the surface of cells. The monolayer was then washed and trypsinized. Serial dilutions of the cell suspensions were plated on GC agar and colony counts were made.

In the other assays bacteria phagocytized by the tissue culture cells were identified and differentiated from extracellular bacteria by a method involving tagging the extracellular bacteria with peroxidase conjugated antigonococcal antibodies as previously described in the Annual Report.

## Results and Conclusions.

The data from the viability studies is represented in Table 10. When antisera was not used to kill extracellular bacteria approximately equal numbers of colony forming units were recovered from untreated and cytochalasin treated tissue cultures. When rabbit antisera was added to the untreated monolayer almost 10,000 bacteria per ml. remained viable. On the other hand, when the rabbit antisera was added to the cytochalasin-B treated tissue culture almost no bacteria remained viable. The low counts of viable bacteria in the cytochalasin-B and rabbit antisera treated cultures suggests that the antisera killed virtually all extracellular bacteria. If this is true, then the viable bacteria recovered from the tissue cultures treated with antibody alone must have been protected from the antisera by being intracellular.

Preliminary data has been generated on the factor which allows the gonococcus to penetrate tissue culture cells. Normal bacteria and bacteria fixed with a formalin-glutaraldehyde solution both adhere to the surface of the tissue culture cells and are phagocytized by the cells. Dead or alcohol fixed gonococci still adhere to the tissue culture cells but are no longer phagocytized by them, suggesting that the substance mediating phagocytosis is somewhat labile and sensitive to alcohol.

Because of parallels drawn above it is felt that the phagocytosis of the gonococci by the tissue culture cells represents one of the first steps in the invasion of a host by these bacteria. This model may, therefore, lend itself to isolating the factor which mediates this phagocytosis. If this substance in turn is stabilized by aldehyde fixation it may be useful in producing immunity against gonococcal infection. Efforts are being made to extract this material from the bacteria and identify it.

### E. Attachment of *Neisseria gonorrhoeae* to human buccal epithelial cells as a model for local immune studies.

Introduction. There is evidence that a local antibody response develops to *N. gonorrhoeae* [12,14]. However, the function of this antibody is not known. Since the initial event in an infection is implantation on mucosal cells of the offending agent the ability of local secretions to inhibit the implantation of gonococci to epithelial (buccal) cells was studied.

### Methods and Materials.

*N. gonorrhoeae*: Gonococcal strains were freshly isolated from patients at WRAMC, Ft. Benning and Korea. They were maintained frozen at -70°C or in a lyophilized state until used. All strains were verified to be *N. gonorrhoeae* by gram stain, oxidase reaction and sugar fermentations.

Table 10. Viability of intracellular gonococci in BHK-21 cells.

Antiserum	Colonies per ml of trypsinized cells	
	Untreated tissue culture	Cytochalasin-B treated <sup>a</sup> tissue culture
None	22x10 <sup>6</sup> <sup>b</sup> (0.8-45x10 <sup>6</sup> )	7.0x10 <sup>6</sup> (0.2-15x10 <sup>6</sup> )
Rabbit anti-GC <sup>c</sup>	9.4x10 <sup>4</sup> (1.4-28x10 <sup>4</sup> )	36 (0-200)

<sup>a</sup> Cytochalasin-B 0.1 mg% added to tissue culture with inoculum of bacteria.

<sup>b</sup> Each number represents the average of four tissue culture wells for each of two separate trials. The ranges are in parenthesis.

<sup>c</sup> Ten percent rabbit hyperimmune serum plus 10% fresh or fresh frozen normal rabbit serum.

Epithelial adhesion: Human buccal epithelial cells were scrapped with a wooden applicator and suspended in PBS pH 8.0 and washed x 2. The buccal cells were enumerated in a hemocytometer and adjusted with Medium 199 (Microbiological Associates, Bethesda, Md.) to  $2 \times 10^5$  cells/ml. Unless otherwise stated equal volumes (0.025 ml.) of buccal cells and gonococci (0.025 ml.) were adjusted in Medium 199 to be at a 50:1 ratio of organism/epithelial cell and incubated at  $37^\circ\text{C}$  x 30 min. on a shaker apparatus. Pooled hyperimmune rabbit antisera (Annual Report, 1974) conjugated with horse radish peroxidase were then used to identify the gonococci by incubating 0.1 ml. of an appropriate dilution of antiserum x 30 min. on a shaker apparatus. The cells were washed in normal saline, centrifuged, resuspended in 0.1 ml. of NaCl, fixed with 95 percent ethyl alcohol x 10, dried onto slides and overlaid with 3'-3'-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, Mo.) in .1M Tris in 50 percent ethanol, pH 7.4. The number of buccal cells with organisms were recorded.

Inhibition of adhesion (EAI): EAI was carried out by mixing an equal volume of antiserum or sections (0.05 ml.) and the reaction mixture of buccal cells and gonococci (final dilution of serum or secretion is 1:2). A  $\geq 50$  percent reduction was considered significant.

### Results.

Previous studies revealed that rabbit antisera and human genital secretions were capable of blocking buccal cell adhesion, and that this property was directed to the greatest extent against the homologous strain [12]. However, the system was based upon the number of bacteria attached per cell and was found not to be as sensitive as needed for detecting local antibody activity. Therefore, the test system was modified.

### Standardization of EA

1. Organism/buccal cell ration: In order to develop a test as sensitive as possible for determining antibody activity the smallest ratio of organisms to epithelial cells which resulted in an adequate number of cells with at least one GC attached was determined (Table 11). A ratio of 50 organisms/epithelial cell was subsequently used.

2. Buffer, diluents: A variety of buffers and diluents were tested in order to determine those which would allow the greatest "stickiness" of *N. gonorrhoeae*. Little difference was noted in pH (6.0-8.0) or diluent (199 with and without BSA)(0.1%-4%). However, a difference was noted when Media 199 from different manufacturers was used. When the secretions were concentrated by lyophilization the tonicity of the secretions became an important variable.

Table 11. Percentage of cells with organisms attached at various organism/cell ratios.

500:1	100:1	50:1	10:1	1:1	0.5:1
98%	80%	80%	52%	35%	16%

Adhesiveness was adversely affected with increased osmolality that was greater than approximately 2x normal saline and 3 percent sucrose. (Table 12).

Table 12. Effect of osmolality on adhesiveness of N. gonorrhoeae.

NaCl or sucrose concentration	% cells with GC attached	
	NaCl	Sucrose
Normal NaCl (0.9%)	41	-
1%	40	38
1.5%	32	-
2.0%	38	40
2.5%	16	-
3%	8	38
4%	8	20
4.5%	4	-
5%	-	18
7%	-	12

3. Buccal cell variation: The percentage of buccal cells having organisms attached varied depending on the donor (Table 13). Buccal cells could be saved in PBS and used up through five days.

Table 13. Variations in buccal cell adhesion<sup>1</sup>.

Donor	Day			
	1	5	7	12
CW	30 <sup>2</sup>	16	24	-
JC	28	10	-	-
LT	24	-	-	-
ET	18	22	20	48
JB	18	14	22	-
BB	10	12	6	-
S	20	28	14	20
AD	2	20	14	34
LS	8	-	22	-
DR	14	16	20	20
RM	-	10	26	-
HD	-	10	16	-

<sup>1</sup> Percentage cells with GC attached.

<sup>2</sup> Different organisms used each day.

4. Gonococcal variation: Organisms picked and transferred from a simple colony also showed variation in attachment (Table 14) despite the fact that they were all of the same colony.

5. Statistics: Because of the day to day variations in the percentage cells with organisms attached a formula was derived to determine the number of cells to count in order that a 50 percent reduction would be significant at a  $P \geq 0.5$  level. The minimal number of cells counted was 50.

Table 14. Variation in ability of T<sub>1</sub> colonies<sup>1</sup>  
to attach to buccal cells<sup>2</sup>.

Colony	% attachment
#1	2
2	14
3	46
4	26
5	30
6	30
i	24

<sup>1</sup>Transferred from one colony.

<sup>2</sup>Same buccal cell source.

#### Blocking of epithelial cell adhesion.

Preliminary studies with vaginal or urethral secretions which were not adjusted before testing to a standard amount of S-IgA concentration resulted in blocking of epithelial cell adhesion of the homologous organisms (Table 15). Percentages refer to the amount of globulin present with regards to the S-IgA standard.

Standardization of vaginal secretions from patient 418 to 50% ± 10% of S-IgA (11S) standard (0.02 mg/ml IgA) was then carried out. Blocking titers over a three week period remained essentially unchanged (Table 15). Results vs. two heterologous organisms and the IgG levels are also shown.

#### Summary:

A sensitive, standardized, reproducible test for measuring blocking of epithelial cell adhesion has been developed. Local secretions are capable of blocking epithelial cell adhesion of gonococci. The class of immunoglobulins responsible for this activity and the gonococcal antigen(s) which mediate this effect will be determined.

Table 15. Blocking EA by vaginal and urethral sections.

Patient	Date	Organism					
		149	400	319	373	418	101
149 <sup>1</sup>		1:4					
400 <sup>1</sup>			1:8 (95%) <sup>3</sup>				
319 <sup>1</sup>				1:2 (<20%) <sup>3</sup>			
373 <sup>1</sup>					1:2 (43%) <sup>3</sup>		
418 <sup>2</sup>	1-29-75			<1:4		1:64	1:64
	1-31					1:64	
	2-2					1:32	
	2-4					1:64	
	2-6					1:32	
	2-8					1:64	
	2-19			1:2		1:32	
	3-4					1:64	
	3-2					1:128	

<sup>1</sup> Not standardized to amount of S-IgA present before testing.

<sup>2</sup> Standardized to 50% of 11S standard (.04 mg/ml IgA).

<sup>3</sup> Percent of S-IgA standard.

F. Antibiotic sensitivities of *Neisseria gonorrhoeae*, Korean strains.

There has been documented increasing resistance of *N. gonorrhoeae* to penicillin over the past 20 years. This has been a particularly worrisome aspect in Korea where treatment failures have been reported for many years. To determine what role an increased resistance to antibiotics might be playing in this problem, the minimal inhibitory concentrations of 69 strains of *N. gonorrhoeae* from Korea were determined.

Methods and Material.

**Gonococcal strains.** Sixty-nine gonococcal strains were isolated in the Republic of South Korea and lyophilized during the time interval April 1974-October 1974. Before testing, all of the isolates were tested for fermentation of glucose, maltose, sucrose and lactose, were gram stained and tested for oxidase production. Eleven American isolates were also tested.

**Antimicrobial sensitivity testing.** A modified agar plate dilution method was employed (Annual Report 1974). The currently recommended antibiotics were tested: penicillin, ampicillin, tetracycline and spectinomycin.

Results.

There was an increased resistance to penicillin, ampicillin and tetracycline of the Korean strains as compared to the U. S. strains (Table 16). The difference is much less marked for spectinomycin.

There is an obvious straight line correlation between penicillin and tetracycline sensitivities. The same does not hold true between spectinomycin and penicillin, ampicillin and tetracycline.

Comments.

Except for spectinomycin the Korean strains were more resistant to the recommended antibiotics than the U. S. strains. This appears to be a plausible explanation for treatment failures with the currently recommended treatment schedules. Whether the relatively high sensitivity to spectinomycin is because it is a newer drug is not known. Thus, the possibility of increased resistance over time is not a mute point and the question of recommending the use of spectinomycin for treatment failures only must be weighed carefully.

Table 16. Minimum inhibitory concentration (MIC) ( $\mu\text{g/ml}$ ) of four antibiotics for Neisseria gonorrhoeae.

Antibiotic	0.025	.05	.1	.2	.4	.8	1.6	3.2	6.4
<b>Ampicillin</b>									
Korean	1	2	12	47	6				
U. S.	3	2	4	1					
<b>Tetracycline</b>									
Korean			2	1	8	19	38		
U. S.			6	2	2	1			
	.0031	.0072	.0156	.0312	.0625	.125	.25	.5	1 2
<b>Penicillin</b>									
Korean			1		1	7	4	8	40 6
U. S.			1	3	2	2	2	1	
	1	2	4	8	10	12	14	16	32
<b>Spectinomycin</b>									
Korean			2	28	27	11	1		
U. S.			10	1					

## II. Hospital Infections

Hospital acquired infections have become a major problem resulting in increased morbidity, mortality and costing been 30-40 million dollars per year [15]. The methods and resources to control these problems have been known for many years [16]. Nevertheless, there exists two major stumbling blocks, 1) the intransigence of human nature to admit that such problems exist on one hand and 2) the overreaction to certain situations, i.e. closing down a nursery because of "staphylococcus", always assuming it to be a point source outbreak.

For the past nine months we have attempted to coordinate the laboratory findings with the clinical situation in an effort to develop rational approaches to solve these problems.

### A. Hospital Epidemiology.

#### 1. Major outbreaks

a. A point source outbreak of staphylococcal infection at Walter Reed Army Medical Center.

#### Problem.

On 21 October 1974 there were two blood cultures isolated from neonates in the nursery which were positive for Staphylococcus aureus. One of the neonates had toxic epidermal necrolysis. The second child had a faint erythematous rash on the third day of life.

The rate of acquisition of S. aureus in the nursery has been monitored since February 1974. While there is often an increase in acquisition rates before a staphylococcal outbreak, none was noted in our nursery. This suggested the introduction of a virulent strain and not an increase in the rate of cross-infection. This was verified when both isolates were of the same bacteriophage type.

#### Methods and Materials.

All personnel associated with Ward 16 patients were cultured nasally for S. aureus on blood agar and mannitol salt agar. Similarly, all neonates in the nursery continued to have nasopharyngeal (NP) and umbilical cultures for staphylococci. Extensive environmental cultures were also taken. All cultures positive for staphylococci were held for bacteriophage typing along with the isoaltes from the index cases.

Bacteriophage typing was carried out as previously described [17]. Medical charts were reviewed as indicated.

### Results.

Of the 59 personnel who had contact with the patients on Ward 16, 57 were cultured and 11 had S. aureus on NP cultures. Six environmental cultures, which included the seam of the mothers' beds and the circumcision board, were also positive. One other neonate was also positive in this survey. Phage typing of the staphylococci revealed that the index cases, a third infant and one house officer all had a similar phage type, 3a/71-3c,70. This implicated the house officer as the carrier of the virulent phage type. Further chart review showed that he had been intimately involved in the care of the three children. The circumcision board, both physicians performing the circumcisions, beds, sheets and floors on Ward 16, and other personnel were either negative for staphylococci or were negative for that phage type.

The implicated house officer was then put on leave from the hospital. He was instructed to bathe twice daily with a hexachlorophene soap and took cephalixin for 10 days. He remained out of the hospital for one week more to allow recolonization with another (less virulent) strain of staphylococcus; however, upon reculture his nasopharynx was negative for staphylococcus.

### Discussion.

This episode is important because it demonstrated that while hospital personnel carry staphylococcal organisms, it is not until a virulent strain disseminates that a problem actually exists. This particular phage type, 3a/71-3c,70, is one such virulent phage type and has been associated with toxic epidermal necrolysis (Ritter's disease) [18]. This was seen in one of our patients.

This outbreak also demonstrates that while airborne spread is recognized, hand to hand spread or close personal contact is the most common means of staphylococcal dissemination in the nursery. Fomite spread is also less important in staphylococcal spread in the nursery [19]. These findings again reemphasize the need for scrupulous hand washing techniques.

#### b. Escherichia coli 0125:B15 outbreak.

### Introduction.

E. coli 0125:B15, a strain which has been associated with diarrhea in neonates [20], was isolated from the nursery on Ward 16 and later on Ward 7. The spread to Ward 7 was believed to have occurred following transfer of an index case from Ward 16 to Ward 7. There had been little diarrheal illness and no child had required therapy for diarrhea due to this particular strain of E. coli.

### Methods.

All neonates on Wards 16 and 7 nurseries had rectal cultures on MacConkey's media for the isolation of E. coli. Similarly all staff members working on these two floors were cultured nasally and rectally for E. coli.

Enterotoxin production was tested for by rabbit ileal loop studies [21].

### Results.

Nasal and rectal cultures were done on 82 of 93 personnel on both floors. No E. coli was found in 121 cultures of 103 patients and personnel; nonenteropathogenic E. coli (EPEC) in 76 cultures of 74 patients and personnel and EPEC of varying serotypes in 45 instances of 39 patients and personnel (six patients had two different serotypes). The EPEC serotypes isolated were:

0125:B15	-	17 isolates	086:B7	-	7 isolates
0126:B	-	2	0112:B11	-	1
019:B14	-	1	0127:B8	-	1
Poly C	-	16			

A total of 121 E. coli were serotyped.

Five staff members on Wards 7 and 16 were identified as carriers of E. coli 0125:B15. They were subsequently treated with oral antimicrobials and all became negative for this serotype on reculture. No evidence of toxin production was found.

### Recommendations.

The spread of E. coli 0125:B15 most likely resulted from a breakdown in good technique. Hand washing before and after the handling of infants was stressed. The use of disposable gloves was also recommended.

Furthermore, we suggested that neonates transferred from the Ward 7 nursery to the Ward 16 nursery not be sent back to Ward 7 in view of the substantial risk of introducing a virulent organism from the more heavily transversed Ward 16 nursery with its ill patients to the Ward 7 well-baby nursery.

### Discussion.

The association of certain strains of E. coli with infantile diarrhea was made over 50 years ago [22]. Solomon et al. have observed, however, that the frequency of the asymptomatic carrier state of pathogenic E. coli makes it impossible to attach causal significance to the

isolation of the strains of these organisms alone [23]. In the outbreak of E. coli 0125:B15 isolations described above it is doubtful that this particular strain was toxigenic. Little clinical disease was observed and rabbit ileal loop studies were negative. However, it did serve notice that had a more pathogenic organism been present in our nursery under similar conditions, the level of nursery technique would have been inadequate to limit widespread morbidity.

## 2. Urinary Catheter Study

### Introduction.

Estimates are that 1.5 million nosocomial infections occur annually in the United States [15]. In one hospital alone there was an excess stay of 21 days per patient involving 2009 patients who had nosocomial infections. The cost of nosocomial infection for that hospital was six million dollars in one year [24].

Urinary tract infections (UTI) are the most common nosocomial infection, comprising 40 percent of all such infections [25]. While urinary catheters are the single most important factor in nosocomial UTI, it has been well shown that careful management of catheters can result in a substantial decrease in nosocomial UTI in the gram negative sepsis that often follows [26].

In January 1975 we began a three month study of urinary catheter use at WRAMC. The purpose was to define the incidence of catheter-induced infection and the clinical setting in which this was likely to occur.

### Methods.

Patient selection. All patients having an indwelling urinary catheter or having an in-and-out catheterization (straight catheterization) were identified either by questioning the head nurse on each floor or by examining the orders or nurses' notes on a daily basis. Patients were admitted to the study once catheterized and were followed through discharge from the hospital.

Data processing. Data was collected on 3x5 cards for each patient and transcribed onto computer program sheets at the time of discharge. Transcription of data was done with the aid of a code.

The program used to analyze the hospital infection data is a Fortran program written for CDC 3500, operating under Master 3.4. The data is stored as a desk file in 80 column card image. Data is stored in 47 fields in one, two or three digit numeric codes. Several generalized search capabilities are available to provide for analyses of the results:

1. A tabulation of the information stored in any one field for all patients.
2. A tabulation of selected fields for a selected group of patients. Selection of the group of patients is accomplished by specifying codes which meet the criteria. Up to 24 acceptable codes may be specific and up to nine fields may be connected with a Binary "and".
3. A tabulation of the duration of catheterization for selected groups of patients.

### Results.

This study began 6 January 1975 and new patients were admitted to the study through 31 March 1975. Thereafter patients were followed until discharge. The data are presently being analyzed in the computer; however, a trial of the computer program was run on the data accumulated through 28 February 1975. The following are some of these data:

There were 153 patients (65 male, 88 female). Sixty-five patients (42 percent) had bacteriuria.

Attack rate by service. General medicine 12/24 (50 percent), general surgery 7/19 (37 percent), obstetrics 5/18 (28 percent), gynecology 15/33 (45 percent) and urology 9/29 (31 percent).

Prophylactic antimicrobials: 36/87 patients on prophylactic antimicrobials had bacteriuria.

Length of hospitalization: Of catheterized patients those with no bacteriuria stayed an average of 14 days and those with bacteriuria stayed 21 days.

Straight catheterization. Twenty-seven percent (4/15) of patients catheterized subsequently acquired bacteriuria. All were women.

Prognosis and outcome: 26/55 patients with rapidly or ultimately fatal disease had bacteriuria but none died with his infection as the primary cause of death. 39/97 patients with nonfatal disease had bacteriuria and one patient died of his infection.

### Discussion.

If the above trends in the data are maintained, one may conclude that the incidence of infection associated with the use of the urinary catheter at WRAMC will approximate 40 percent. While this incidence is similar to those now being reported in other hospitals [25], Garibaldi, et al.[26] stress that this is far above the 10 percent incidence of infection which has been reported with scrupulous attention to catheter care. They demonstrated in their hospital

that the excess incidence of infection was due in large to poor catheter care. Sixty percent of our catheters were handled in an aseptic fashion and 17 percent had multiple breaks in sterile technique detected.

The 27 percent incidence of infection following straight catheterization far exceeds the reported 1-5 percent rate reported in other series [28,31].

The cost of urinary catheter associated infections may be calculated by the cost of hospitalization for seven extra days for each patient, or seen in the death from infection of one patient who entered the hospital with a nonfatal disease.

In the absence of demonstrable efficacy of prophylactic antimicrobials in this study the only means of reducing costs incidental to urinary catheterization is through judicious use and improved catheter care.

We plan to use these data to support efforts at improving catheter care in the hospital through in-service educational programs.

#### B. Cephapirin/Cephalothin Study

##### Introduction.

Cephapirin is a new cephalosporin whose activity is comparable to cephalothin [27], however, its cost is considerably less. Phlebitis is a common and troublesome side effect of intravenous cephalothin therapy. Consequently, if the incidence of phlebitis with cephapirin were significantly less, as has been claimed [28,29], then we would recommend that the pharmacy replace cephalothin with cephapirin. We decided to study whether there is a difference in the incidence of phlebitis when cephapirin is used by ward physicians.

##### Method.

Patients receiving cephapirin or cephalothin were identified by the pharmacy. These patients were then seen daily by Infectious Disease Service physicians. The drug preparation that the patient was receiving was not known until after the patient was taken off therapy. The dose of antimicrobial was not controlled since the purpose was to study the drugs as they were being used in the hospital by ward physicians.

The degree of phlebitis was recorded as follows:

- 0 - no erythema or tenderness
- 1+ - erythema and/or mild tenderness extending along the vein less than 2.5 cm above the infusion site.
- 2+ - erythema and/or mild tenderness extending along the vein more than 2.5 cm above the infusion site.
- 3+ - erythema and/or severe tenderness extending along the vein greater than 2.5 cm.

The age of the patient, length of time of IV infusion, whether a scalp vein or IV catheter was used and whether other medications were infused through the IV were also noted.

#### Results.

The data are summarized in Tables 17 and 18. Seventy-seven patients received cephalothin and 43 patients received cephalpirin by the intravenous route. There was no significant difference in the incidence of reactions. Both groups of patients had a high rate of phlebitis associated with cephalosporin use.

Cephalothin therapy did, however, result in a greater severity of phlebitis. Twenty-three percent of those receiving cephalothin had 2+ and 3+ reactions as opposed to 12 percent receiving cephalpirin.

The likelihood of reactions to both drugs increased with increasing duration of catheterization, however, this was probably not true of the severity of reaction although the numbers are too small for statistical analysis.

#### Discussion.

Cephalpirin sodium is a cephalosporin for parenteral use that has a spectrum and an activity equivalent to those for cephalothin [29]. The dosage, blood levels, MIC's and excretion patterns are also similar [27]. Cephalpirin has been offered as a less expensive alternative to cephalosporin as well as a better tolerated cephalosporin preparation in terms of phlebitis [29,30].

There are conflicting data on the incidence of phlebitis with cephalpirin, however, most studies do agree that it is as efficacious as cephalothin. Quintiliani, et al. gave cephalpirin intravenously to 22 patients with a wide spectrum of infectious disease and found no phlebitis and no gross organ toxicity from the drug [27]. In a single blinded study of 10 healthy subjects cephalothin caused phlebitis more

Table 17. Incidence of phlebitis with cephalothin.

Days	Degree of phlebitis				Total no. reactions/No. pts.	2+, 3+ phlebitis/No. pts.
	0	1+ (Pain)	2+	3+		
1	16	6	3	2	11/27	5/27
2	12	7	0	5	12/24	5/24
3	4	3	3	3	9/13	6/13
4	3	6	1	1	8/11	2/11
>4		2			2/2	0/2
<b>Total</b>	<b>35</b>	<b>24</b>	<b>7</b>	<b>11</b>	<b>42/77 (55%)</b>	<b>18/77 (23%)</b>

Table 18. Incidence of phlebitis with cephalirin.

Days	Degree of phlebitis				Total no. reactions/No. pts.	2+ phlebitis/No. pts.
	0	1+ (Pain)	2+	3+		
1	15	8	0	0	8/23	0/23
2	4	5	1	0	6/10	1/10
3	3	0	1	1	2/5	2/5
4	0	2	1	1	4/4	2/4
>4		1			1/1	0/1
<b>Total</b>	<b>22</b>	<b>16</b>	<b>3</b>	<b>2</b>	<b>21/43 (50%)</b>	<b>5/43 (12%)</b>

frequently and more severely than cephalosporins [30]. These findings were confirmed in other studies [27].

A double-blind study of 20 patients found no difference in either the incidence or the degree of phlebitis with the two cephalosporins [31]. Inagaki and Bodey reported that while the overall incidence of phlebitis was similar for both drugs, severe phlebitis occurred twice as often in the cephalothin treated group [28].

Our study is in agreement with those of Lane, et al. [30] and Inagaki and Bodey [28]. The overall incidence of phlebitis was similar in both groups, however, unlike Lane, et al., we did find that patients treated with cephalosporins had less severe reactions but the difference was not very striking.

We conclude that while the two antimicrobials are similar in efficacy, and while cephalosporins are less expensive and perhaps cause less severe episodes of phlebitis, cephalothin should remain in our formulary. The differences were not that great to forego the wide experience and extensive literature that already exists for cephalothin. Unless further investigation reveals a new property of the drug, cephalosporins will not be in our formulary.

### C. Pseudomonas aeruginosa Epidemiology

#### Introduction.

Each year for the past five years P. aeruginosa isolates have been collected from clinical specimens submitted to the microbiology laboratory. The purpose of the collection was to study the epidemiology of P. aeruginosa at WRAMC by correlating the bacteriology of the organisms with the clinical course of the patient from whom it was isolated.

Results of the pyocin typing, serotyping and antibiotic sensitivity testing of the organisms from the first three collection periods have been reported [32,33]. In order to examine those factors that may be important in the resistance of P. aeruginosa to gentamicin, tobramycin and carbenicillin, the charts of all patients from whom a resistant organism was isolated were reviewed.

#### Methods.

The methods of collection and storage of strains, as well as the method of antibiotic sensitivity testing, have been previously described [33]. Charts were reviewed on each of the patients for the following information: age, sex, underlying disease or conditions, course of disease and outcome. In addition, the date of Pseudomonas isolation, anatomic site of isolation, ward location, serotype, clinical antibiogram, prior and current antibiotic therapy were noted.

These data for the year 1973-74 are still being accumulated and will be programmed on a computer when all the charts are available. The same will be done for the 1974-75 collection.

Results.

A report of the epidemiologic features of Pseudomonas isolated from 1970-73 has recently appeared [33].

Antimicrobial sensitivity testing. Table 19 shows the MIC's of gentamicin for Pseudomonas organisms. An MIC of 10 µg/ml or greater was defined as resistant. The rate of gentamicin resistance has been relatively constant over the four years except for 1972. The sharp increase in 1972 is not readily explainable, however, it dropped sharply in 1973.

Table 19. Gentamicin sensitivity.

Year	No. of strains with indicated MIC					Total	% resistant*
	1.25	2.5	5	10	>10		
1970	0	43	46	4	0	93	4.3
1971	3	61	32	3	2	101	4.9
1972	0	27	62	11	3	103	21.4
1973	0	32	65	5	2	104	6.73

\* Resistance -  $\geq 10$  µg/ml

Tobramycin sensitivity is shown in Table 20. An organism with an MIC of 5 µg/ml or greater is defined here as resistant. Data from the first two collection periods are incomplete. In 1972 and 1973 the resistance to tobramycin has been low and relatively constant.

Resistant of Pseudomonas to carbenicillin is demonstrated in Table 21. An MIC of 125 µg/ml or more defines a resistant organism. In 1971 the data are incomplete. The last collection period showed a somewhat decreased rate of carbenicillin resistance when compared to 1972, but no significant difference from the first collection year in 1970.

Gentamicin use. Gentamicin use at WRAMC during each of the last three years is shown in Table 22. Gentamicin use in 1972 did not increase over the previous year. Nevertheless, an increase in

Table 20. Tobramycin sensitivity.

Year	No. of strains with indicated MIC										Total	% resistant*	Not done
	0.25	0.5	1	2	5	10	>10						
1970	2	5	8	6	0	0	0	0	21		76		
1971	0	5	4	6	1	0	1	17		89			
1972	2	3	77	22	0	1	1	106	1.89	0			
1973	0	5	84	14	1	0	0	104		0			

\* Resistance =  $\geq 5$   $\mu\text{g/ml}$

Table 21. Carbenicillin sensitivity.

Year	No. of strains with indicated MIC								Total	% resistance*	Not done
	15.6	31.2	62.5	125	250	500	>500				
1970	0	6	35	10	1	0	0	52	1.92	46	
1971	1	0	2	1	0	1	0	5		103	
1972	14	46	33	7	1	0	5	106	5.66	0	
1973	9	60	27	5	3	0	0	104	2.88	0	

\* Resistance = >125 µg/ml

gentamicin resistance was noted in 1972. In 1974 the use of gentamicin appeared to be greatly increased.

Table 22. Gentamicin use at WRAMC.

Year	Amount used (in grams)	Cost <sup>1</sup> (in dollars)
1971	437	21,164.92
1972	701	33,709.12
1973	701	34,090.64
1974 <sup>2</sup>	506 <sup>2</sup>	22,806.72

<sup>1</sup> Prices changed each year

<sup>2</sup> Through 22 Jul 1974

Chart review. Twenty-one patients had resistant *Pseudomonas* on initial isolates. There was no correlation between resistance and age, ward location, underlying disease, site of isolation or survival. Although 6/14 isolates whose pathogenicity were known were regarded as pathogens, there did not appear to be any effect on hospital course or survival. Prior antimicrobial therapy had no effect on pathogenicity. Of those known not to have prior antimicrobial therapy none had persistent organisms as part of their hospital course. It is interesting to note that these organisms were relatively benign. No instances of blood-borne disease or death was observed. Moreover, multiply resistant organisms did not appear to be any more virulent.

Those 31 patients who developed resistant *Pseudomonas* from initially sensitive *Pseudomonas* had organisms that appeared to be more virulent. Two patients died and sepsis with the resistant *Pseudomonas* occurred in two patients.

These organisms tended to develop in patients who were more ill, regardless of age. While those in the third decade were more numerous in this group they also had a higher percentage of sick patients who did poorly as judged by resistance to therapy. While no one site predominated, urinary isolates were more likely to be pathogens and perhaps more resistant to antibiotics. Sepsis occurred in two such patients and one patient died.

Isolates in this group were more likely to be pathogen (58% vs. 29%), resistant to therapy and patients with these organisms were less

likely to recover from their infection. While prior antimicrobial therapy in general seemed to have no effect on the likelihood of an organism becoming a pathogen or developing resistance, being on an aminoglycoside in particular may have increased the chances of gentamicin and tobramycin resistance. The hospital course was not related to age, pathogenicity, underlying disease or being on at least one effective anti-Pseudomonas drug.

#### Discussion.

The results of our antimicrobial sensitivity testing revealed a striking increase in gentamicin and carbenicillin resistance in 1972. The increased resistance was concurrent with the increased use of these two drugs that year, however, the number of resistant organisms decreased the next year in spite of a continued increase in gentamicin use. This suggests that the level of resistance seen in 1972 was not secondary to increased gentamicin use alone. The ominous trend that seemed to be developing never materialized for some yet unknown reason.

In the first half of 1974 the use of gentamicin significantly increased over past years at WRAMC. It will be important to watch for increased levels of resistance among current Pseudomonas isolates since resistant Pseudomonas in the past has been associated with widespread antimicrobial use.

If one excludes 1972 isolates, the incidence of resistance to gentamicin, carbenicillin and tobramycin has been about 5%, 2% and 1%, respectively, which approximates the rates reported in the literature [34]. As in our experience, Greene, et al. did not find that prior therapy with carbenicillin led to resistance [35]. In our series carbenicillin resistance developed in patients who were not even on carbenicillin. While Greene, et al. noted that carbenicillin resistant Pseudomonas isolates tended to be more virulent (than gentamicin resistant organisms) we did not observe this in our series. Resistance to all three drugs tended to be unusual in other series and occurred once in ours. This was not associated with increased virulence.

Greene, et al. found resistance to be mostly an in vitro phenomenon and correlated little with tendency to invasion [35]. Development of resistance was not dependent on prior carbenicillin therapy but was correlated with nonparental gentamicin [36]. Our experience was essentially similar to that of Greene, et al. with one exception: Pseudomonas organisms which were initially sensitive and then became resistant during the hospitalization seemed to be somewhat more virulent than Pseudomonas that was resistant on the initial isolate. While our data do not lend itself to statistically significant statements, nevertheless the trend is suggestive and bears watching in subsequent collections.

D. Susceptibility of vascular grafts to infection of bacterial origin.

Infection of vascular grafts is infrequent, however, when it does occur loss of life or limb is a common complication. The present study compares the resistance of dacron grafts, autogenous veins and bovine heterografts to infection by a strain of S. aureus isolated from a case of subacute bacterial endocarditis. The organism could be identified by possession of the typical characteristics as well as phage type and antibiogram.

Adult mongrel dogs weighing 22-30 kg (mean 26 kg) were anesthetized with phenobarbital (30 mg/kg IV). Using aseptic technique, three cm segments of the infra-renal abdominal aorta were excised and replaced with knitted dacron grafts in 12 animals, autogenous jugular veins in 10 animals and bovine heterografts in nine animals. Immediately after closure of the midline abdominal incision,  $3 \times 10^7$  S. aureus were suspended in 500 ml. of normal saline and infused intravenously over a two hour period. Three weeks later the grafts were recovered aseptically and cultured thoroughly. Thus far, 8/12 dacron grafts, 2/10 autogenous veins and 1/9 bovine heterografts were positive for the organism injected IV. At this point it would appear autogenous veins or bovine homografts are equally resistant to infection by a bacterial agent while dacron grafts are significantly more susceptible.

E. Occurrence of bacteremia after esophageal dilation.

It has been found that 100 percent of patients undergoing esophageal dilation exhibited a bacteremia post-dilation. Thirteen of 18 patients had positive blood cultures immediately after dilation. Seventeen of 18 had positive cultures within five minutes. Although the incidence of bacteremia was reduced at 10 and 15 mm post-dilation, four were still positive at 30 min.

The fact that S. aureus, S. epidermidis and Bacillus subtilis were the organisms found suggested that the dilators themselves were the source of the contamination.

The incidence of bacteremia occurring after the use of sterilized dilators was then determined. A single gas-sterilized dilator was used in one patient and all blood cultures were negative. Subsequently, the dilators were scrubbed with 7.5 percent povidone-iodine for 5, 4, 3, and 2 min. Following preparation these dilators were handled in a sterile manner, i.e., sterile rubber gloves, towel, barrier drape and K-Y jelly, to avoid accidental contamination. No positive cultures were obtained following dilation. In order to determine the minimum time required for preparation to prevent bacteremia, the dilators were scrubbed with povidone-iodine for 15, 30, 45, 60 and 90 seconds. The dilators were immersed in 500 ml. of TSB for

2 min. and the flasks were incubated at 37°C for 2 days. It was determined that 30 seconds was the minimal time required for sterilization.

Two patients who were dilated with a dilator, scrubbed for 30 seconds, developed bacteremia. Four patients were dilated with a dilator and scrubbed for 30 seconds. All cultures from these patients were negative.

While the bacteremia induced in our patients was not clinically significant it cannot be ignored since certain individuals may be more susceptible to septic complications. Esophageal dilation probably results in breaks in the esophageal mucosa. In this respect, it might be compared with the biopsy procedures which have been reported to be associated with bacteremia.

Patients receiving immunosuppressive drugs are more susceptible to infection and have developed septicemia following esophageal biopsy and brushing through a contaminated endoscope. Patients with diabetes mellitus or those receiving steroids also might be regarded as increased risk. The question of prophylaxis against subacute bacterial endocarditis might be raised in appropriate patients.

F. Analysis of amniotic fluid taken during intrauterine fetal monitoring.

The use of the intrauterine fetal monitor has become an accepted obstetrical procedure. Since it involves passing a catheter through the nonsterile vagina there may be an increased mortality due to maternal and prenatal infections. It has been suggested in several studies that evaluation of the amniotic fluid by culture and microscopic examination for the presence of PMNs may aid in the prediction of when a mother and fetus are at risk. As a result the advanced identification of those patients who should receive prophylactic antibiotics would be possible. However, a specific criticism can be made of each previous study (i.e., no anaerobic cultures done, not randomized, excessive morbidity, narrow patient population, etc.).

Procedure.

The fetal monitor is offered as a routine procedure to all patients in labor at WRAMC. Seventy-five patients undergoing fetal monitoring have been studied to date. The monitoring system consists of (1) a scalp electrode which is attached to the infant's head and used to measure pulse rate and (2) a triple lumen catheter which is placed in the amniotic sac after rupture of membrane and is used to measure intrauterine pressure. The catheter is inserted vaginally through a sleeve tube. The fluid for analysis was obtained from the aspirating channel of the catheter at time of insertion and on some patients every three hrs. during the monitoring and at the termination

of the procedure. Fluid that was obtained during standard working hours was handled as follows:

1. Two slides were made at the bedside for Wright's and Gram's stain.
2. A syringe with amniotic fluid was capped and carried immediately to the laboratory.
3. The specimen was plated on the following media.
  - a. Aerobic - blood, phenethylalcohol. Agar, chocolate and MacConkey's plates.
  - b. Anaerobic - HM (blood agar supplemented with hemin and menadione), VK (as with HM, plus vancomycin and kanamycin) plates and a thioglycolate tube.

The thioglycolate tube was subcultured to the above aerobic media at 24 hr. and to the anaerobic media at 48 hr. Anaerobic cultures were incubated in a gaspak (BBL, Baltimore, Md.) jar at 37°C. The blood, phenylethylalcohol agar and MacConkey's plates were incubated at 37°C in a standard incubator, the chocolate plates at 37°C in a CO<sub>2</sub> incubator.

Fluid that was obtained after normal working hours was processed as follows:

1. Slides made as above.
2. Fluid was injected into a thioglycolate tube on the obstetrical service and placed in the 37°C incubator overnight. The next day it was then taken to the laboratory and processed in the manner previously described.

Organisms isolated from these fluid samples were then identified by standard techniques for the first 30 specimens. Thereafter, all specimens were processed as above but only specimens from patients demonstrating a clinical infection, febrile morbidity (temp. of 100.4° or greater x 2 at least 6 hr. apart), premature removal of the membrane (PROM) or undergoing a cesarian section, were evaluated bacteriologically. Smears were made and analyzed on all patients.

Both mothers and their neonates were followed carefully post-delivery. An infection was defined as an obvious site of clinical inflammation with an accompanying positive culture for a recognized pathogen.

### Results.

A total of 70 patients have been studied, resulting in 137 specimens of which 58 were cultured. Organisms were seen on Gram stains in 70 percent of the patients. Fifty percent of the specimens had at least one polymorphonuclear leukocyte seen in the slide, while of those 20 percent had at least one PMN/oil field.

Ninety-one percent of the specimens were culture positive and a wide variety of organisms were identified (Table 23).

Of the patients with either a positive culture or a positive smear, only one developed clinical illness, an incidence of four percent. This patient had only a mild fever without symptoms. Conversely, no patients with  $<1$  PMN/oil field developed clinical disease.

### Discussion.

"Contamination" of the amniotic fluid with vaginal flora during fetal monitoring is quite common.

The Gram stain is not useful as a prediction of morbidity and mortality.

Although patients with  $>1$  PMN/oil field were at increased risk,  $<5$  percent of the mothers or their neonates developed clinical infection or a febrile episode and hence the use of "prophylactic" antibiotics in such a situation is not indicated.

Table 23. Bacteriology (34 patients).

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Growth - 91%

Organisms

Aerobic - 1.	<u>Staph. epidermidis</u>	- 34
2.	Streptococci (non-group A, B or D)	- 27
3.	Streptococci (group D, non-enterococcal	- 15
4.	Streptococci (group B)	- 11
5.	Diphtheroids	- 10
6.	Lactobacillus	- 9
7.	Yeast	- 9
8.	<u>E. coli</u>	- 8
9.	Citobacter	- 4
10.	Streptococci (enterococcal)	- 4
11.	Streptococci (group A)	- 2
12.	Listeria	- 1
13.	Enterobacter	- 1
14.	Klebsiella	- 1
15.	<u>Staph. aureus</u>	- 1

Anaerobic - 40 specimens - identification pending

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### Summary and conclusions.

The outer cell wall membrane antigens of Neisseria gonorrhoeae have been isolated and characterized. The lipopolysaccharide is serologically related to a common antigenic determinant of N. meningitidis as demonstrated by hemagglutination inhibition assays. The protein forms the basis of an immunologic typing scheme developed for N. gonorrhoeae based on inhibition of a solid phase radioimmunoassay. Clinical strains of gonorrhea have also been typed using meningococcal antisera in a bactericidal system. A previously undetected capsule has been identified in N. gonorrhoeae. Lipids extracted from pathogenic Neisseria and other gram negative organisms were toxic for N. gonorrhoeae. Gonococci invading tissue cultures were demonstrated to have intracellular viability. Clinical laboratory support of infectious diseases problems has included studies on the epidemiology and antibiotic sensitivity patterns of Pseudomonas aeruginosa, a study demonstrating increased susceptibility of dacron grafts to bacterial infection as compared to autogenous vein or bovine heterografts, and demonstration that the Wright's stain and culture of amniotic fluid is of limited use in predicting infection secondary to intrauterine monitoring.

Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable Disease and Immunology

Work Unit 168 Bacterial diseases of military importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6529	75 07 01	DD DR&E(AR)6J6	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DIB'S INSTR'	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61102A	3A161102B71Q	00	169			
B. CONTRIBUTING							
C. <del>XXXXXXXX</del>	CARDS 114F						
11. TITLE (Precede with Security Classification Code)							
(U) Field Studies of Leishmaniasis and Other Tropical Diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
002600 Biology 010100 Microbiology 003500 Clinical Medicine							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD			
75 07	CONT	DA		C. In-House			
17. CONTRACT GRANT				18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (in thousands)	
A. DATES/EFFECTIVE: NA EXPIRATION:				PREVIOUS			
B. NUMBER:				FISCAL YEAR			
C. TYPE: 4. AMOUNT:				75	3	240	
E. KIND OF AWARD: f. CUM. AMT.				76	4	390	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: US Army Med Rsch Unit - Panama			
ADDRESS: Washington, DC 20012				ADDRESS: Box 1809 APO New York 09826			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: Walton, B. C. COL			
TELEPHONE: 202-576-3551				TELEPHONE: 82-3017			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Intermill, R. MAJ			
				NAME: Hendricks, L. P. MAJ			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Leishmaniasis (U) Latin America (U) Epidemiology (U) Chemotherapy							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. (U) With emphasis on Leishmaniasis, the objective is the acquisition of data concerning prevalence and distribution of parasitic diseases in Latin America; recognition of infectious diseases of actual or potential military importance; increase of knowledge of reservoirs and vectors involved, and improvement of diagnosis, treatment and control of these diseases.</p> <p>24. (U) Toward development of a screening program for new antileishmanial drugs, efforts focused on use of axenic amastigotes in <u>in vitro</u> system, and available strains/species of rodents were tested for susceptibility to infection for a new animal model. Strains of hemoflagellates are cryopreserved at low passage level to compare characteristics. Laboratory raised sandflies were produced from eggs of wild-caught females and forced feeding techniques used for experimental infection of the insect vector. Newly initiated biochemical studies focused on oxidative metabolism of <u>Leishmania</u>.</p> <p>25. (U) 74 07 - 75 06. Effects of known antileishmanial drugs were demonstrated with axenic amastigote system, but undefined media elements require study before system can be standardized. A new rodent model of cutaneous infection was developed which proved to be of value in drug testing. Epidemiological study was initiated of kala azar in Honduras where it was not previously known and isolates from patients afforded first KA strains from Central America for laboratory study. Capability for experimental sandfly infection was established. A Biochemistry section was initiated and differences in O<sub>2</sub> respiration between life cycle stages of <u>Leishmania</u> have been demonstrated. The monkey coccidian <u>Isospora arctopithecii</u> was further characterized and found to cause patent infections in an unprecedented number of host species.</p> <p>For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1974 - 30 June 1975.</p>							

PII Redacted

Project 3A161102B71Q : COMMUNICABLE DISEASES AND IMMUNOLOGY  
Task 00 : Communicable Diseases and Immunology  
Work Unit 169 : Field Studies of Leishmaniasis and Other  
Tropical Diseases

Investigators:

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Description

The program of USAMRU-Panama involves field and laboratory studies to elucidate certain aspects of the relationships of vectors, reservoir hosts, and human host factors to the manifestations of parasitic disease, with particular emphasis on the forms of leishmaniasis occurring in the New World, some of which constitute a current military medical problem. Because of the lack of a completely effective drug to treat leishmaniasis, the major effort is toward development of in vitro models for screening compounds for antileishmanial activity and animal models for testing drug efficacy against infection.

Progress

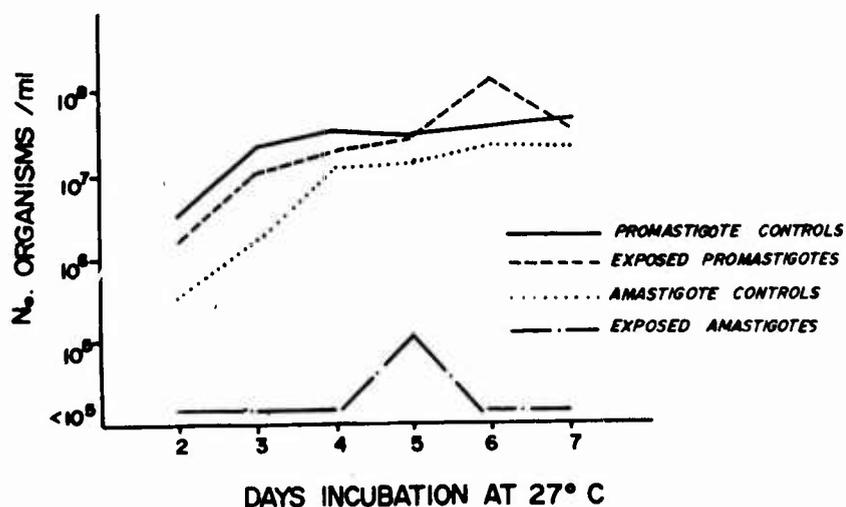
A. Leishmaniasis

1. Systems for screening drugs for anti-leishmanial activity.

a. In vitro systems. Axenic amastigotes of Leishmania braziliensis produced extracellularly by temperature/media-induced conversion from promastigotes (Annual Report 1973) were evaluated as a possible test system to screen for antileishmanial activity. A recent human isolate from Panama was mass produced and preserved in liquid nitrogen to provide a uniform stock of organisms at low passage level for the trials. The clinically active pentavalent antimonial, Glucantime<sup>(®)</sup> (methylglucamine antimoniate) served as the reference drug. Eight compounds received from the Division of Medicinal Chemistry which represented several classes of compounds with a known degree of anti-leishmanial activity were tested in this system. The test compounds were screened at 3 concentration levels; 0.1 µg,

10  $\mu$ g, and 100  $\mu$ g/ml, and amastigotes were exposed to the drug for 24, 48, and 96 hours in liquid medium at 34°C. After the exposure period, leishmaniae were inoculated into fresh liquid medium and incubated at 27°C to assess drug effect. Failure to multiply as promastigotes was regarded as indicating drug action. In most cases where LD<sub>100</sub> was not attained with an active compound, the effect was demonstrated by delayed growth, since the number of viable organisms in the inoculum is an inversely proportional function of the drug effect.

Representative growth curves from media inoculated with amastigotes after exposure to active and inactive drugs are shown in figures 2-3. The activity of the clinically active reference drug was apparent



Figur 1. Comparative effect of 96 hr exposure to 100  $\mu$ g/ml methylglucamine antimoniate on promastigotes and axenic amastigotes of *L. braziliensis*.

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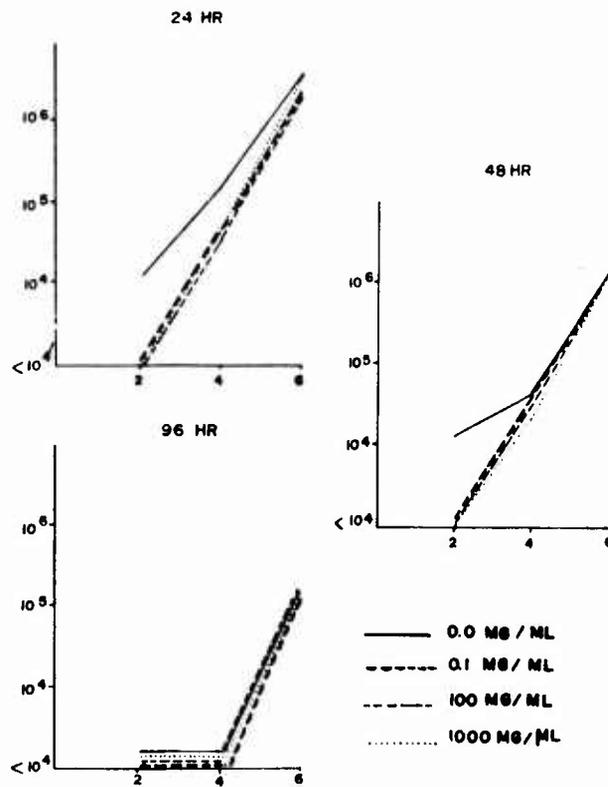


Figure 2

Pattern of growth in culture of Leishmania braziliensis after exposure of axenic amastigotes to a compound with no antileishmanial activity.

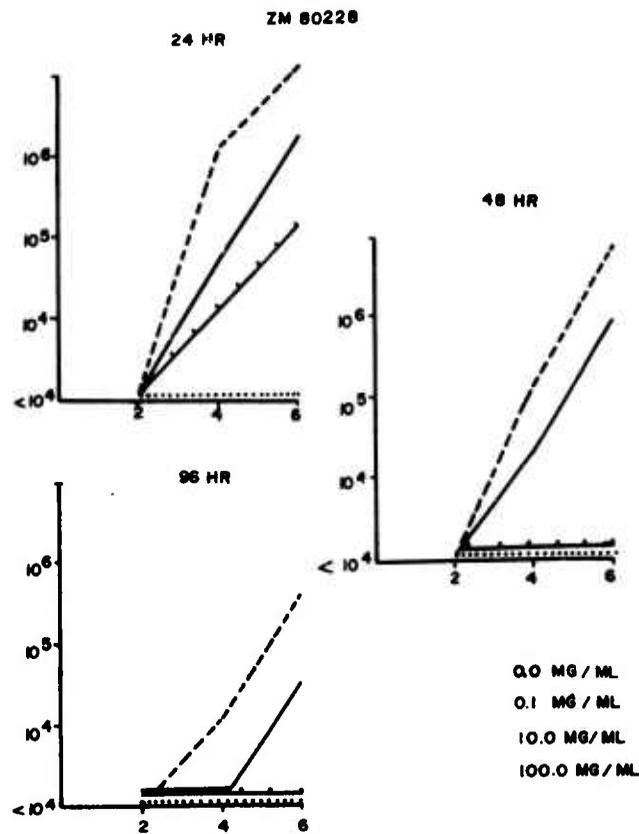


Figure 3

Pattern of growth in culture of *Leishmania braziliensis* after exposure of axenic amastigotes to a compound with antileishmanial activity.

at the 100 µg/24 hour exposure level, but LD<sup>100</sup> was not achieved until 96 hours exposure, when both 10 µg and 100 µg/ml yielded negative cultures. (Table 1). Of the compounds tested, 4 demonstrated antileishmanial activity greater than that of the reference drug, one had activity at a lower level, and 3 no activity. (Table 1).

Certain anomalies in the pattern of results, particularly in the failure to reach LD<sup>100</sup> in some cases, are apparent in the system. Some of these may be explained by the incomplete conversion of the total population to the amastigote form. Since the promastigote stage is not affected by drug concentrations which are effective against amastigotes (Figure 1), any residual promastigotes in the population tested would result in positive back culture, hence false negative results, with concentrations which would be 100% effective for the amastigotes. This imposes limitation, but does not preclude the use of this as a screening system. The most serious problem encountered with the system concerns the inconsistencies in achieving consistent growth and conversion of test organisms in the liquid medium used. After considerable experimentation, the problem now appears to be pinpointed upon variations in characteristics of various lots of fetal calf serum, with the adverse effect possibly due to a contaminant introduced during processing to eliminate a bovine virus threat. Identification of a large volume lot of suitable reagent has only been possible by time consuming empirical tests, which has seriously delayed further evaluation of this potentially useful system. Initially 4 drug concentrations were tested, 0.1, 1.0, 10.0 and 100 µg/ml, but technical difficulties encountered prompted reduction to only 3, and the 1.0 µg/ml concentration was arbitrarily eliminated. Inspection of the results indicates that even effective compounds show little activity at 0.1 µg/ml, and maximum effect is reached at 10 µg, indicating the 1.0 µg level would be of considerable more interest. In future testing, concentrations of 1.0, 10 and 100 µg/ml will be used.

The antileishmanial activity demonstrated in this system correlates very well with known clinical activity results in other tests. Of the 4 compounds showing the highest activity 2, Lampit and Diamidine, have been used successfully clinically, while Astiban is an antimony compound, and Primaquine, an 8-aminoquinoline, both known antileishmanial groups. This correlation indicates the validity of this system as a primary screening system to detect antileishmanial effectiveness.

b. Laboratory models. With the objective of developing an animal model which produces discrete lesions paralleling the human disease which would serve a secondary drug screening system, the search for animals susceptible to *L. braziliensis* infection continued (Annual Report 1973), with efforts focused on rodents. A two avenue approach was fol-

Table 1  
In Vitro Antileishmanial Activity of Compounds Against  
L. braziliensis in Axenic Amastigote System

Compound	E X P O S U R E P E R I O D								
	24 Hours			48 Hours			96 Hours		
	0.1 µg	10.0 µg	100 µg	0.1 µg	10.0 µg	100 µg	0.1 µg	10.0 µg	100 µg
Glucantime	*	+	+++	+	++	+++	-	++++	++++
Nifurtimox BD 54239 (Lampit)	-	++++	++++	-	++++	++++	-	++++	++++
Diamidine AX 37252	-	++++	++++	-	++++	++++	-	++++	++++
Astiban ZM 80228	+	++	++++	+	++++	++++	+	++++	++++
Primaquine WR 2975	-	+	++++	-	+	++++	-	++++	++++
Nystatin WR 6183	-	-	+	+	-**	+++	+	-**	+++
Sulfadiazine BB 58906	-	-	-	-	-	-	-	+	+
Tryparsamide ZL 12010	-	-	-	-	-	-	-	-	-
Quinazoline ZM 86024	-	-	-	-	+	-	*	*	*

\*No results - lost culture  
 \*\*More growth than control

lowed, with attempts to produce a uniformly susceptible strain of cotton rat by selective breeding, and to screen a variety of other rodents for susceptibility to infection. All rodent inoculations were made intradermally on the shaved dorsum just anterior to the base of the tail. In the selective breeding program, all weanling animals from the MARU Sigmodon hispidus (Florida strain) colony were challenged, and those developing a papule or ulcer were retained for breeding, and those demonstrating no evidence of infection discarded. As opportunities arose, small numbers of untested species or special strains were acquired for testing for susceptibility. Two candidate strains were obtained because of published reports of unusual susceptibility to visceral leishmanial infection; an inbred mouse strain from the University of Montana, and the African white-tailed rat Mystromys albicaudatus from AFIP.

The weanlings of Sigmodon hispidus from the colony exhibited the previously observed pattern of sporadic susceptibles, with 24 of 83 (28%) exhibiting papules or lesions. Ten pairs have been selected for breeding and the resulting F-1 offspring will be challenged as weanlings to compare susceptibility to that of the unselected population. A summary of the results of challenge of 13 rodent strains is presented in Table 2. Of the various species/strains screened, only the African white-tailed rat, Mystromys albicaudatus demonstrated a higher rate of cutaneous lesions than did Sigmodon. All of 23 Mystromys inoculated developed papules which became discrete ulcers within 2 weeks, most with the circular form, raised borders, and sharply incised margins characteristic of leishmanial infection. (Fig. 4a,b). The evolution of the lesion was similar to that seen in Sigmodon, but in contrast to that host's tendency for spontaneous healing after several weeks, the lesion continued to progress, some reaching over 2 cm in diameter. Five of six animals untreated at 105 days had open lesions. Additional breeding pairs have been requested from AFIP and it is planned to establish a breeding colony to permit further evaluation of this model.

c. Drug testing in Mystromys. The availability of these Mystromys with active progressive cutaneous L. braziliensis lesions from the screening program provided the opportunity to observe the effect of the current drug of choice, Glucantime, in this model, and to test the effect of an 8-aminoquinoline, AG 75499, which had been reported to have exceptional antileishmanial activity in a hamster/L. donovani system at the University of Georgia. Twenty surviving Mystromys infected 95 days previously were divided into 3 groups, for roughly equivalent distribution by sex and size of lesion, and individually caged.

Table 2

Development of cutaneous ulcers in Various Species and Strains  
of Rodents Inoculated with Leishmania braziliensis (Parana)

Scientific Name	Common Name	No. Lesion/ No. Exposed	Comments
Family Cricetidae			
<u>Tylomys panamensis</u>	Climbing rat	2/16	Ulcers <2mm, 2 wks duration
<u>Zygodontomys microtinus</u>	Cane rat	0/40	No reaction
<u>Calomys callosus</u>	Vesper mouse	5/19	Ulcers 1-2mm, closed within 4 wks
<u>Sigmodon hispidus</u>	Cotton rat	24/83	Ulcers 1-10mm, closed within 1 month
<u>Mesocricetus auratus</u>	Syrian hamster	0/20	No lesions, positive cultures in 11/11 after 3 months
<u>Mystromys albicaudatus</u>	African white-tailed rat	23/23	Ulcers at 2 wks, enlargement thru 90 days, some to 2 cm
Family Muridae			
<u>Mus musculus</u>	Laboratory mouse WRAIR (outbred)	0/20	Few with slight swelling
<u>Mus musculus</u>	Laboratory mouse NIH (outbred)	0/20	No reaction
<u>Mus musculus</u>	Laboratory mouse U. Montana (inbred)	1/10	Ulcer 1mm, closed within 2 wks
<u>Rattus norvegicus</u>	Laboratory rat (outbred)	0/40	Slight transient swelling in 10/40
<u>Rattus rattus</u>	Laboratory rat Fisher (inbred)	0/39	Few with slight transient swelling
<u>Rattus rattus</u>	Laboratory rat WRAIR (outbred)	0/25	No reaction
Family Caviidae			
<u>Cavia porcellus</u>	Guinea pig (outbred)	1/10	1 with <2mm ulcer, closed within 4 wks
TOTAL		<u>56/463</u>	

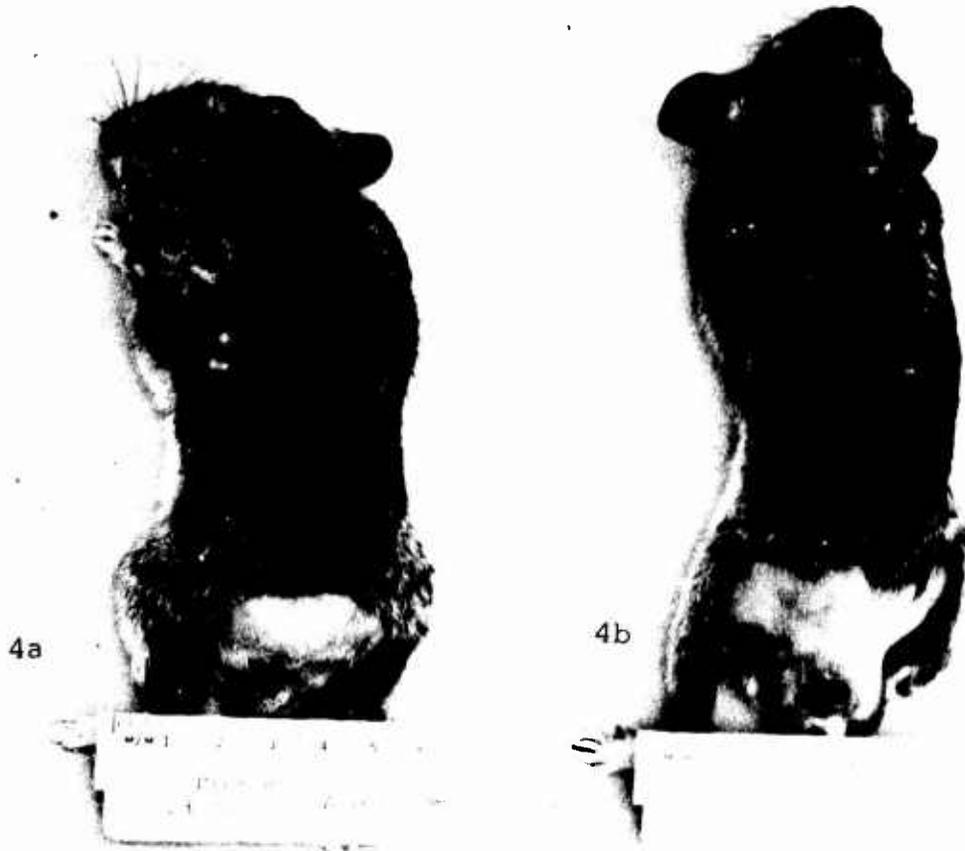


Figure 4a,b. Cutaneous lesions of 3 months evolution produced by inoculation of L. braziliensis panamensis strain intradermally in Mystromys albicaudatus.

Group I - 7 animals received AG 75499, 0.1 ml(=2.5 mg) daily  
x 10

Group II - 7 animals received Glucantime, 0.1 ml(=10 mg) daily  
x 10

Group III - 6 animals received sham inoculation of suspending  
agent

Glucantime (N-methylglucamine antimoniate) provided as liquid in ampules containing 300 mg/ml was diluted in Veronal buffered saline (VBS) with 0.1% Tween 80 to facilitate administration of dose equivalent to that (100 mg/kg) recommended for clinical use. AG 75499 was suspended in VBS/Tween 80 to a concentration of 25 µg/ml. All injections were given intramuscularly in the hind limbs for the first 5 days, and intraperitoneally for the remaining 5 days. Lesions were measured at the start and termination of treatment. The results are summarized in Table 3. Response to AG 75499 was noted on day 5, with all lesions dry and apparently starting to epithelialize. The lesions were all discolored, having the iodine-like color of the drug. However, all animals showed evidence of extreme irritation from the drug and 4 exhibited severe wounds from chewing on their limbs at the injection sites. The route of injection was changed to ip. at this time. Three animals died on day 7, apparently from trauma from chewing their hind limbs, and possibly, drug toxicity. Two more deaths occurred on day 9 from the same causes. Of the 2 surviving, both had lesions reduced in size, but still open. Response to Glucantime was also noted at day 5, when lesions of 2 animals had dried. There was no ill effect noted from the intramuscular injections, and no lesions from chewing on the injection site. On the 10th treatment day lesions were no longer visible on 2 animals, 3 had completely reepithelialized with only borders detectable, 1 was greatly reduced and covered with a scab, and only 1 was still open, although of reduced size. Within 2 weeks, all lesions had healed completely, while average lesion size of the untreated control animals remained stable (Fig. 5).

A second trial of AG 75499 was conducted using the 6 untreated control animals of the first trial. The dosage of 2.5 mg daily x 10 was again used, but administered in 1.0 ml dose by gavage to eliminate the extreme irritation caused by intramuscular injection. At day 5 some effect was again seen in the drying of 3 lesions and all lesions were discolored with the iodine-like stain of the drug. On day 6 one animal died from aspiration of drug during gavage. At the end of the treatment period all lesions were essentially unchanged. During the first 4 days of the 10 day post treatment observation period 3 animals

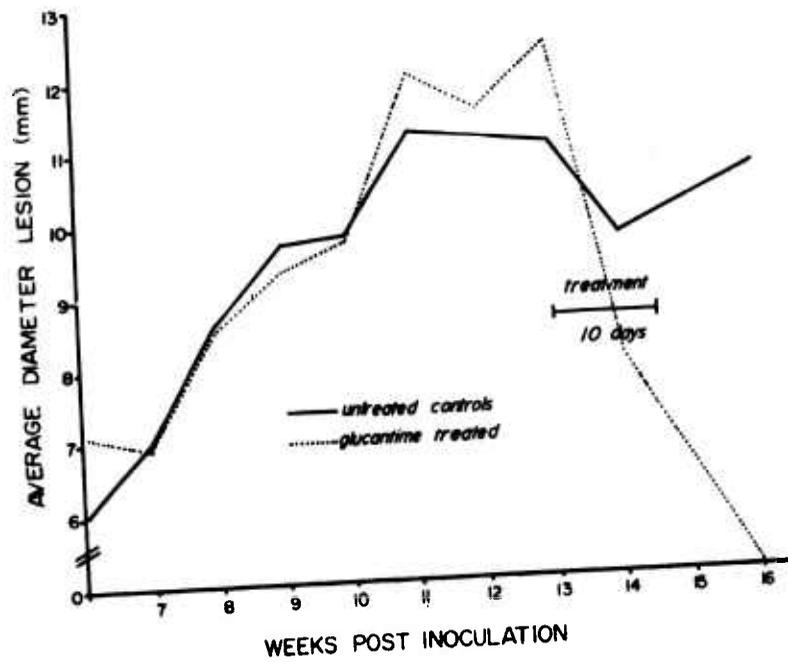


Figure 5

Effect of methylglucamine antimoniate on size of cutaneous ulcers of Mystromys albicaudatus experimentally infected with Leishmania braziliensis.

Table 3

Effect of Drug on Size of Experimental L. braziliensis  
Lesions in Mystromys Tested with Glucantime and AG 75499

	Animal No.	Sex	Weight (Grams)	Lesion Size (mm)	
				Pre-R <sub>x</sub>	Day 10
Group I AG 75499	1612	♀	72	8 x 2	D
	1776	♀	72	20 x 6	D
	1778	♀	58	4 x 5	D
	1782	♀	70	16 x 14	11 x 11
	1783	♂	84	17 x 18	D
	1784	♂	78	40 x 10	25 x 12
	1786	♀	66	4 x 3	D
Group II Glucantime	1613	♂	90	3 x 2	Scar
	1614	♀	74	9 x 6	Healed
	1779	♀	58	8 x 7	Scab 6 x 4
	1780	♂	92	14 x 18	12 x 7
	1791	♀	96	15 x 14	Scar 7 x 3
	1792	♂	114	18 x 19	Dry 15 x 6
	1793	♀	78	12 x 6	Dry 4 x 2
Group III Untreated	1619	♀	88	9 x 5	8 x 8
	1777	♂	116	14 x 6	15 x 6
	1781	♀	90	4 x 3	Dry 4 x 3
	1789	♂	112	9 x 4* 15 x 8	9 x 5* 15 x 8
	1795	♂	100	13 x 11	14 x 10
	1796	♂	96	15 x 8	15 x 8

\*Two Lesions

died, apparently of drug toxicity. The 2 remaining animals exhibited some reduction of lesion size, but healing did not occur. The failure to effect healing of cutaneous lesions in these 2 trials is not in accord with the antileishmanial activity of 400X that of antimonial compounds reported in the 8-day hamster/L. donovani test. However, this may well be due to differences in the 2 parasite models used, L. braziliensis being notoriously difficult to treat. If these differences are confirmed, it will emphasize the need for a L. braziliensis model to test candidate drugs against that infection.

The 100% healing achieved with a dosage and regimen of Glucantime approximating that in clinical use, strongly suggests that the L. braziliensis/Myiostomys model will be extremely useful for drug testing.

## 2. Biological characteristics of three strains of New World Leishmania causing mucocutaneous disease.

The lack of consistent morphological differences has been largely responsible for the lack of clear taxonomic relationships among the Leishmania infecting man in the New World. The propensity of the South American form to develop the highly characteristic mucocutaneous disease, known as espundia, was early recognized as being distinct from the Old World cutaneous form and it was designated as a separate species, L. braziliensis. All Leishmania from areas where human espundia was known to occur came to be regarded as L. braziliensis, although differences were recognized among isolates from different areas. Hertig acknowledged these differences by his use of the designation "L. braziliensis sensu lato". The distinction as a separate species was later conferred upon the parasite of the Yucatan peninsula - British Honduras area, primarily upon the basis of its apparent failure to ever cause mucocutaneous lesions, and it was called L. mexicana. Taxonomic separation upon the basis of clinical manifestations also was proposed for L. pifanoi, which causes generalized disseminated cutaneous lesions in Venezuela, and L. braziliensis guyanensis also causing disseminated cutaneous lesions by metastatic spread, although these have been less widely accepted. In 1972, Lainson and Shaw (8) proposed a systematic classification scheme based upon biologic characters for grouping New World forms into 2 groups, the L. braziliensis complex and the L. mexicana complex. This scheme is based upon growth in in vitro culture and pathogenesis in the hamster, as well as location of infection in the sandfly gut, a differential feature which had been noted earlier by others (7). The variation among populations within the complexes was acknowledged by the use of trinomials, e.g. L. braziliensis panamensis and L. mexicana amazonensis, which has been criticized by some

systematists. The characteristics used to separate the 2 complexes are indicated in the following scheme.

Characteristic	<u>L. braziliensis</u> Complex	<u>L. mexicana</u> Complex
1. Clinical features	Has propensity to develop delayed mucocutaneous lesions.	Does not develop secondary mucocutaneous lesions.
2. Growth in culture	Grows poorly or not at all in blood agar medium. Promastigotes in primary culture often cannot be subpassaged.	Rapid and profuse growth in blood agar medium.
3. Pathogenesis in hamsters	Grows slowly in skin, parasites sparse, no visible swelling for 9-12 months, no metastases, does not visceralize.	Grows rapidly in skin, gross deformations start 2-3 months. Profuse parasites, metastasizing in cutaneous nodules, invades viscera and gonads.
4. Location in vector gut	Established principally in hind gut, particularly hind triangle, with or without accompanying midgut infection.	Almost exclusively midgut infection.

Three isolates from human espundia cases, and all initially presumed to be L. braziliensis braziliensis upon this basis, were characterized upon the basis of growth in cultures and behavior in hamsters. They were 1.) a strain (1114) from Brazil received from Lainson and Shaw and classified as L. b. braziliensis, 2.) a strain (1128) isolated in our laboratory from a nasal biopsy of a case of espundia originating in eastern Peru, 3.) a strain (1156) isolated by M. E. Hajduk from a nasal biopsy of the first autochthonous case of espundia in the U.S., apparently acquired in Texas (2).

The results are summarized in Table 4. The strain from Brazil conformed to the predicted pattern for L. b. braziliensis. However, the strain from Peru consistently grew very well in culture, although the slow evolution of lesions in hamsters was not distinguishable from that of the Brazil isolate. This pattern also applies to the great majority of strains isolated from humans in Panama which have been tested. However,

Table 4

Differences in Biologic Characters of Three Strains  
of Leishmania Causing Mucocutaneous Disease

Characteristic	Origin and Strain Number		
	Brazil 1114	Peru 1128	Texas 1156
1. Clinical feature	Mucocutaneous lesions	Mucocutaneous lesions	Mucocutaneous lesions
2. Growth in culture	None	Rapid, profuse	Rapid, profuse
3. Pathogenesis in hamster	Evolution slow, slight swelling in 8 months, parasites sparse	Evolution slow, slight swelling in 11 months, parasites moderate	Evolution rapid, swelling 1 month, parasites profuse, metastases, visceralizing



Primary and metastatic (arrow) lesions produced in 3 months  
of inoculation of hamster with isolate from an autochthonous espundia  
case from Texas.

subjectively the promastigotes of the Peru strain from culture are markedly different, and readily distinguishable by their smaller size and rapid, frenetic motion from any other strain in our laboratory. The Texas isolate conforms in each regard to the criteria for L. mexicana, except for the clinical form of the disease elicited. However, this might be explained upon the basis of cobalt irradiation received for treatment of carcinoma a few years before onset of symptoms, which quite possibly altered the immune state of the patient and hence the disease course. It is of interest that the only other probable autochthonous case of leishmaniasis from the U.S., (9) also from Texas, likewise presented as an atypical clinical entity in the form of non-ulcerating, disseminated lesions. Geographically, the nearest known endemic area to Texas is Yucatan, where only L. mexicana is known to occur. With no intent to imply an argument for subspecies status, this isolate will henceforth be referred to as L. mexicana texana as a designation of convenience.

### 3. Biochemical studies on Leishmania.

Assignment of a biochemist to this unit for the first time necessitated the expenditure of a considerable period of time, not yet complete, to accumulate minimum essential instrumentation and reagents to establish a laboratory. The arrival of a polarographic oxygen monitor has provided a potential for studies on the oxidative metabolism of Leishmania. Arrival of a UV recording spectrophotometer is anticipated and will be used with the oxygen monitor for combination analysis which is expected to provide important information on specific metabolic pathways subject to drug action. Preliminary results with the oxygen monitor suggest that amastigote and promastigote forms are metabolically quite different, supporting observations made in other studies that drugs having an effect on one form may be entirely without effect on the other. Investigation of species specific biochemical markers has been initiated. Differentiating parameters include oxygen utilization, presence of terminal electron transport oxo-reductases, and effects of drugs on specific metabolic pathways. Preliminary results suggest significant differences between pathogenic and non-human Leishmania species, and may yield important clues for approaches to chemotherapy. Techniques have been explored for obtaining preparations of amastigote forms of L. braziliensis essentially free of tissue culture host elements, and considerable success has been achieved with DEAE-cellulose and Sephadex G-100 column separation.

The reports (1,4) that the plant lectin, Concanavalin A, specifically agglutinates Leishmania donovani promastigotes and Trypanosoma cruzi epimastigotes, while not agglutinating T. cruzi trypomastigotes, suggested that Concanavalin A might be employed as a biochemical marker to differentiate various pathogenic Leishmania species. Seven species of Leishmania, 2 stages of T. cruzi, and several types of vertebrate and invertebrate cell types were tested for Concanavalin A (Con A) effect.

Table 5

Effect of Concanavalin A on  
Parasitic and Non-Parasitic Cells

<u>Leishmania</u> Species:		Agglutination
<u>L. panamensis</u>	promastigotes	yes
<u>L. panamensis</u>	amastigotes (Vero)	yes
<u>L. tropica</u>	promastigotes	yes
<u>L. hertigi</u>	promastigotes	yes
<u>L. donovani</u>	promastigotes	yes
<u>L. braziliensis</u>	promastigotes	yes
<u>L. mexicana</u>	promastigotes	yes
<u>L. chagasi</u>	promastigotes	yes
Insect Cells:		
<u>Aedes albopectus</u>		no
<u>Antheraera eucalyptii</u>		no
<u>Aedes albopectus</u> (trypsinized)		yes
Mammalian Cells:		
Monkey kidney (Vero)		yes
Pig kidney		yes
Mouse fibroblasts		yes
Trypanosomes:		
<u>T. cruzi</u>	epimastigotes	yes
<u>T. cruzi</u>	trypomastigotes	yes

A summary of the results of the studies is presented in Table 5. The finding by Dwyer (4) that Con A specifically agglutinates L. donovani promastigotes was confirmed. Additionally it was found that this lectin agglutinated all Leishmania species, as well as all mammalian cells tested. In contrast to the report of Alves and Colli, (1) we found it to agglutinate both trypomastigotes and epimastigotes of T. cruzi. In fact, the only cells upon which Con A had no demonstrable effect were two insect cell types. We interpret these studies as evidence that Concanavalin A is probably not a useful tool for the differentiating pathogenic Protozoa being studied in this laboratory.

#### 4. Phlebotomine sandfly vectors.

a. Colonization and mass rearing of Phlebotomine sandflies. In a renewed effort to develop a capacity for laboratory rearing of a sufficient number of Phlebotomine sandflies for laboratory experiments, the effect of external conditions for rearing offspring of wild-caught females was studied. Prior studies in this laboratory (Annual Report 1972) and others (3) indicated that other containers were superior to the traditional clay bean pot to provide a suitable micro-environment for sandfly development because they reduced or eliminated the tedious time consuming daily examination of rearing vessels. During this year, a study was conducted to determine the importance of ambient conditions in the external environment on the ability of the container to provide the micro-climate required for the development of the immature stages of Lutzomyia trapidoi. The variable factors included temperature, humidity, and light. Three locations within the insectary were utilized to provide the variety of conditions; outer insectary with uncontrolled conditions from 25-30°C and 50-70% humidity, semi-controlled insectary room of 27-29°C and 55-60% humidity range, and a Hotpoint controlled environmental chamber at 25°C with 75-80% humidity. The insectary areas had indirect sunlight with the natural photoperiod, and the environmental chamber was regulated to provide 12 hour light cycles. Plastic circular food containers 16 x 8 mm and 12 x 8 mm in diameter, lined with plaster of paris as a moisture retaining substrate, were used. Larvae were provided leaves of the Anacardium tree minced in a blender, and supplemented by liquefied yeast. Rearing containers were prepared 24 hours in advance to stabilize the micro-environment within the container before the blood-fed females were introduced. During the study, the containers were inspected once or twice weekly to observe the development of the larvae, and other critical factors such as humidity, and presence of molds, fungi, mites, etc.

The results are summarized in Table 6. These data indicate that of the 3 environments, the unregulated ambient of 25-30°C and 50-70% humidity is by far the best suited to rearing of L. trapidoi. Further

confirmation of the better suitability of these conditions was provided when first generation adults (F-1) were recovered only from containers maintained under these conditions. Little difference was noted between the semi-controlled and controlled environments in number of sandflies emerging, or in time of development. Sandflies reared in semi-controlled conditions emerged in 44-52 days, and those in the environmental chamber in 42-64 days. The initial emergence period of 44-45 days from containers under uncontrolled conditions in the insectary was significantly more synchronous. It is not known if the variation in conditions per se favored better development or whether the conditions selected in the regulated areas were not optimum. However, unless it can be shown that significantly better development could be obtained under other conditions in a fixed and regulated environment, it would appear advantageous not to rely on mechanical and physical regulation. Mite infestations in the non-regulated environment were more prevalent, but molds and drying conditions were more frequent in the 2 regulated areas. Further studies on influence of external conditions are continuing.

b. Experimental Leishmania infections in Phlebotomines. In early work on visceral and cutaneous leishmaniasis in the Old World, the incrimination of Phlebotomine sandflies as vectors was established, and it was assumed that these insects would ultimately be shown to transmit the leishmaniasis of the New World. The association of sandflies with endemic disease areas and finding of naturally infected insects has been amply demonstrated, but the achievement of experimental infections in the laboratory has been accomplished in only rare and sporadic instances in this hemisphere. Infection of laboratory reared sandflies by feeding on experimental animals is difficult (7) and holding them alive and inducing them to take subsequent blood meals has proved a difficult barrier. Experimental infection of hamsters with L. braziliensis via a vector is known from a single report and L. mexicana could be transmitted to man only once in 332 attempts, and only by using wild-caught sandflies. A clear need exists for additional information on the biology of vector-parasite relationships between Leishmania spp. and Phlebotomines.

Hertig and colleagues have used an artificial feeding technique to infect Panamanian sandflies with in vitro culture forms to study the distribution of promastigote infections established in the insect gut, and showed that this parameter is of value in classifying Leishmania (6).

The basic technique of Hertig has been adapted to provide the capability to artificially feed and infect adult sandflies. The basic feature of the original technique, a glass capillary tube with an orifice of 0.04 or 0.05 mm, depending upon the species of sandfly to be fed, has been retained, but the handling and holding techniques have been improved. In place of holding the sandflies by compressing the wings into a groove,

the adult fly is held in position against a fine gauze by negative air pressure. Mechanical devices allow the position of both the fly and the capillary tube to be manipulated to achieve the entry of the stylets into the orifice, and bend the labium back to simulate the normal feeding position of the mouth parts. The proper position triggers a feeding reaction and the fly rapidly engorges with the defibrinated rabbit blood in the capillary tube. Engorged sandflies are individually held in plastic holding vessels. In initial trials sandflies engorged rapidly, and preliminary results from feeding over 150 Lutzomyia gomezi and L. trapidoi indicate that the technique can be used successfully for artificial feeding experiments. The availability of amastigote forms produced in tissue culture which can be administered in the artificial blood meal will provide an experimental situation more closely simulating nature, since amastigotes are the stage acquired in feeding on a vertebrate reservoir host. This tool, which has not previously been available to investigators, is ready for preliminary trials.

Table 6  
Results of Rearing Lutzomyia trapidoi  
Under Three Laboratory Conditions

Insectary Conditions	No. Experiments	Original No. of Females	Ave. No. Females/ Container	No. Emerged/ Female
25-30°C 50-70% humidity	1	40	13.3	10.6
27-29°C 55-60% humidity	2	36	9.0	<1
25°C 75-80% humidity	6	517	36.9	<1

### B. Toxoplasma/Isospora Infections.

It was the classical concept of early workers that the coccidian genus Isospora is characterized by a direct life cycle and marked host specificity. World-wide research on this and related genera of sporozoans received great impetus when Toxoplasma gondii was found to be an intestinal coccidian of felines, with morphological characteristics of the genus Isospora. These recent investigations of isosporan-like life cycles cast little doubt on the accepted concept of a narrow range of hosts for this parasite.

However, Hendricks (5), in the redescription of the primate coccidian Isospora arctopitheci, reported natural and experimental infections in two families of Panamanian primates. Successful cross transmission experiments with this parasite in other New World primates and several species of wild and domestic carnivores were achieved during the year which indicate that this parasite does not have a limited host range.

Rhesus monkeys procured from a dealer in New York, 6 species of Panamanian monkeys, and 5 species of wild carnivores procured locally, as well as domestic dogs and cats were tested for susceptibility to infection by this parasite by administration of 100,000 oocysts orally. Fecal samples were examined daily from day 3 to day 14 post-infection. Appearance of oocysts was considered as evidence of completion of the endogenous cycle in the intestinal epithelium. The results are summarized in Table 7.

Isospora arctopitheci produced patent infections with oocyst production in 12 of 16 species of animals administered oocysts by the oral route. The taxonomic range of hosts producing the endogenous cycle of this parasite covered 2 families of non-human primates, representing all of the 6 species of primates native to Panama, and 5 families of carnivores, 2 domestic and 3 sylvatic. The animals that did not become patent included the Old World rhesus monkey, Macacca mulatta and one carnivore, the olingo, Bassuricyon gabbii (Table 7). Two widely divergent species of experimentally exposed animals, domestic chickens and laboratory mice, not represented in the table, did not become oocyst patent, but were found to act as intermediate hosts for this parasite as reported earlier (Annual Report 1973). The prepatent period on 29 susceptible primates varied from 6 - 10 days (average 7.24), with the patent period ranging from 3 to 55 days. Among the 12 carnivores, the prepatent period varied from 7 - 11 days (average 8.2) and the patent period from 2 - 8 days. In some of the experimental infections large quantities of oocysts were recovered and sporulated. In these cases, Koch's postulates were confirmed when marmosets which

Table 7

Susceptibility of Experimentally Exposed Animals to  
Infection with Isospora arctopithecii

Scientific Name	Common Name	Exposed/ Patent	Prepatent Period (Days)	Patent Period (Days)
<u>PRIMATES</u>				
Family Cebidae				
<u>Aotus trivirgatus</u>	Night monkey*	2/2	6-10	3-5
<u>Ateles geoffroyi</u>	Spider monkey	2/3	8	3
<u>Cebus capucinus</u>	White face*	2/2	8	23
<u>Alouatta villosa</u>	Howler monkey*	2/2	7-8	13
<u>Saimiri oerstedii</u>	Squirrel monkey	2/2	6	3
Family Callithricidae				
<u>Saguinus geoffroyi</u>	Marmoset*	20/22	7-9	55
Family Cercopithecidae				
<u>Macaca mulatta</u>	monkey	0/2	-	-
<u>CARNIVORES</u>				
Family Didelphidae				
<u>Didelphis marsupialis</u>	Common opossum	2/2	8	2
Family Canidae				
<u>Canis familiaris</u>	Dog*	2/2	7-8	2-3
Family Procyonidae				
<u>Nasua nasua</u>	Coatimundi*	2/2	9	1-7
<u>Potos flavus</u>	Kinkajou*	2/2	8-9	2-7
<u>Bassaricyon gabbii</u>	Olingo	0/1	-	-
Family Mustelidae				
<u>Eira barbara</u>	Tayra*	1/1	7	8
Family Felidae				
<u>Felis catus</u>	Cat*	3/4	7-11	1-4

\* Animals yielding oocysts which induced infections in recipient marmosets.

had previously been determined not to be shedding oocysts were administered the recovered oocysts. As indicated in Table 7, in all instances these exposures resulted in patent oocyst infections in the recipient marmosets. It was determined that an inoculum of 100,000 oocysts constituted a marmoset LD<sub>50</sub>.

The present data extend the host range to 4 more primate genera and at least 5 families, representing 6 genera and species of carnivores which would also produce oocyst infections. This lack of host specificity for the endogenous cycle make it unique among coccidian parasites described to date.

Since many of the animals used in these studies were wild caught, the possibility that their exposure to I. arctopitheci "triggered" natural occult isosporan infections cannot be ruled out in all cases. However, the consistent prepatent period would make this appear unlikely. The short sporulation and prepatent period, direct mode of transmission, the wide host range and the often fatal consequences make this a dangerous parasite which could present a hazard of disastrous proportions in institutes that house large numbers of primates and/or other animals.

The potential public health aspects of this parasite should not be overlooked. Zaman, 1967 reported the gibbon, Hylobates klossi to be a susceptible experimental host for I. belli of man. With the wide host range now known for I. arctopitheci, man may also be a potential susceptible host for the intestinal stage of this unique parasite. In view of the ability of this and related parasites to produce extra-intestinal infections in widely divergent orders of vertebrates, the possibility that they might produce toxoplasmosis-like infections in man must also be considered.

#### C. Other Parasitic Infections.

1. Paragonimus. Dr. Ichiro Miyazaki, Professor Emeritus, Kyushu University, Fukuoka, Japan, again studied New World lung flukes as a Visiting Scientist for a month in this laboratory, as well as in other laboratories in Ecuador and Colombia. In Panama, 273 fresh water crabs of 3 species were examined, 52 (19%) were positive for metacercariae, of which 45 were the B-form, 9 the A-form and one C-form, including 3 crabs with dual infections. Experimental infections of cats and rats were carried out in an attempt to verify identities of the 3 morphological types. A-form metacercariae yielded adult Paragonimus peruvianus, confirming the identity of the life cycle stages of this

parasite. No adult forms could be obtained from cats infected by similar administration of B-form metacercariae, nor in one group of rats. It is considered that the B-form probably represents P. caliensis. The identity of the single C-form is likewise not known, but quite likely represents an undescribed species, distinct from the 3 species now shown to occur in Parana: P. peruvianus, and P. caliensis, P. mexicanus.

Project        3A16110PB/1Q : COMMUNICABLE DISEASES AND IMMUNOLOGY  
Task                00 : Communicable Diseases and Immunology  
Work Unit        169 : Field Studies of Leishmaniasis and Other  
                                 Tropical Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA OA 6447	75 07 01	DD-DR&E(AR)436	
3. DATE PREV. SUPPLY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8. DESPN INSTR <sup>f</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUP
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>g</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	61102A	3A161102B71Q	00	170			
11. CONTRIBUTING							
12. CRYPTOCODE	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Zoonotic Diseases of Military Importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
62 06		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRE. YRS		B. FUNDS (in thousands)	
B. NUMBER:				FISCAL YEAR		75	
C. TYPE:				CURRENT		3.5	
D. KIND OF AWARD:				76		4	
E. CUM. AMT.						298.5	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Marvin Rogul, Ph.D.			
				NAME: MAJ Michael G. Groves, DVM			
23. KEYWORDS (Precede with Security Classification Code) <sup>j</sup>							
(U) Zoonoses; (U) Yersinia enterocolitica; (U) Escherichia coli; (U) Rocky Mt. Spotted Fever; (U) Pseudomonas; (U) Mycoplasmales							
23. TECHNICAL OBJECTIVE, 24. APPROV. CH., 25. PROGRAM (Paraphrase individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To study zoonoses of real or potential military significance, to determine the natural history, biological characteristics of agents, to develop suitable diagnostic procedures, treatment and control measures.							
24. (U) Conventional microbiological and chemical technics are used. New procedures are developed as needed.							
25. (U) 74 07 - 75 06 "Wet tail" disease in hamsters and antibiotic "toxicity" may be related in that both were associated with large numbers of E. coli in the small intestine, both could be prevented by oral neomycin, and "wet tail" survivors were not susceptible to lethal doses of penicillin. The intermittent isolation of Yersinia enterocolitica from the feces of experimentally infected puppies for varying periods up to 59 days post-infection affirms that dogs can be infected and serve as potential reservoirs of the agent. Using a monocyte culture technique, Rocky Mt. Spotted Fever was diagnosed in experimentally infected rhesus monkeys as early as the fourth day of febrile illness, even in the presence of mild or inapparent infection. Studies to define the nutritional requirements of the Mycoplasmales revealed that glyoxalate suppressed urease activity and growth of Ureaplasmas, and more quickly sterilized cultures of Acholoplasma and Mycoplasma than Ureaplasmas. The acid end product of S and R strains of Pseudomonas pseudomallei when grown in the presence of glucose was identified as gluconic acid, an intermediate in the glucose oxidation pathway. Under most cultural conditions, only R strains produce an acid end product, oxalic acid. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Zoonotic Diseases of Military Importance

Investigators:

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Description.

Studies are conducted on zoonotic diseases of real or potential military importance and entail epidemiological investigations to define natural history and occurrence of diseases, basic and applied research which bear on development of suitable diagnostic, treatment, and control measures. Principal efforts are with Escherichia coli, Yersinia enterocolitica, Pseudomonads, Mycoplasmatales, and Rickettsia rickettsi.

1. Hamster diarrhea study.

Outbreaks of a watery diarrhea are the principal causes of morbidity and mortality in hamster colonies. Typically, young hamsters which have undergone a stress such as weaning or shipping are most often affected. The disease syndrome has been variously described as "wet tail" after its principal sign of diarrhea, or histopathologically as "proliferative ileitis". Most attempts to establish an etiologic agent for hamster diarrhea have been unsuccessful because organisms isolated from cases of wet tail have failed to reproduce the disease in hamsters. Recently, however, Japanese workers (1) have shown that Bacillus piliformis (Tyzzer's organism) can produce a transmissible enterocolitis in weanling and 8-week-old hamsters. Despite this, it appears probable that other organisms, in addition to B. piliformis, may be responsible for outbreaks of diarrhea among hamsters.

The enzootic nature of wet tail, its predilection for the young, and the sporadic occurrence of this disease following stress situations suggests an indigenous etiology such as Escherichia coli, an organism commonly incriminated as a cause of hamster wet tail. We, as well as others (2), have observed that the intestinal contents of affected hamsters frequently contain very large numbers of E. coli. Furthermore, similarities between the clinical signs of wet tail and a phenomenon in the hamster and guinea pig known as antibiotic "toxicity" have been noted (3). An increased concentration of Gram-negative flora in the gastrointestinal tract of these animals following the

administration of penicillin or other antibiotics primarily effective against Gram-positive organisms appears to be responsible for the antibiotic "toxicity".

Investigations into hamster diarrhea and its possible relationship to *E. coli* were begun this year after 3 outbreaks of wet tail resulted in the deaths of several hundred weanling hamsters received by the WRAIR. Results of these continuing studies are detailed here.

A. Hamster small intestine Escherichia coli concentrations. The small intestines were removed from "normal" hamsters (controls), hamsters with wet tail, and hamsters which had survived an outbreak of wet tail 2 weeks previously. The entire gut contents were removed and serial, 100-fold dilutions of gut contents were prepared in tryptic soy broth. Dilutions were incubated at 37 C for 18-24 hours and then streaked on MacConkey's agar and incubated 18-24 hours at 37 C. Representative lactose-positive colonies were identified using standard techniques. Results of the study showed a striking increase in the small intestinal *E. coli* concentrations of the wet tail group when compared to the normal and survivor groups (Table 1).

B. Effect of prophylactically administered neomycin on the lethal effects of penicillin. Orally administered neomycin has been reported to be effective in reducing the mortality of wet tail (4). Because of the similarities between wet tail and antibiotic "toxicity", we administered 0.5% neomycin ad lib in the drinking water to hamsters given 100,000 units of potassium penicillin G subcutaneously to ascertain if neomycin could prevent the lethal effects of penicillin. Hamsters were divided into four groups. One group received neomycin starting 7 days and a second group received neomycin starting 1 day prior to penicillin treatment. Two control groups, one receiving neomycin and no penicillin and one receiving penicillin and no neomycin, were used. All hamsters receiving neomycin prophylactically starting 7 days prior to penicillin treatment were protected (Table 2). In contrast, hamsters which received no neomycin or neomycin starting 1 day prior to penicillin inoculation died. In 89% of the latter animals small intestine *E. coli* concentrations were  $10^9$  organisms/ml or greater. All hamsters in the 7 day neomycin group had  $10^5$  or less organisms/ml of small intestine contents 2 weeks post inoculation with penicillin.

An additional observation was made which further emphasized the possibility of a relationship between wet tail and antibiotic "toxicity". Three survivors of a wet tail outbreak were inoculated with 100,000 units of penicillin and all three survived. As reported above, this dosage level of penicillin was lethal to 100% of the hamsters (19 of 19) not receiving neomycin treatment.

C. Detection of Escherichia coli enterotoxin using a hamster intestinal loop test. An intestinal loop test for the detection of

E. coli enterotoxin was evaluated in hamsters. The technique used was a modification of one described for the mouse by Punyashthiti and Finkelstein (5). The method was unsatisfactory, however, due to the high number of false positive reactions. Isolates will be evaluated in the rabbit ileal loop test.

In summary, small intestinal concentrations of E. coli are greatly increased in hamsters with wet tail. Similarities between wet tail and so-called antibiotic "toxicity" were observed. Both conditions could be prevented with oral neomycin, both produced similar changes in the small intestinal flora, and hamsters recovered from wet tail were not susceptible to the lethal effects of penicillin.

## 2. Experimental Yersinia enterocolitica infections in dogs.

Yersinia enterocolitica infections range from asymptomatic to septicemic but most commonly present themselves as a diarrhea or mesenteric lymphadenitis. Large outbreaks of diarrheal disease among school children in Japan have been attributed to Y. enterocolitica (6), and clusters of cases within families have also been reported. The organism has been recovered from a variety of wild and domestic animals. Human infections have, in at least one instance (7) and probably a second (8), been associated with Y. enterocolitica infections in young puppies.

Because the dog may be a potentially important transmitter of Y. enterocolitica infections to man, a cooperative study was conducted with the Division of Pathology defining the infection in young dogs. The microbiological aspects of this study are reported here.

Ten 6 to 8-week-old puppies of mixed breeding were infected with  $10^{12}$  Y. enterocolitica organisms each. Three puppies each were inoculated subcutaneously and intratracheally, and 4 puppies were infected per os. Feces were cultured twice weekly beginning 2-weeks prior to infection.

Results of fecal cultures are summarized in Table 3. Seven of the puppies had intermittently positive cultures through the 27th to the 59th day post infection. One puppy had a single positive on day 6 post infection and two puppies remained negative throughout the entire experiment. Only 8% of the fecal cultures from the subcutaneously infected dogs yielded Y. enterocolitica compared to 31% of the dogs infected orally and 42% of the dogs infected intratracheally. Synovial fluid, liver, mesenteric and prescapular lymph nodes, thyroid gland, duodenum and ileum were cultured at postmortem, 8 to 10 weeks post infection. One puppy (#9) had positive cultures from both sets of lymph nodes, the duodenum, and the ileum. Three puppies (#2, #4, and #7) had Y. enterocolitica cultured from only the ileum, and 6 puppies had no positive postmortem cultures.

In summary, the intermittent isolation of Y. enterocolitica from the feces of experimentally infected puppies for varying periods up to 59 days post infection affirms that dogs can be infected and serve as potential reservoirs of the agent. Infection via oral or respiratory routes appeared to be more efficient than subcutaneous infection.

### 3. Early diagnosis of Rocky Mountain spotted fever using the primary monocyte culture technique.

Rickettsial disease continues to be of great actual and potential significance in the world today. During the last 6 years the incidence of Rocky Mountain spotted fever (RMSF) in the United States has increased from an average of 400 cases per year in the 1960's to 774 cases in 1974 (3,10). Five to 7 percent of the infected patients died (11). As with other rickettsial diseases, diagnosis is based initially on clinical findings because serological confirmation is available only late in the illness. The development of a rapid laboratory diagnostic technique for RMSF would be of great immediate usefulness.

Buhles et al. (12) identified Rickettsia rickettsi in cultures of circulating monocytes from 4 experimentally infected guinea pigs at 10 to 14 days after inoculation. Gambrill and Wisseman (13) have described in detail both the morphology of cultured macrophages and the growth of typhus rickettsiae in experimentally infected human macrophage cultures. The characteristic adherence of monocyte cells to a glass surface was used by both groups of investigators to select and cultivate this subpopulation of circulating cells.

This study was performed to further investigate the usefulness of the monocyte culture technique as a diagnostic procedure for human rickettsial disease.

Four, 10 to 12 pound male rhesus monkeys (Macaca mulatta) were divided into 2 groups and inoculated subcutaneously with 1 ml of a yolk sac suspension of R. rickettsi (Sheila Smith strain) adjusted such that 2 received  $10^5$  and 2  $10^4$  plaque forming units (PFU). Monkeys were monitored by daily physical examination and measurement of rectal temperatures. Complete blood count, platelet count, serology by complement fixation (14), monocyte culture, and plaque assay (15) were performed prior to inoculation and on the 2nd, 4th, and 10th days after the onset of fever. Because temperature variation with excitement is seen in monkeys, the designation of "second febrile day" was made only after 2 consecutive days of a rectal temperature greater than 104 F.

The monocyte culture was performed basically as described by Nyindo et al. (16). Sixteen ml of blood were drawn aseptically from each monkey into a heparinized (20 units/ml) syringe containing 8 ml of 3 % dextran (Pharmachem) in normal saline. The syringe was inverted

with the needle end up and allowed to stand for 30 to 60 min until the supernatant was clear. The supernatant fluid, consisting of a mixture of dextran and white blood cells, was transferred to a 2nd syringe and agitated to distribute the cells throughout the mixture. One ml of this mixture was placed into each of 8 Leighton tubes containing alcohol-washed coverslips. Culture tubes were incubated at 35 C in an atmosphere of 5% CO<sub>2</sub> for 24 hrs, then washed 3 times with Hanks' balanced salt solution to remove unattached cells. Cultures were maintained in Eagle's minimum essential medium with Earle's balanced salt solution supplemented with 1% glutamine and 20% homologous heat inactivated monkey serum. Cultures were refed every 3 days. No antibiotics were employed. Two coverslips were removed from Leighton tubes on the 2nd, 5th, 8th, and 10th day of culture. One coverslip was stained by the Gimenez (17) technique and 1 was stained with specific fluorescein-conjugated anti-*R. rickettsi* rabbit serum. Specificity of the antiserum was established before use in the study. Uninfected monocyte cultures were used for control purposes. Identification of rickettsiae was based on typical morphological and tinctorial or immunofluorescent characteristics of organisms observed intracellularly. When possible, 250 cells were counted before calling a culture negative.

Both monkeys receiving the higher dose of rickettsiae (1 and 3) became febrile by the 4th post-inoculation day (Fig. 1). Monkey 1 remained febrile for 3 days, showed slight decreases in appetite and activity, but did not develop a rash. Monkey 3 remained febrile, became increasingly lethargic and anorectic, and developed a generalized petechial rash on the 4th febrile day. The monkey died 1 day later. Postmortem histological studies demonstrated diffuse necrotizing vasculitis, which is typical of *R. rickettsi* infection.

In the lower dose group, monkey 2 was febrile by the 4th post-inoculation day, but became afebrile within 4 days. No clinical signs of the disease were noted other than the fever. Monkey 4 had an elevation in temperature for only 1 day and did not become clinically ill. Since the hyperthermia could have been induced by excitement, this monkey was not considered to have become febrile.

The estimated levels of rickettsemia by the plaque-formation technique are shown (Table 4). Transiently febrile monkeys 1 and 2 had approximately 300 rickettsiae/ml on the 2nd febrile day and none subsequently. The monkey that succumbed (#3) had a persistent rickettsemia. Monkey 4, that never became febrile, was not rickettsemic when tested on day 10.

All of the surviving monkeys developed specific complement fixing antibody titers by the 10th febrile day (Table 5).

Moderate anemia, leukopenia, and thrombocytopenia developed in the 3 febrile monkeys, while monkey 4 never showed any significant hematologic changes.

The results of staining of coverslip cultures of monocytes by the Gimenez and direct immunofluorescent techniques are shown (Table 6). Rickettsiae were demonstrated by fluorescent staining as early as the 4th and not later than the 6th day after the onset of fever. The fluorescent antibody technique was more sensitive for observing rickettsiae very early in the disease, but by 7 to 9 days into the illness, organisms were clearly discernable by the Gimenez staining technique. Surprisingly, rickettsiae also were demonstrated from a culture taken the 13th post-inoculation day from monkey 4, which never became febrile.

In summary, the 2 levels of rickettsial inoculum used produced a full spectrum of clinical illness from asymptomatic to fulminating and fatal. In the fatal case, clinical and histological observations supported a diagnosis of RMSF similar to that seen in seriously ill humans. The asymptomatic infection seen in monkey 4 was substantiated by significant increases in specific complement fixing antibodies and demonstration of organisms in monocyte culture preparations. The period of rickettsemia detected by plaque assay correlated well with the febrile response. This technique may have minimized levels of rickettsemia, since detection of small numbers of plaques in undiluted blood was obscured by the presence of red blood cells.

The small size of the monkeys used and attendant problems with anemia limited the volume of blood obtained and the frequency of sampling for monocyte culture. Nevertheless, the animals receiving  $1 \times 10^5$  PFU of rickettsiae had positive cultures which allowed a diagnosis of RMSF to be made as early as 4 days into their illness. The group receiving  $1 \times 10^4$  PFU was more difficult to diagnose but was positive by febrile day 6. It is possible that with more frequent sampling and larger numbers of replicates, the time required for diagnosis could be reduced further.

Our studies concentrated on diagnosis, from the onset of febrile illness to days 9 to 12 when serological data becomes clinically useful. Although all animals had positive cultures by 12 days, data from this time onward is not reported because it lacked diagnostic significance.

#### 4. Nutritional requirements of the Mycoplasmatales.

Ureaplasma urealyticum is an organism which is primarily isolated from the urogenital tracts of man and animal. The organism is suspected of being an opportunist implicated in the production of non-specific urethritis, bladder stones, infertility and infant weight loss (18). The strains of this species are distinguished in the

Mycoplasmatales by the production of a urease and their tiny colonial size on agar media and that is why they were originally designated as T (for tiny)-PPLO or T-mycoplasmas. But, they are similar to the Mycoplasmas because they lack a cell wall and because both need cholesterol for growth.

Except for urea and cholesterol, there are no other defined nutrients which are known to provide energy or sustain these organisms. When urea is incorporated into culture broths, it is quickly hydrolysed and copious amounts of free ammonia are released into the medium. This rapid production of ammonia raises the pH of the media and is so toxic to the organisms that these so-called "suicidal" broth cultures must be subcultured every 18-24 hours. Nonetheless, good use can be made of this urease to detect growth. For when ammonia is released after urea hydrolysis, the pH indicators incorporated into growth media demonstrate a striking color change. Quite often microbial cultures which produce copious quantities of ammonia are better maintained on media containing a carbohydrate energy source, such as a hexose. The acidic end products of carbohydrate metabolism are then able to scavenge, incorporate or neutralize the toxic ammonia.

It was the purpose of this project to find some nutrient which would prolong and increase the growth of the Ureaplasmas in broth cultures. The strain T-960 was used in these studies. Growth was qualitatively monitored by observing a color change of the phenol red pH indicator incorporated into broth cultures. For quantitative studies, two methods were used. In the first method, serial ten-fold dilutions of broth cultures were inoculated onto A-6 agar. The assay was based on the counting of colony forming units (CFU) (19). In the second method 0.2 ml amounts of broth cultures were serially diluted in 1.8 ml of pH6 urea broth containing phenol red. The last tube to show an alkaline reaction was designated the end point color changing unit (CCU).

A survey was first conducted with a variety of potential energy sources incorporated into the pH indicator media of Taylor-Robinson, et al. (20). Any departure from the pH progression of the control broth was investigated. The organisms in the aberrant cultures were recultured in experimental broths. They were also recultured in the control broths to determine viability. The experimental media were:

3% glucose	Initial pH	6.7
3% xylose	"	6.6
3% ribose		6.6
1% sodium pyruvate		6.7
1% sodium succinate		6.7
1% 2-oxoglutarate		6.5

It was found that both ribose and sodium pyruvate retarded pH increases in the media. On subculture into plain CCU broth, it was found that ribose was immediately toxic to the organism, but pyruvate kept the pH down without killing the organisms for 2-3 days. At that time, we assumed that this was a prolongation of the culture although it could also have been considered a suppression of growth increase. The pyruvate effect was considered the most interesting phenomenon to investigate. Broth cultures containing 1% pyruvate at pH6 and 6.4 were compared to plain broth pH6 for 50 hours. The growth was assayed by counting CFUs. Pyruvate was assayed enzymatically (Calbiochem pyruvic acid determination Cat. No. 8852). The results are shown in Figs. 2 and 3. It was obvious that pyruvate could increase the length of the lag phase and simultaneously depress the pH rise for 30-50 hours. During this time the pyruvate steadily disappeared, but obviously hindered multiplication. On subsequent pyruvate assays as well as this one we found that over a period of hours or days the amount of pyruvate in uninoculated broth was not always what had been the initial concentration, and the pH of the medium increased with the decrease in pyruvate. The unpredictable disappearance of pyruvate caused erratic CFU assays. It was later found that horse sera varied in their content of lactic acid dehydrogenases and other enzymes acting on pyruvate, e.g. some pyruvate transaminases. It was also found that when either pyruvate or 2-oxoglutarate was added to the media and adjusted to pH6, the pH subsequently rose and if readjusted rose again. Heating sera to partial denaturation (64 C/30 min) still did not destroy the enzymes acting on pyruvate. A number of animal fluids were tested (Table 7) and it was found that the agamma globulin horse serum caused hardly any rise in pH for either pyruvate or 2-oxoglutarate. Interestingly, the oxamate treatment of the horse serum seemed to extract more 2-oxoglutarate enzymes than pyruvate enzymes. However, some enzyme activity did remain. The only animal fluid which did not cause any increase in pH was bovine serum fraction A (Difco). Since this component does not support consistent T-960 growth, pyruvate and 2-oxoglutarate were left out of the subsequent metabolite surveys.

A search of the literature revealed that in those cases where pyruvate was known to inhibit bacterial growth (21) it was thought to be due to inhibition of isocitrate lyase, the first enzyme of the glyoxalate cycle. To pursue this hypothesis, intermediates of the Tricarboxylic acid (TCA) and glyoxalate cycles were introduced into the standard Shepard media pH6, containing urea (Table 8). In accordance with our suspicions, the three compounds which depressed or prolonged the Ureaplasma growth were glyoxalate, succinate and malate. These compounds or their physiological derivatives are also known to depress isocitrate lyase activity. Glyoxalate was especially effective in suppressing the pH (inhibition of urease) and growth, although viable organisms were evident for a number of days.

As a note of caution, it should be mentioned that glyoxalate catabolite repression may be involved in glycine synthesis. It is also feasible that glyoxalate feedback inhibits purine degradation, because glyoxalate and urea are major end products of purine catabolism.

The effect of glyoxalate was then tested on representatives of physiologically different Mycoplasma and Acholeplasma species represented as follows:

Carbohydrate fermenting; non-sterol requiring

A. laidlawii A  
A. laidlawii PG9

Carbohydrate fermenting; sterol requiring

M. pneumoniae FH  
M. pneumoniae Mac  
M. fermentans PG 18

Non-fermenting; sterol requiring

M. hominis PG21  
M. arthritidis PG6

These organisms were inoculated into a Hayflick's broth (70 parts PPLO broth base, 20 parts horse serum, 10 parts of 25% yeast extract, 1,000 units/ml penicillin final concentration) containing 1% glyoxalate. Horse serum was omitted from the Acholeplasma cultures. An effort was made to maintain the initial pH at 7. Ten-fold dilutions of the test media were made in appropriate CCU media (1-arginine pH6 for non fermenters and glucose pH 7.8 for fermenters). Although the uninoculated glyoxalate media usually stayed at pH7, the control media often rose from pH7 to pH7.8. Readjustment was only temporarily effective; the pH eventually rose to 7.8 again. All of the Mycoplasma and Acholeplasma cultures were auto-sterilized by 1% glyoxalate within 18-24 hrs. after inoculation; whereas, the control cultures remained viable for days. This immediate inhibition was in marked contrast to the Ureaplasma cultures where increases in growth were inhibited but culture viability was maintained for days at its initial inoculated level in glyoxalate broth.

The minimal inhibitory concentrations (MIC) of glyoxalate were assayed for a number of Mycoplasmatales (Table 9). It would appear from this assay that the Ureaplasma strains are among the more resistant organisms and the M. pneumoniae among the more susceptible.

Future experiments will be concerned with determining the metabolic or biochemical site(s) on which glyoxalate is acting.

In summary, glyoxalate was found to suppress the urease activity and growth of Ureaplasmas. It was also found to more quickly sterilize Acholeplasma and Mycoplasma cultures than Ureaplasmas. The probability that different metabolic sites are being repressed in the three genera is currently under investigation.

#### 5. Regulatory mechanisms in smooth and rough strains of Pseudomonas pseudomallei.

The different biochemical activities of smooth and rough strains of Pseudomonas pseudomallei are now known to affect the proper laboratory diagnosis of isolated strains. Throughout these and previous investigations, strains 165 and 7815 were studied as culturally stable types of the smooth and rough strains, respectively. Previously it was shown that the so-called "suicidal" (smooth) strains were killed by the accumulation of ammonia in the culture medium, whereas the "non-suicidal" (rough) strains detoxified the ammonia by excreting oxalic acid. The major metabolic pathway for assimilation of  $\text{NH}_4^+$  in P. pseudomallei appeared to be via the enzymatic reaction of pyruvate with  $\text{NH}_4^+$  to form alanine. Further, the rough strain 7815 was able to reduce nitrate to a gas, whereas the smooth strain 165 was unable to reduce nitrate beyond nitrite. Investigations of the metabolic pathways of  $\text{NH}_4^+$  as they relate to the laboratory phenomenon of smooth and rough colonies continued. Using a variety of nutritional substrates, investigations were designed to define the transaminase and nitrite and nitrate reductase activity of the two strains.

A. Nitrogen metabolism of P. pseudomallei. The presence or absence of selected transaminases in sonicated enzyme preparations of both strains was determined by thin-layer chromatography after incubation with the proper amino acid and keto-acid combination (Table 10). As expected, the amino group was transferred between the precursors of the major amino acid pathways as outlined in Fig. 4. However, the absence of alanine-oxoglutarate and alanine-oxalacetate aminotransferases in strain 165 was surprising. The initial amino disseminating step (Fig. 4) after its incorporation into alanine is at this time unclear. The crude assay procedure may not have detected low levels of alanine transaminase activity or a more circuitous route may be involved in transferring the amino group from alanine to the biosynthetic pathways.

Differentiation of strain 7815 from strain 165 by their utilization of nitrogenous compounds was investigated. Sixty-four nitrogen-containing compounds were tested for their ability to support the growth of the two strains of P. pseudomallei. The growth media consisted of a chemically defined basal medium in which the test compound was the sole carbon source, the sole carbon and nitrogen source, or the sole nitrogen source. Growth was monitored by following the daily change in optical density at 660 nm of broth

cultures of both organisms. Utilization of the compound was defined as an increase in O.D. which continued after three or more serial passages in the same medium. Thirty-six of the 60 compounds tested were used as a carbon source by both strains (Table 11). In these assays  $(\text{NH}_4)_2\text{SO}_4$  was incorporated in the medium as a nitrogen source. Only one compound, DL-amino-N-butyric acid, supported the growth of strain 7815 and did not support the growth of strain 165. The only other differences between the strains was that strain 7815 generally appeared to grow faster and to greater cell mass in the 36 media containing the growth sustaining compounds than strain 165; and when D-glucosamine was the substrate, strain 7815 grew immediately, whereas, strain 165 grew after a lag of 2 or 3 days. Twenty-two amino acids and D-glucosamine were then tested as carbon and nitrogen sources (Table 12), eliminating  $(\text{NH}_4)_2\text{SO}_4$  in the medium. Only glycine and leucine failed to support the growth of both strains.

Ammonium, nitrate and nitrite were also tested as sole nitrogen sources utilizing lactate as a carbon source. Growth of both strains occurred in all three types of media.

Utilization of nitrogen-containing substrates by both strains in these experiments appeared to be identical. The failure of both strains to utilize glycine as a sole carbon and nitrogen source was not surprising, since both organisms lack transaminases for glycine-oxalacetate, glycine-oxoglutarate and serine-pyruvate, which are necessary for the utilization of the amino group from glycine.

Since previous reports (22) indicated that there was a strain difference in the utilization of nitrate and nitrite, and since no strain differences were observed using nitrate and nitrite as sole nitrogen sources, investigation of the enzymes nitrate reductase and nitrite reductase are currently in progress.

B. Carbohydrate metabolism of P. pseudomallei. It had been previously found that the acidic end product of rough strain 7815 was oxalic acid. Under most cultural conditions only the rough strain (7815) produced this metabolite; however, when glucose was present, both the rough strain (7815) and the smooth strain (165) produced an acid product other than oxalic acid.

Purification of this acid end product of glucose-grown Pseudomonas pseudomallei for identification by UV and IR spectroscopy or gas chromatography was fraught with technical difficulties. It is known that 2-ketogluconate accumulates in the media of many Pseudomonads when they are grown in the presence of glucose. Previously, 2-ketogluconate was sought and not found as the acid product of the two strains under study; however, this did not exclude another intermediate of the hexose oxidation pathway from accumulating in the medium. The only likely candidate was the immediate precursor of 2-keto-

gluconate, gluconate. When the compounds 2-ketogluconic acid and gluconic acid were used as standards and were chromatographed on two different types of TLC plates and in two different solvent systems, in all cases only gluconic acid migrated like the unknown. Thus strains 7815 and 165 of Pseudomonas pseudomallei accumulate gluconic acid in the media when grown in the presence of glucose.

In summary, investigation of the nitrogen metabolism of a smooth and a rough strain Pseudomonas pseudomallei was continued. There was no significant difference between the two strains in their carbon and nitrogen source utilization. Alanine transaminases which were present in strain 7815 were undetected in strain 165; however, both strains possessed a variety of transaminases for disseminating amino groups into synthetic pathways. Nitrate or nitrite was metabolizable as a sole nitrogen source for either strain.

The acid end product of glucose-grown Pseudomonas pseudomallei strains 7815 and 165 was identified by thin layer chromatography to be gluconic acid, an intermediate in the glucose oxidation pathway.

Table 1. Concentrations of Escherichia coli in the small intestines of hamsters

Minimum number of <u>E. coli</u> /ml of small intestine contents	Hamster group		
	Normal	Wet tail	Wet tail survivors
$10^1$ or less	14/21 (67%)	2/28 ( 7%)	6/9 (67%)
$10^3$	4/21 (19%)	4/28 (14%)	2/9 (22%)
$10^5$	2/21 (10%)	11/28 (39%)	1/9 (11%)
$10^7$	1/21 ( 5%)	3/28 (11%)	0/9 ( 0 )
$10^9$ or greater	0/21 ( 0 )	8/28 (29%)	0/9 ( 0 )

Table 2. Prevention of the lethal effects of penicillin in hamsters with neomycin

Days on neomycin prior to treatment with penicillin	Penicillin <sup>1</sup> treatment	Number of survivors/total
0	+	0/19
1	+	0/8
7	+	11/11
7	-	5/5

<sup>1</sup>.100,000 units subcutaneously.

Table 3. Results of fecal cultures for Yersinia enterocolitica

Day post infection	Route of infection									
	Subcutaneous			Intratracheal			Per os			
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
3				+				+		
6		+	+	↓	+		+	↓		+
10			↓	↓	↓	+	↓	↓		
13				↓	↓	↓	↓			+
17				↓						↓
20				↓	+		+			
24			+				↓			
27			↓	+	+	+	↓	↓		↓
31							↓	↓		↓
34							↓			
38							↓			
41										
45					+		+			
48				+	↓		↓			
52	PM				↓		↓		PM	PM
55		PM	PM	PM	↓					
59					PM	PM		PM		
62										
66										
69							PM			

PM - Postmortem examination performed and organs cultured.

Table 4. Blood titers<sup>1</sup> of *Rickettsia rickettsi* in experimentally infected monkeys by day after onset of illness

Monkey number	Infecting dose (PFU) <sup>1</sup>	Day after onset of febrile illness when culture taken		
		2	4	10
1	1 x 10 <sup>5</sup>	3 x 10 <sup>2</sup>	0	0
3	1 x 10 <sup>5</sup>	9 x 10 <sup>2</sup>	2 x 10 <sup>2</sup>	monkey died
2	1 x 10 <sup>4</sup>	3 x 10 <sup>2</sup>	0	0
4	1 x 10 <sup>4</sup>	ND <sup>2</sup>	ND	0

<sup>1</sup>•Titers expressed as plaque forming units per ml of whole blood  
<sup>2</sup>•ND = not done

Table 5. Serum antibody titers to *Rickettsia rickettsi* by days after onset of febrile illness

Monkey number	Reciprocal of complement fixing antibody titer				
	Control	Days after onset of febrile illness			
		2	4	10	20
1	< 10	<20	≥ 20	≥ 320	≥ 320
3	< 10	<10	10	monkey	died
2	< 10	20	20	≥ 320	≥ 320
4	< 10	<10	ND <sup>1</sup>	≥ 320	≥ 320

<sup>1</sup>•ND = not done

Table 6. Detection of Rickettsia rickettsi in experimentally infected monocyte cultures by two different staining techniques and time after onset of illness

Infecting dose	Monocyte staining technique	Days after onset of febrile illness <sup>1,2</sup>					
		C <sup>3</sup>	4	6	7	9	12
1 x 10 <sup>5</sup> plaque forming units	Gimenez	-	-	-	+	ND	+
	Fluorescent	-	+	+	+	ND	+
1 x 10 <sup>4</sup> plaque forming units	Gimenez	-	-	+	ND	+	+
	Fluorescent	-	-	+	ND	+	+

<sup>1</sup>. Cultures read on days 4 and 7 were planted on day 2, whereas cultures read on days 6, 9, and 12 were planted on day 4 after onset of febrile illness.

<sup>2</sup>. + indicates morphologically distinct organisms observed intracellularly; - indicates no organisms observed; and ND indicates not done.

<sup>3</sup>. Control

Table 7. Changes in pH of media containing various animal fluids and 1% pyruvate or 2-oxoglutarate

Time <sup>1</sup> hr	pH of media containing 1% pyruvate and 20% of						
	Horse serum	Agamma globulin calf serum	Bovine amniotic fluid	Fetal bovine serum	Agamma globulin horse serum	"Oxamate" absorbed horse serum	Bovine serum fraction A
0	6.0	6.0	6.0	6.0	6.0	6.0	5.9
6	6.7	6.4	6.7	6.6	6.1	6.5	5.8
	pH of media containing 1% 2-oxoglutarate						
0	6.0	6.0	6.0	6.0	6.0	6.0	5.9
6	6.5	6.3	6.5	6.4	6.1	6.2	5.9

<sup>1</sup>. Incubation time at room temperature (25 C)

Table 8. Effect of TCA or glyoxalate cycle intermediates on growth and pH of Ureaplasma urealyticum strain T-960 cultures

1% Metabolite in medium <sup>1</sup>	Effect on pH	Effect on culture growth
Sodium acetate	No effect	None
Sodium glyoxalate	pH does not rise	Growth remains stationary. No increase over initial inoculum for 3 to 4 days.
Sodium citrate	No effect	No effect
Pottasium DL-isocitrate	No effect	No effect
Succinic acid	pH rise initially retarded	Late growth was slightly prolonged.
Fumaric acid	No effect	No effect
L-Malic acid	pH rise is retarded	Early growth was prolonged.
Control <sup>1</sup>	Rapid pH rise	Rapid growth. Cultures were generally sterile in 24-36 hr.

<sup>1</sup>. Basal medium was Shepard's urea broth pH6. Growth was assayed by CCU.

Table 9. The minimal inhibitory concentrations of glyoxalate for Ureaplasma and Mycoplasma broth cultures

Strain	Concentration of organisms	Minimal inhibitory concentration glyoxalate
	CCU/ml	mg/ml
<u>U. urealyticum</u>		
T-960	6	5
T-58CB4	4	5
<u>M. hominis</u>		
PG 21	6	2.5-5
<u>M. arthritidis</u>		
H 606	5	2.5
PG 6	4	0.6-1.25
<u>M. pneumoniae</u>		
FH	6	0.6
Mac	6	0.6

Table 10. Transaminase activity of sonicated preparations from Pseudomonas pseudomallei strains 7815 and 165

Transaminase	Soluble fraction		Particulate fraction	
	165	7815	165	7815
L-alanine - 2-oxoglutarate aminotransferase	-	+	-	-
L-alanine - oxalacetate aminotransferase	-	+	-	-
glutamate - pyruvate aminotransferase	-	-	-	-
glutamate - glyoxylate aminotransferase	-	-	-	-
glutamate - oxalacetate aminotransferase	+	+	-	-
aspartate - pyruvate aminotransferase	-	-	-	-
aspartate - glyoxylate aminotransferase	-	-	-	-
aspartate - 2-oxoglutarate aminotransferase	+	+	-	-
glycine - 2-oxoglutarate aminotransferase	-	-		
glycine - oxalacetate aminotransferase	-	-		
serine - pyruvate aminotransferase	-	-		

+ = transaminase activity present  
 - = transaminase activity not detected

Table 11. Growth of smooth (165) and rough (7815) strains of *Pseudomonas pseudomallei* on nitrogen containing compounds used as a sole source of carbon in broth cultures<sup>1</sup>

Growth		No growth	
Simple amino acids	Others	Simple amino acids	Others
B-alanine L-alanine L-arginine L-aspartic acid L-asparagine cysteine cystine L-djenkolic acid L-glutamic acid D-glutamic acid L-glutamine glycine L-4-hydroxyproline L-isoleucine DL-kynurenine leucine L-lysine DL-phenylalanine L-proline L-serine L-threonine L-tryptophan L-tyrosine DL-valine	allantoin anthranilic acid betaine choline 2,6-diaminopurine D-glucosamine guanine hippuric acid hypoxanthine protamine uric acid xanthine	L-canavanine 3,5-diiodo-L-tyrosine L-histidine DL-homocystine DL-hydroxylysine DL-methionine DL-norleucine	acetamide adenine p-aminobenzoic acid p-aminohippuric acid p-aminosalicylic acid citrulline creatinine cytosine p-dimethylaminobenzaldehyde glycylglycine methylamine niacinamide spermine thiourea thymine tryptamine

<sup>1</sup>.  $(\text{NH}_4)_2\text{SO}_4$  incorporated in the medium as a nitrogen source.

Table 12. Growth of smooth (165) and rough (7815) strains of Pseudomonas pseudomallei on nitrogen containing compounds as nitrogen and carbon sources in broth cultures

<u>Growth</u>	<u>No growth</u>
B-alanine	glycine
L-alanine	leucine
L-arginine	
L-aspartic acid	
L-asparagine	
cysteine	
cystine	
L-djenkolic acid	
D-glucosamine	
L-glutamic acid	
D-glutamic acid	
L-glutamine	
L-4-hydroxyproline	
L-isoleucine	
L-lysine	
DL-phenylalanine	
L-proline	
L-serine	
L-threonine	
L-tryptophan	
L-tyrosine	
DL-valine	

Figure 1. Febrile response of four monkeys experimentally inoculated with Rickettsia rickettsi

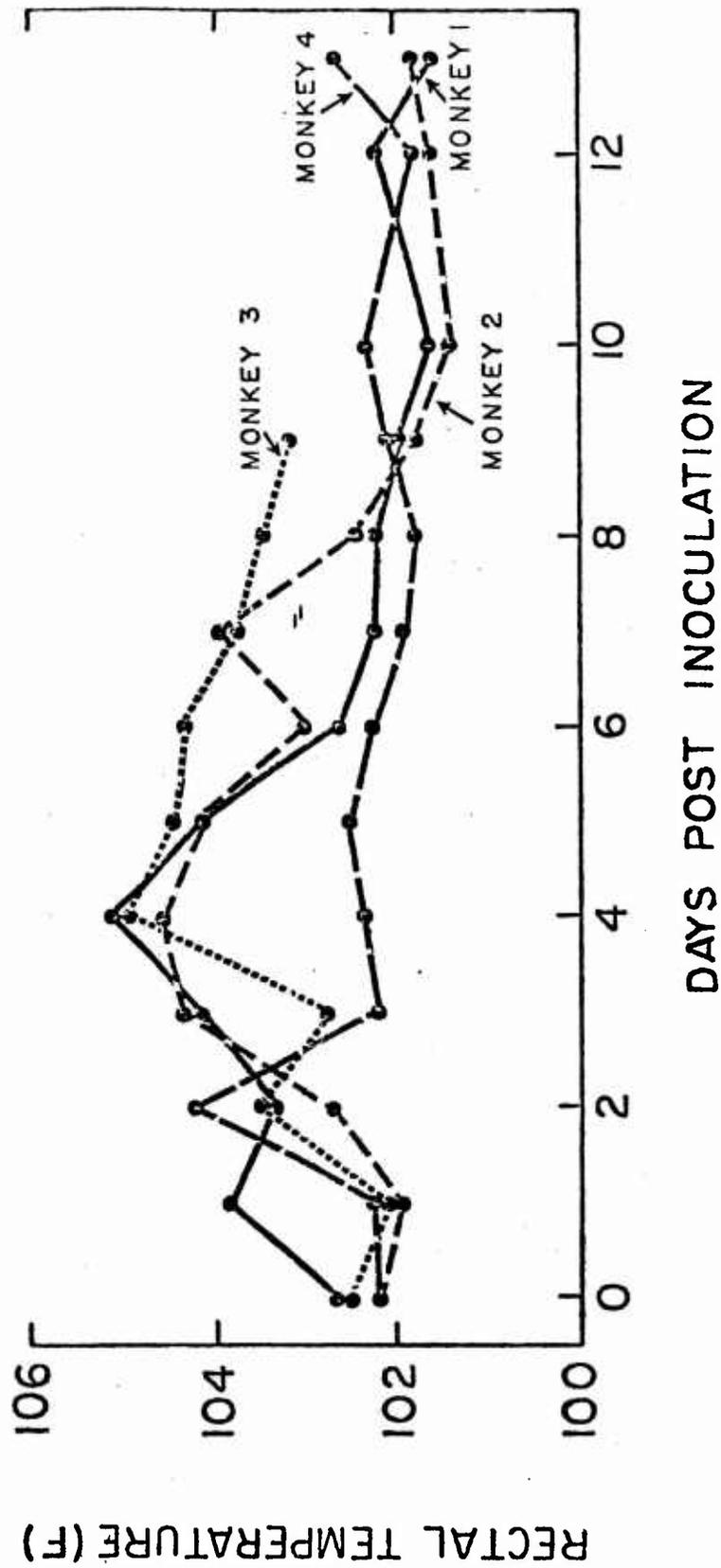


Figure 2. The growth and pH of *U. urealyticum* T-960 cultures in broth with and without pyruvate.

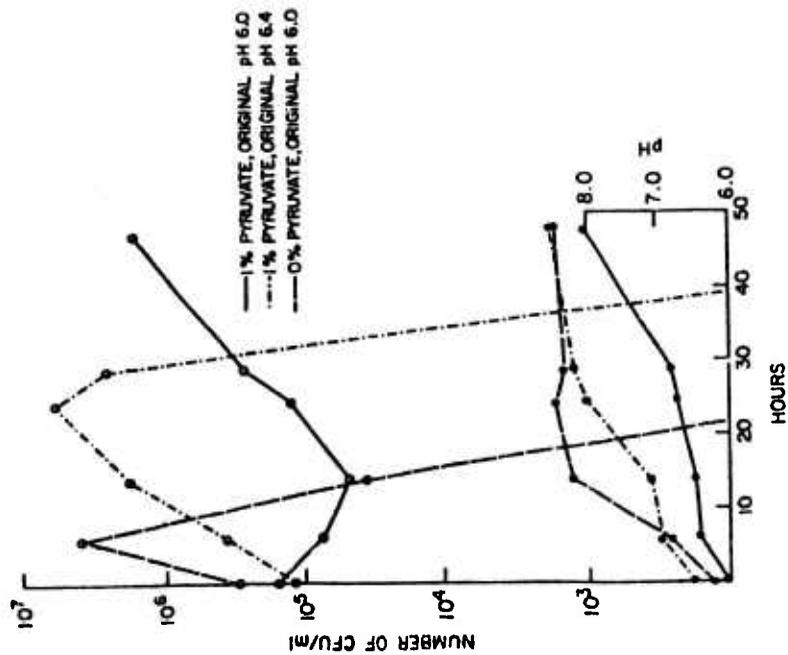


Figure 3. The assay of pyruvate in the pyruvate containing cultures shown in Figure 2.

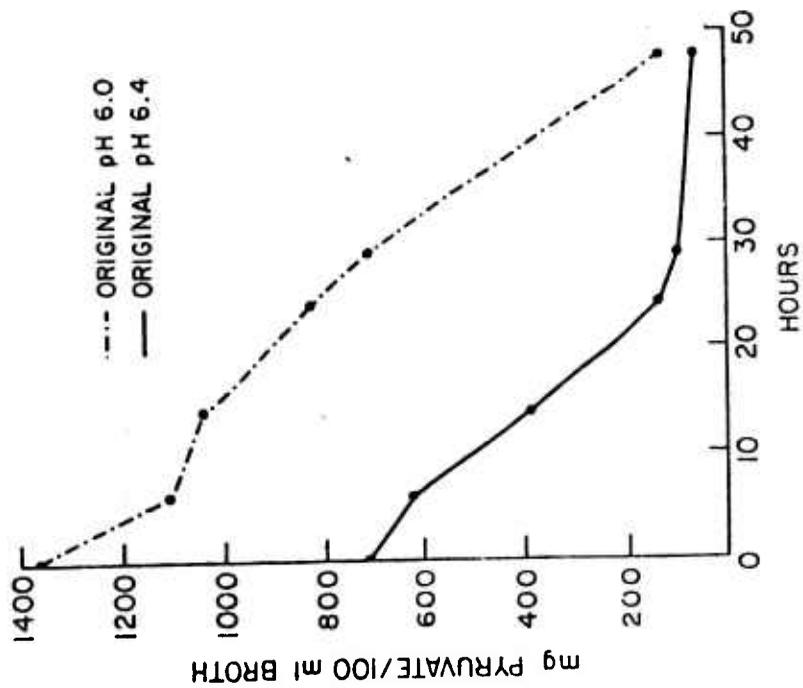
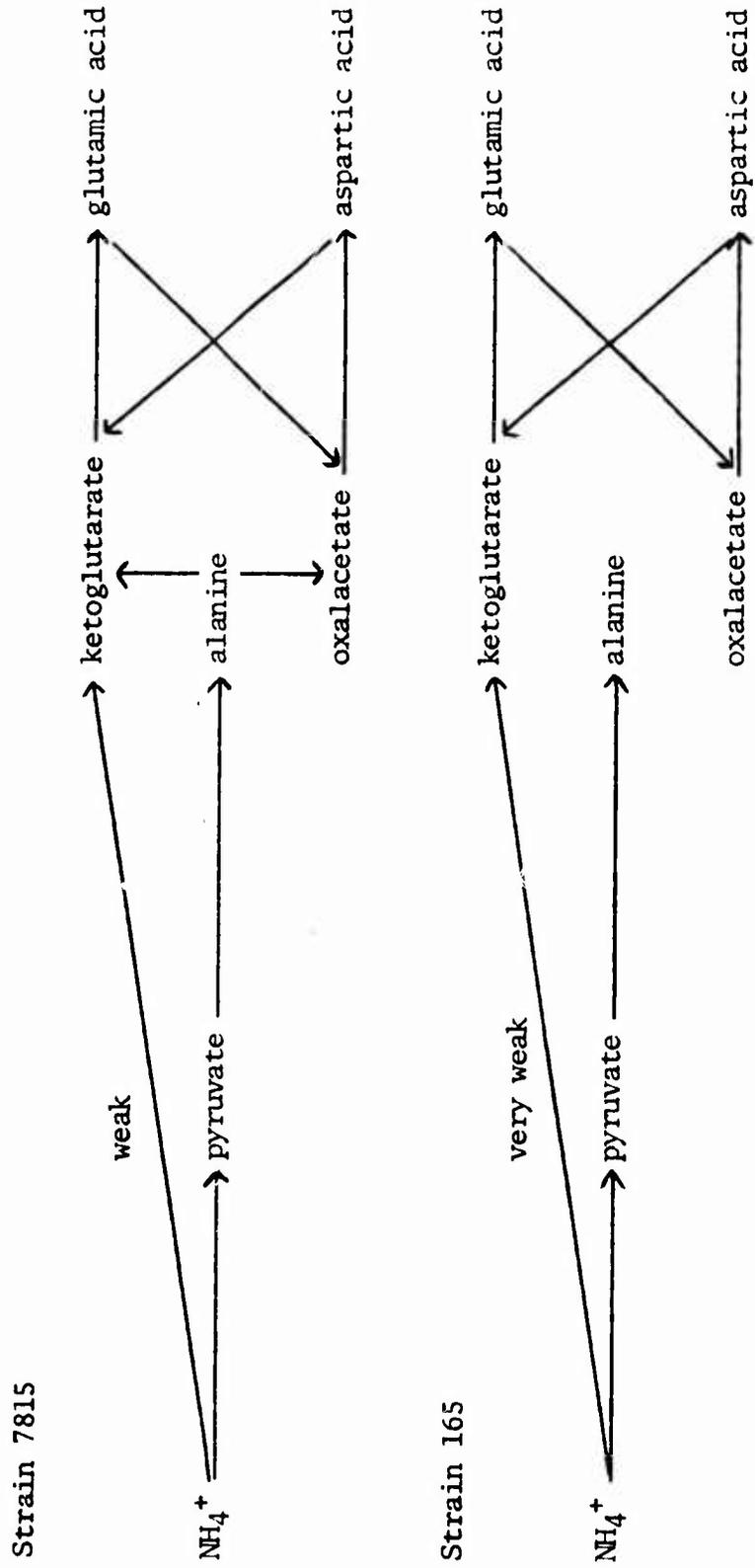


Figure 4. Pathways of ammonium incorporation in Pseudomonas pseudomallei strains 7815 and 165



Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Zoonotic Diseases of Military Importance

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
				DA OA 6448	75 07 01	DD DR&E(A)R)6.10	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCT <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8A. DDU'S INSTN	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>f</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
6. PRIMARY	61102A	3A161102B710	00	171			
7. CONTRIBUTING							
8. CONTRACTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>g</sup>							
(U) Development of Biological Products							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>h</sup>							
010100 Microbiology							
13. START DATE	14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
58 05	CONT		DA		C. In-House		
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				b. PREVIOUS		c. FUNDS (in thousands)	
d. NUMBER: NA				e. FISCAL YEAR		75	
f. TYPE:				g. CUM. AMT.		5	
h. KIND OF AWARD:				76		5	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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24. KEYWORDS (Precede each with Security Classification Code)							
(U) Bioassays; (U) Biological Products; (U) Dengue Virus Vaccine; (U) Febrile Antigens; (U) Meningococcal Vaccines; (U) Plague Vaccine; (U) Vaccines							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Precede individual paragraphs identified by number. Precede rest of each with Security Classification Code)							
23. (U) This work unit is concerned with the development of manufacturing methods and the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.							
24. (U) Increased effectiveness and reduced reactivity are pursued by use of new physical and chemical methods for processing. Improvement in stability and reduction of logistic requirements are achieved by application of modern freeze-drying and packaging techniques.							
25. (U) 74 07 - 75 06 Investigations have continued on the development of new and improved biological products for military use. - 1. Studies on meningococcal vaccines have been directed toward the improvement of the yield of the group A polysaccharide, and the development of pilot scale methods for the preparation of purified cell wall antigens from the group B organism. 2. Experimental studies were initiated on the preparation of an inactivated plague vaccine from attenuated strains of the plague bacillus. 3. Investigations on the development of a dengue virus type 2 vaccine have continued. 4. A study was initiated on the feasibility of preparing freeze-dried antigens for use in diagnostic tests for the febrile diseases. 5. A search for a virulence-enhancing medium to substitute for mucin in the mouse potency assay for the evaluation of bacterial vaccines has been carried out. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 171 Development of biological products

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Description.

This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

Progress.

1. Meningococcal Vaccine.

During the past year additional studies have been carried out on the development of pilot methods for the preparation of protein antigens from Neisseria meningitidis group B, for possible use in the immunization of man against this type of meningococcal meningitis. Investigations were also initiated on the utilization of a new strain and a new purification method for the production of group A meningococcal polysaccharide that has shown promise of considerably greater yields.

a. The work on N. meningitidis group B protein antigen during this period was limited to the production of two 30 liter lots of 12 hour cultures. The organisms were inactivated with 0.5% phenol and were collected through a Sharples centrifuge. The sedimented organisms were then fractionated by Dr. Wendell Zollinger of the Department of Bacterial Diseases, WRAIR, with the intent of obtaining purified cell wall fractions to be used for immunization by the intranasal route. Some difficulty has been encountered in obtaining a sterile product without denaturing or removing a good portion of the purified cell wall material. However, the work is continuing with attempts to produce the cell wall antigen under sterile conditions or by rendering the cell wall fractions small enough to pass through a

sterilizing membrane filter.

b. During this period investigations were initiated to determine the effect of the use of a new strain and a new purification procedure on the production of group A meningococcal polysaccharide. Studies were carried out to compare the yield of high molecular weight polysaccharide produced by *N. meningitidis* strain A-4 (M1027) with that produced by strain A-1, previously used in the preparation of group A meningococcal vaccine. Also studied was the effect of the addition of 0.2% yeast extract dialysate to the modified Franz growth medium.

Fifteen hour meningococcal cultures, in 15 liter batches, were inactivated with Cetavlon, and the sediments collected by Sharples centrifugation were extracted with 1 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  followed by the chloroform and ethanol fractionation steps (Gotschlich 1973) to a final purity that complies with the chemical and pyrogenic requirements established for the group A meningococcal vaccine (FDA, 1973). The comparison is shown in Table I.

TABLE I

Effect of Strain and Growth Medium on Production of Purified Group A Meningococcal Polysaccharide

	<u>Modified Franz Medium</u>		<u>Modified Franz + Yeast Dialysate</u>
	<u>Strain A-1</u>	<u>Strain A-4</u>	<u>Strain A-4</u>
Yield, mg/L	14.8	35.5	55.5
Protein, %	0.35	0.83	0.64
Nucleic Acid, %	0.91	0.42	0.41
Phosphorus, %	7.99	8.06	8.10
Pyrogenicity in Rabbits, 2.5 µg/kg	Non- pyrogenic	Non- pyrogenic	Non- pyrogenic
Molecular Weight	>100,000	>100,000	>100,000

From the above results it appears that strain A-4 produces more than twice the yield of polysaccharide as strain A-1, and that the addition of 0.2% yeast extract dialysate to the Franz medium further increases the yield. Additional studies are planned to determine the reproducibility of these findings under pilot scale production conditions.

In an attempt to reduce the incubation period with the A-4 strain, comparisons of yields from 15 hour cultures versus 12 hour

cultures in modified Franz medium were studied. Typical results are shown in Table II.

TABLE II

Effect of Incubation Time with Strain A-4 on Production of Purified Group A Meningococcal Polysaccharide

	<u>12 Hr Culture</u>	<u>15 Hr Culture</u>
Yield, mg/L	29.6	35.5
Protein, %	0.53	0.83
Nucleic Acid, %	0.45	0.42
Phosphorus, %	8.33	8.06
Pyrogens in Rabbits, 2.5 µg/kg	Pyrogenic	Non-pyrogenic
Molecular Weight	>100,000	>100,000

Although the 12 hour culture yielded a polysaccharide which meets the chemical requirements for the group A meningococcal vaccine, the product failed to pass the pyrogen test. On the other hand, the 15 hour culture provided a product which was satisfactory in all respects.

During these studies, it was observed that the pH of the 12 hour cultures was 5.9 to 6.1 as compared with 5.1 to 5.3 for the 15 hour cultures. Additional work is planned to determine whether or not the pH of the culture has a direct effect on the pyrogenicity of the final purified polysaccharide.

Studies were also initiated on the use of phenol for extraction of the crude polysaccharide obtained from a 15 hour culture of the A-4 strain grown in modified Franz medium with 0.2% yeast extract dialysate (Gotschlich, 1973). The crude material in 1/10 saturated, neutral sodium acetate was extracted three times with half volumes of phenol (100 gm crystalline phenol dissolved in 40 ml of 1/10 saturated, neutral sodium acetate). The aqueous-salt phase was dialysed against 0.1 M CaCl<sub>2</sub> for 24 hours at 5 C. The contents of the dialysis bag were precipitated with ethanol; the precipitate was dissolved in water and centrifuged at 100,000 x g for 4 hours. However, at this stage the product did not meet the phosphorus content requirement of 8%. Further processing included three ethanol opalescences and one extraction with chloroform. The values for the phenol extracted material, those after further purification, and for a comparable portion processed by the standard ethanol and chloroform procedure are shown in Table III.

TABLE III

Phenol Purification vs Ethanol and Chloroform Purification  
of Group A Meningococcal Polysaccharide

	<u>Phenol</u>	<u>Phenol + Ethanol and Chloroform</u>	<u>Ethanol and Chloroform</u>
Yield, mg/L	62.5	40.7	55.5
Protein, %	0.66	0.44	0.64
Nucleic Acid, %	0.23	0.22	0.41
Phosphorus, %	6.96	8.08	8.10
Pyrogens in Rabbits, 2.5 µg/kg	N.D.	Non- pyrogenic	Non- pyrogenic
Molecular Weight	N.D.	>100,000	>100,000

As indicated in the data above, the phenol extracted material did not meet the requirement of 8.0% phosphorus. However, with further purification (ethanol and chloroform) all results were in compliance.

Additional studies are in progress to determine the effect of polysaccharide and impurity concentration on the phenol extraction, in an attempt to eliminate the need for additional purification procedures.

## 2. Plague Vaccine.

The U.S. Army killed plague vaccine has been available from only one licensed commercial source. The vaccine is prepared from a virulent strain of the plague bacillus, Yersinia pestis, presenting serious safety problems and requiring special licensed facilities to insure that the work areas are suitable for plague work.

There are a number of attenuated strains of the plague bacillus currently available that could be safely grown and processed under less stringent laboratory safety requirements and with minimum hazard (Annual Report, 1970). Although they proved unsuitable as living, attenuated vaccines, these strains possibly could be used for the production of inactivated vaccine, either individually or in combinations to insure the incorporation of the necessary antigenic components. Accordingly, a collaborative study was initiated with the Department of Hazardous Microorganisms, WRAIR, to investigate the optimum conditions for the production of inactivated harvests of these attenuated strains of the plague bacillus, and to evaluate the immunogenicities of the inactivated preparations in experimental animals.

The All22 strain of Y. pestis, an attenuated strain previously used for the preparation of F-1 antigen, was grown for 72 hours on Bacto Blood Agar Base (Difco) agar at each of two temperatures (28 C and 37 C). The harvest pools at each temperature were subdivided into four aliquots and different concentrations of formalin (0.1%, 0.2%, 0.4% and 0.65%) were added. The inactivating harvests were held at room temperature and sampled at 24, 48 and 72 hours. The samples were streaked on brain heart infusion agar plates and inoculated into brain heart infusion broth. The agar plates and broth were incubated at 37 C and observed for 72 hours. The results are summarized in the following Table.

TABLE IV

Formalin Inactivation of Y. pestis (Strain All22)  
Harvests Grown at 28 C and 37 C

Formalin (%)	Inactivation Time					
	24 Hrs		48 Hrs		72 Hrs	
	28 C	37 C	28 C	37 C	28 C	37 C
0.1	-*	-	-	-	-	-
0.2	-	-	-	-	-	-
0.4	-	-	-	-	-	-
0.65	-	-	-	-	-	-

\* No apparent growth on agar or in broth.

Both the 28 C and 37 C harvests were completely inactivated by 0.1% formalin within 24 hours. These inactivated preparations and those prepared from several other selected attenuated strains of the plague bacillus will be tested for immunogenicity in animals, and compared with the current U.S. Army plague vaccine.

3. Dengue Virus Vaccine.

During this year studies have continued on the development of a dengue virus type 2 vaccine for human use. Investigations have been carried out in a continuous line of fetal rhesus monkey lung (FRhL) cells and a human cell culture strain (WI-38).

a. Growth curves of dengue virus type 2, strain PR159, seed material (Annual Report, 1974) were obtained in FRhL cells. To determine whether satisfactory yields of virus can be attained in sub-confluent rather than confluent monolayers, flasks (75 cm<sup>2</sup>) having different cell densities were washed with 25 ml of Hanks Balanced Salt Solution (HBSS) and then inoculated with virus at a multiplicity of infection (MOI) of 1. Virus was adsorbed at 35 C for approximately 90 minutes, followed by removal of the inoculum and three rinses of the cell monolayers with 25 ml of HBSS. After the addition

of 25 ml of maintenance medium (Eagle's Minimum Essential Medium containing 2% fetal bovine serum, 1% glutamine and 0.1% Streptomycin and Neomycin), the flasks were incubated at 35 C. At 0, 1, 2, 3, 4, 5, 6 and 7 days post inoculation, 0.3 ml samples from each of two flasks were removed and mixed with an equal volume of inactivated fetal bovine serum (FBS) and frozen at -70 C. Samples were assayed by plaquing in LLC-MK<sub>2</sub> cell monolayers. The results are presented in Table V.

TABLE V

Growth Curves of Dengue Type 2 Virus in FRhL Cells  
at Different Cell Densities

	1.0x10 <sup>6</sup> *	1.5x10 <sup>6</sup>	2.0x10 <sup>6</sup>	2.8x10 <sup>6</sup>	3.6x10 <sup>6</sup>
Day 0	2.0x10 <sup>2</sup> **	3.3x10 <sup>2</sup>	3.2x10 <sup>2</sup>	2.9x10 <sup>2</sup>	1.8x10 <sup>2</sup>
1	2.8x10 <sup>5</sup>	8.1x10 <sup>4</sup>	7.2x10 <sup>4</sup>	8.5x10 <sup>4</sup>	1.0x10 <sup>5</sup>
2	1.2x10 <sup>6</sup>	1.2x10 <sup>6</sup>	1.2x10 <sup>6</sup>	2.6x10 <sup>6</sup>	2.4x10 <sup>6</sup>
3	1.2x10 <sup>6</sup>	4.2x10 <sup>6</sup>	6.4x10 <sup>6</sup>	8.3x10 <sup>6</sup>	5.1x10 <sup>6</sup>
4	6.8x10 <sup>5</sup>	4.0x10 <sup>6</sup>	7.4x10 <sup>6</sup>	7.1x10 <sup>6</sup>	8.4x10 <sup>6</sup>
5	5.7x10 <sup>5</sup>	4.1x10 <sup>6</sup>	5.8x10 <sup>6</sup>	5.5x10 <sup>6</sup>	5.1x10 <sup>6</sup>
6	N.D.	2.3x10 <sup>6</sup>	3.6x10 <sup>6</sup>	4.3x10 <sup>6</sup>	3.1x10 <sup>6</sup>
7	N.D.	2.8x10 <sup>6</sup>	3.2x10 <sup>6</sup>	3.3x10 <sup>6</sup>	3.2x10 <sup>6</sup>

\* Cells/flask

\*\* PFU/0.2 ml

As can be seen from the above table, the titers of infectious virus did not differ significantly in the flasks containing 2.0x10<sup>6</sup>, 2.8x10<sup>6</sup> and 3.6x10<sup>6</sup> cells per flask, where the monolayers were confluent. The sub-confluent monolayers (1.0x10<sup>6</sup> and 1.5x10<sup>6</sup> cells/flask) did not yield more infectious virus, as was expected; in fact, the titers of infectious virus from these flasks were slightly lower. On the basis of these results, in all subsequent experiments a cell density of 3.0 - 4.0x10<sup>6</sup> cells per 75 cm<sup>2</sup> flask was used.

Since fetal bovine serum (FBS) in the maintenance medium is undesirable in vaccines for human use, additional growth curves of the same seed material were obtained in 75 cm<sup>2</sup> flasks of FRhL cells to determine the optimal concentration of human serum albumin (HSA), in lieu of FBS, in the maintenance medium. The same procedures as above were used, except that different concentrations of HSA were utilized in the maintenance medium. The results of these growth curves are shown in the following table.

TABLE VI

Growth Curves of Dengue Type 2 Virus in FRhL Cells with Different Concentrations of HSA in the Maintenance Medium

	<u>0.25% HSA</u>	<u>0.5% HSA</u>	<u>1% HSA</u>	<u>2% HSA</u>
Day 0	$3.0 \times 10^{2*}$	$1.6 \times 10^2$	$1.1 \times 10^2$	$2.5 \times 10^2$
1	$6.2 \times 10^4$	$5.1 \times 10^4$	$3.9 \times 10^4$	$5.0 \times 10^3$
2	$2.6 \times 10^5$	$2.7 \times 10^5$	$1.2 \times 10^5$	$6.1 \times 10^4$
3	$1.8 \times 10^6$	$1.2 \times 10^6$	$8.1 \times 10^5$	$5.1 \times 10^5$
4	$2.8 \times 10^6$	$2.1 \times 10^6$	$1.0 \times 10^6$	$6.3 \times 10^5$
5	$2.2 \times 10^6$	$2.1 \times 10^6$	$2.0 \times 10^6$	$9.0 \times 10^5$
6	$1.8 \times 10^6$	$8.0 \times 10^5$	$9.7 \times 10^5$	$8.8 \times 10^5$

\* PFU/0.2 ml

The results indicate that the yields of infectious virus with different concentrations of HSA in the maintenance medium are basically the same, except possibly for the 2% HSA concentration, where the titers are lower. Furthermore, the cells in the uninfected control flask containing 2% HSA in the maintenance medium showed patches of rounded and granular cells throughout the monolayer, indicating that the 2% HSA concentration is toxic for the cells. On the basis of the results obtained in this experiment, it was decided that 0.25% HSA would be included in the maintenance medium. This concentration of HSA has been satisfactorily used in the production of other viral vaccine for human use.

It appears from the above results that the yield of infectious virus with HSA in the maintenance medium is lower than that attained with FBS (See Table V). Therefore, the following growth curves were obtained to compare the effects of HSA versus FBS on virus yield. At the same time, maintenance medium without any serum additive was included in the experiment. The same procedures as previously described were used for the growth curves. The results are shown in Table VII.

TABLE VII

Comparison of the Effects of FBS, HSA and No Serum Additive  
in the Maintenance Medium on the Replication of  
Dengue Virus Type 2 in FRhL Cells

	<u>2% FBS</u>	<u>0.25% HSA</u>	<u>No Serum Additive</u>
Day 0	$2.6 \times 10^2$ *	$2.5 \times 10^2$	$1.9 \times 10^2$
1	$6.0 \times 10^4$	$3.4 \times 10^4$	$2.8 \times 10^4$
2	$9.8 \times 10^5$	$2.1 \times 10^5$	$1.5 \times 10^5$
3	$4.6 \times 10^6$	$1.4 \times 10^6$	$1.4 \times 10^6$
4	$4.0 \times 10^6$	$2.3 \times 10^6$	$2.4 \times 10^6$
5	$3.7 \times 10^6$	$1.6 \times 10^6$	$2.5 \times 10^6$
6	$3.1 \times 10^6$	$5.2 \times 10^5$	$1.7 \times 10^6$
7	$1.0 \times 10^6$	$2.3 \times 10^5$	$5.8 \times 10^5$

\* PFU/0.2 ml

From the above results, it appears that the maintenance medium without any serum additive is as good as with HSA in the maintenance medium. The effect of 2% FBS in the maintenance medium was superior over-all. However, since the U.S. Food and Drug Administration requirements for vaccines for human use specify that levels of added serum be less than one part per 1,000,000 parts of vaccine, further investigations will be made with maintenance medium containing no serum additives.

In an attempt to improve the titer of infectious virus with maintenance medium containing no serum additives growth curves were obtained at different incubation temperatures, in order to determine whether less heat inactivation and/or a greater yield of virus would result at a lower temperature. The same procedures were used as previously described. Table VIII shows the results of these growth curves.

TABLE VIII

Growth Curves of Dengue Virus Type 2 in FRhL Cells  
at Different Incubation Temperatures

	<u>28 C</u>	<u>31 C</u>	<u>33 C</u>	<u>35 C</u>	<u>37 C</u>
Day 0	6.5x10 <sup>1</sup> *	7.0x10 <sup>1</sup>	1.2x10 <sup>2</sup>	1.2x10 <sup>2</sup>	1.0x10 <sup>2</sup>
1	(Acid)	6.5x10 <sup>3</sup>	2.0x10 <sup>4</sup>	3.0x10 <sup>4</sup>	1.9x10 <sup>4</sup>
2	1.3x10 <sup>5</sup>	2.8x10 <sup>5</sup>	3.0x10 <sup>5</sup>	2.4x10 <sup>5</sup>	1.2x10 <sup>5</sup>
3	7.4x10 <sup>4</sup>	7.3x10 <sup>5</sup>	1.1x10 <sup>6</sup>	4.4x10 <sup>5</sup>	(Acid)
4	1.3x10 <sup>5</sup>	1.6x10 <sup>6</sup>	1.5x10 <sup>6</sup>	8.2x10 <sup>5</sup>	1.9x10 <sup>5</sup>
5	4.8x10 <sup>5</sup>	2.9x10 <sup>6</sup>	5.1x10 <sup>6</sup>	2.6x10 <sup>6</sup>	1.2x10 <sup>5</sup>
6	4.4x10 <sup>5</sup>	1.7x10 <sup>6</sup>	3.1x10 <sup>6</sup>	1.4x10 <sup>6</sup>	6.7x10 <sup>5</sup>

\* PFU/0.2 ml

From the above results, it appears that 33 C is the optimal temperature of incubation. However, these growth curves will be repeated since there were some unavoidable fluctuations in the temperatures of some of the incubators used for this experiment.

b. Dengue virus type 2 (strain PR159) was previously demonstrated to replicate in WI-38 cells (Annual Report, 1974). Growth curves in this cell system were obtained to compare the effects of FBS, HSA and no serum additive in the maintenance medium on virus yield. The same procedures as previously described were used for the growth curves. The results are presented in Table IX.

TABLE IX

Comparison of the Effect of FBS, HSA and No Serum Additive  
in the Maintenance Medium on the Replication of  
Dengue Virus Type 2 in WI-38 Cells

	<u>2% FBS</u>	<u>0.25% HSA</u>	<u>No Serum Additive</u>
Day 0	5.3x10 <sup>3</sup> *	4.7x10 <sup>3</sup>	4.0x10 <sup>3</sup>
1	3.0x10 <sup>4</sup>	2.7x10 <sup>4</sup>	1.2x10 <sup>3</sup>
2	5.0x10 <sup>4</sup>	1.0x10 <sup>4</sup>	2.4x10 <sup>3</sup>
3	4.2x10 <sup>4</sup>	5.3x10 <sup>3</sup>	9.5x10 <sup>3</sup>
4	1.0x10 <sup>5</sup>	3.2x10 <sup>3</sup>	1.6x10 <sup>4</sup>
5	2.5x10 <sup>5</sup>	2.1x10 <sup>3</sup>	2.0x10 <sup>4</sup>
6	1.6x10 <sup>5</sup>	1.7x10 <sup>3</sup>	2.3x10 <sup>4</sup>

\* PFU/0.2 ml

From the above results, it appears that 2% FBS in the maintenance medium is superior to HSA and no serum additive. Further investigations will be carried out with WI-38 cells to determine whether these cells can produce sufficient virus to serve as a suitable substrate for the preparation of dengue vaccines.

c. Currently, a stock of certified WI-38 cells is being prepared in this laboratory for further experimentation and as a possible substrate for dengue vaccines for human use without further sub-cultivation. One confluent 4 oz. prescription bottle of WI-38 cells (passage 13) was received from Dr. Leonard Hayflick (Stanford University School of Medicine, Stanford, CA). Cells were passaged in split ratios of 1:2. At passage 16, the cells were stabilized with a cryoprotective medium (E-MEM, 10% FBS, 10% glycerol, 2% glutamine, 0.1% S&N), frozen and stored in liquid nitrogen. The following table shows the passages and harvesting of WI-38 cells starting from one frozen ampoule of passage 16 cells.

TABLE X

Passage and Harvesting of WI-38 Cells

<u>Day</u>	<u>Date</u>	<u>Passage</u>	<u>No. &amp; Size of Container</u>
		16	Frozen ampoule
0	15 April 1975	17	2x75 cm <sup>2</sup>
6	21 April 1975	18	4x75 cm <sup>2</sup>
10	25 April 1975	19	4x150 cm <sup>2</sup>
14	29 April 1975	20	8x150 cm <sup>2</sup>
20	5 May 1975	21	3 rollers* + 4 amps
23	8 May 1975	22	6 rollers
27	12 May 1975	23	12 rollers
33	18 May 1975	24	24 rollers
37	22 May 1975	Harvest, ampouling & freezing	

\*Rollers = 690 cm<sup>2</sup>

The monolayers from passage 24 were washed twice with HBSS, trypsinized for approximately 2 minutes at 37 C, decanted and the cultures returned for 15-20 minutes at 37 C. The cells were then suspended in growth medium containing 10% glycerol, pooled and distributed into ampoules at a cell concentration of  $1.5 \times 10^6$  cells per ml. Two ml aliquots were placed into 156 ampoules and 4 ml aliquots were placed into 17 ampoules. The ampoules were flame sealed and held at 4 C for 3 hours and then placed for 3 hours into

an alcohol bath held in a -70 C dry ice chest, before storing in liquid nitrogen.

All growth media from passage 24 cells were saved, pooled and tested for sterility. Aliquots of the pool were taken and frozen at -70 C and will be tested for adventitious agents and mycoplasma.

d. Studies were also initiated on the development of a plaquing assay for dengue viruses in FRhL cells. Initially the same plaquing medium that was used for LLC-MK<sub>2</sub> cells was tried for FRhL cells. The titer of dengue virus material was approximately  $1 \times 10^3$  PFU/0.2 ml in FRhL cells whereas in LLC-MK<sub>2</sub> cells the titer was  $2 \times 10^6$  PFU/0.2 ml. Thus the plaquing efficiency was very poor in FRhL cells as compared to LLC-MK<sub>2</sub> cells.

Various plaquing media and procedures were then investigated in order to increase the efficiency, and the distinctiveness of the plaques. Up to the present time, the best results were obtained with the following plaquing media and procedures. Cells were grown to confluency in 25 cm<sup>2</sup> flasks, growth medium was removed and 0.2 ml of inoculum was used in which the diluent consisted of 0.5% Bovine Albumin Fraction V in buffered physiological saline. The virus was adsorbed for 1.5 hours at 35 C and then overlaid with 5 ml of BME containing 7% FBS, 0.5% agarose and antibiotics. The flasks were incubated at 35 C for 6 days and then received 5 ml of a second overlay containing 0.5% agarose and Neutral Red at a final dilution of 1:10,000 in HBSS solution. The flasks were reincubated at 35 C for an additional 3-4 hours, removed and placed at room temperature overnight before plaques were counted. By using this technique, the titer of the dengue 2 material was increased to  $3 \times 10^5$  PFU/0.2 ml. However, the plaquing system in FRhL cells is still not as sensitive, nor are the plaques as discernible, as in the LLC-MK<sub>2</sub> plaquing system. Furthermore the monolayer of FRhL cells undergoes rapid deterioration after the addition of Neutral Red. Further studies are being carried out in an attempt to improve the plaquing in FRhL cells.

#### 4. Diagnostic Antigens.

A study was initiated on the feasibility of preparing freeze-dried bacterial antigens for use in diagnostic tests for the febrile diseases. Antigens prepared in the dried form would in all probability be more stable, even under adverse storage conditions, than the presently available fluid antigen preparations. However, dispensing of dried material into glass containers the same size as required for diluted fluid antigen would be undesirable from a

logistic viewpoint. On the other hand, the preparation of dried products packaged in small plastic envelopes, or compressed into tablets, would permit shipment and storage of many units in one container, thus conserving space and reducing costs. The reconstituting fluid and containers required are much less critical and can be obtained from local sources when needed.

Studies carried out during this period were directed at determining the most satisfactory method of inactivating the bacterial harvests in order to yield the most stable active antigen preparations in the dried state. The typhoid somatic ("O") antigen, prepared from Salmonella typhi, strain 0901, was used as a model.

The concentrated harvest of S. typhi, strain 0901, grown in Kolle flasks on trypticase soy agar and suspended in buffered physiological saline, was divided into six 50 ml portions. Each portion was treated differently to determine the most satisfactory method for inactivating the organisms and preserving the antigenic activity. The methods employed are as follows:

- I. Bacterial cells sedimented by centrifugation and re-suspended in 50 ml 95% ethyl alcohol. Stored at room temperature for 1 hour, then refrigerated.
- II. Bacterial cells sedimented by centrifugation and re-suspended in 50 ml 95% ethyl alcohol. Placed in 60°C water bath for 1 hour, then cooled and refrigerated.
- III. Bacterial cell suspension treated with formaldehyde (final formalin concentration 0.5%), stored at room temperature for 1 hour, then refrigerated.
- IV. Bacterial cell suspension treated with formaldehyde (final formalin concentration 1.0%), stored at room temperature for 1 hour, then refrigerated.
- V. Bacterial cells sedimented by centrifugation and re-suspended in 50 ml acetone. Stored at room temperature for 1 hour, then refrigerated.
- VI. Bacterial cell suspension treated with Cetavlon (final Cetavlon concentration 0.1%), stored at room temperature for 1 hour, then refrigerated.

At varying time intervals (from 1 hour to 48 hours) each cell suspension was tested for inactivation by culturing on trypticase soy broth. The results are recorded in Table XI.

TABLE XI

Inactivation of Treated S. typhi 0901 Suspensions

<u>Time</u>	<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>	<u>V</u>	<u>VI</u>
1 hr	-	-	+	±	-	+
2 hrs	-	-	+	-	-	+
3 hrs	-	-	-	-	-	+
5 hrs	-	-	-	-	-	+
24 hrs	-	-	-	-	-	±
48 hrs	-	-	-	-	-	±

- = No growth on solid or liquid media.  
 + = Growth on solid and liquid media.  
 ± = Growth in broth, no growth on agar.

The above results demonstrate that all of the methods employed were effective in inactivating the organisms within three hours, with the exception of the Cetavlon treatment (Method VI). This was unexpected inasmuch as 1% Cetavlon has been used in this laboratory to rapidly and effectively inactivate other bacterial suspensions, including Neisseria meningitidis, Vibrio cholerae, and S. typhi strain Ty 2.

At the same time intervals all cell suspensions were checked for agglutinability in a specific antiserum. In each instance 2 ml of the suspension were centrifuged and the sedimented cells were resuspended in the proper amount of phenolized saline for use as antigen in the slide agglutination test against a known positive antiserum, and a saline control to detect auto-agglutinability. The results are given in Table XII.

TABLE XII

Slide Agglutination Tests of Treated S. typhi 0901 Suspensions

<u>Time</u>	<u>I</u>		<u>II</u>		<u>III</u>		<u>IV</u>		<u>V</u>		<u>VI</u>	
	C	T	C	T	C	T	C	T	C	T	C	T
1 hr	-	4	-	4	-	4	-	4	1	2	-	4
2 hrs	-	4	-	4	-	4	-	4	±	2	-	4
3 hrs	-	4	-	4	-	4	-	4	-	2	-	4
5 hrs	-	4	-	4	-	4	-	4	±	2	-	4
24 hrs	-	4	-	4	-	4	-	4	1	2	-	4
48 hrs	-	4	-	4	-	4	-	4	1	2	-	4

C = Saline control, T = test with homologous antiserum.  
 Readings indicate degree of agglutinability, ranging from - (negative) to 4 (maximum).

The above results show that all of the treatments provided antigens with good agglutinability, with the exception of the acetone treatment (Method V), which produced a slight auto-agglutinability of the cells and a reduced agglutinability in specific antisera. However, this appears to be due to the presence of residual acetone in the antigen preparation. Additional washing of the cells eliminated this effect.

Preparatory to freeze-drying, each suspension was centrifuged, the sedimented cells were washed three times with buffered physiological saline (BPS) and then resuspended with sufficient BPS to yield 78% ( $\pm$  0.5%) transmittance in the Coleman Jr. Spectrophotometer. Each adjusted suspension was distributed in 1 ml amounts into 10 ml vaccine bottles. Samples of the bottled antigen suspensions were stored at 4 C (fluid) and at -60 C (frozen) and the remainder of the bottles were freeze-dried. The freeze-dried material was stoppered and sealed under vacuum and samples were placed in storage at 4 C, 37 C, 45 C and 56 C. Periodically during storage samples were removed, rehydrated and diluted to the proper volume, and were tested quantitatively for agglutinability in homologous antiserum by the standard tube-type agglutination test. The results are recorded in the following table.

TABLE XIII

Stability of *S. typhi* 0901 Antigen Suspensions  
Upon Storage

<u>Storage Conditions</u>	<u>Time</u>	<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>	<u>V</u>	<u>VI</u>
Frozen, -60 C	2 wks	640	1280	320	160	1280	640
	6 wks	1280	1280	640	320	1280	640
	3 mos	1280	1280	640	320	1280	640
	6 mos	1280	1280	640	320	640	640
Fluid, +4 C	2 wks	1280	640	40	40	640	640
	6 wks	1280	1280	<40	<40	640	640
	3 mos	1280	1280	<40	<40	640	1280
	6 mos	1280	640	320	160	640	640
Freeze-Dried, +45 C	2 wks	1280	1280	1280	1280	640	640
	6 wks	1280	1280	320	640	1280	320
	3 mos	640	1280	640	640	640	320
	6 mos	640	640	160	320	320	320

TABLE XIII  
(continued)

<u>Storage Conditions</u>	<u>Time</u>	<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>	<u>V</u>	<u>VI</u>
Freeze-Dried, +56 C	2 wks	1280	640	640	640	640	320
	6 wks	1280	1280	320	640	640	320
	3 mos	1280	640	160	320	640	320
	6 mos	-	auto-agglutinable		-		

These results indicate that the formalin inactivated antigen preparations (III and IV) are the least stable, with evidence of loss of agglutinability as early as two weeks under some storage conditions (frozen at -60 C, fluid at 4 C). The other preparations appear to be more stable, but some loss of agglutinability is apparent in the freeze-dried samples which have been stored for 6 weeks at 45 C and 56 C.

The results obtained thus far suggest that the alcohol treated antigen preparations (Methods I and II) are the most satisfactory from the standpoint of ease of inactivation and stability of the antigen. Surveillance of these preparations will continue, to determine the effect of prolonged storage at the various temperatures on agglutinability.

Additional studies will be carried out to determine the effect on the stability of the antigen of compression of the dried powder to form tablets. The application of these procedures to other febrile antigens will also be studied.

##### 5. Vaccine Potency Assays.

Investigative studies involving the evaluation of experimental typhoid, cholera and meningococcal vaccines by mouse protection assays were curtailed as the result of the depletion of our supply of gastric mucin, which is required to enhance the virulence for mice of the bacterial challenge suspensions employed in these assays.

Wilson Laboratories of Chicago, IL, the principal supplier of mucin to biological laboratories, has announced that they will no longer produce granular mucin for sale or distribution. A search for other manufacturers to provide satisfactory mucin has proven futile. In order to continue the performance of mouse potency assays, for the evaluation of experimental vaccines as well as for the monitoring of the standard vaccines for the Defense Supply Agency, two parallel studies were initiated to find a virulence-enhancing medium: a) the

addition of a supplement to the available gastric mucin preparations to improve the virulence-enhancing properties, and b) the replacement of mucin by another, more readily available product.

a. In recent years considerable attention has been drawn to the role that iron and its compounds play in enhancing the virulence of certain organisms (Weinberg, 1974). Recently, Fusillo, Smith and Reednick (1974) reported their findings concerning the involvement of the gastric mucosa in iron transfer in mammals. By assaying several different lots of gastric mucin for iron content, they were able to show that the number of mice killed by Staphylococcus aureus suspended in these mucin preparations was directly related to the iron concentration of the mucin. On the basis of these reports, it was considered desirable to determine whether the addition of iron to a sub-standard lot of gastric mucin would sufficiently enhance the virulence of challenge organisms to permit its use in mouse potency assays for the vaccines of interest to us. Salmonella typhi, strain Ty 2, was used as the model challenge organism since U.S. Food and Drug Administration regulations state that a challenge suspension of this organism, suspended in mucin, shall have an LD<sub>50</sub> for mice of no more than 10 colony forming units.

In a pilot experiment iron in the form of ferric ammonium citrate (5 mg Fe<sup>+++</sup>/kg mouse body weight) was added to a 5% suspension of mucin. Appropriate dilutions of S. typhi strain Ty 2 were prepared in the mucin-iron suspensions and in mucin alone, and these were injected intraperitoneally into mice weighing approximately 20 gms. The resulting titrations showed a definite increase in virulence of strain Ty 2 in the iron supplemented mucin preparation.

In a subsequent experiment, increasing concentrations of iron were added to a mucin suspension to determine whether higher iron concentrations would result in a further enhancement of virulence of strain Ty 2 (Table XIV).

TABLE XIV

The Effect of Iron on the Virulence of S. typhi,  
Strain Ty 2 for Mice

<u>Amount of Fe<sup>+++</sup> added to Mucin*</u>	<u>LD<sub>50</sub> Value</u>
NONE	1000 organisms
5 mg Fe <sup>+++</sup> /kg mouse body weight	130 organisms
10 mg Fe <sup>+++</sup> /kg mouse body weight	2.6 organisms
20 mg Fe <sup>+++</sup> /kg mouse body weight	2.6 organisms

\* Wilson Laboratories #96181

Ferri: Ammonium Citrate used as source of Fe<sup>+++</sup>.

The results demonstrate that, as the iron concentration was increased, there was a further increase in virulence to a maximum level. The LD<sub>50</sub> value attained with concentrations of 10 mg Fe<sup>+++</sup>/kg mouse body weight or above, was well within the limit required for potency assays.

In testing several different lots of mucin, with and without the addition of 10 mg Fe<sup>+++</sup>/kg mouse body weight, wide ranges of LD<sub>50</sub> values were obtained. However, with every mucin lot tested an enhancing effect of iron on virulence was demonstrated with reductions in LD<sub>50</sub> values ranging from 5-fold to greater than 100-fold. However, certain lots of mucin had relatively poor virulence enhancing activity, so that the addition of 10 or 20 mg Fe<sup>+++</sup> per kg of mouse body weight was insufficient to lower the LD<sub>50</sub> value to 10 organisms or less. The addition of higher concentrations of iron to these mucin suspensions succeeding in reducing the LD<sub>50</sub> value to an acceptable level, as shown in Table XV.

TABLE XV

The Effect of Various Concentrations of Iron ( $\text{Fe}^{+++}$ )  
on the Virulence of S. typhi, Strain Ty 2

<u>Iron (<math>\text{Fe}^{+++}</math>) Concentration</u>	<u>LD<sub>50</sub> Value</u>	
	<u>Mucin Wilson Lot #96181</u>	<u>Mucin NBC Lot #8840</u>
<u>Experiment #1</u>		
NO IRON	70 organisms	>8500 organisms
5 mg/kg body weight	7.5 organisms	5000 organisms
10 mg/kg body weight	6.0 organisms	750 organisms
20 mg/kg body weight	5.0 organisms	180 organisms
<u>Experiment #2</u>		
20 mg/kg body weight	N.D.	160 organisms
40 mg/kg body weight	N.D.	18 organisms
80 mg/kg body weight	N.D.	8.0 organisms

The above results demonstrate that the various mucin preparations require different amounts of added iron to bring the LD<sub>50</sub> value to an acceptable level (10 organisms or less). Additional experiments are planned to determine the relationship between the available iron content of the mucin preparation and the LD<sub>50</sub> value. A comparison will also be made of vaccine potency assays with the challenge suspension in an iron-supplemented mucin and in a mucin preparation which does not require additional iron.

b. In 1973, Kreeftenberg, van Ramshorst and Loggen reported that a 5% suspension of dried inactive baker's yeast ("Engevita", produced in the Netherlands) can be used as a substitute for 5% mucin in the typhoid mouse protection test. Attempts in this laboratory to reproduce these results, using "Engevita" and several domestic yeast preparations, both active and inactive, were only partially successful. While 5% suspensions of some yeast preparations did enhance the virulence of S. typhi, strain Ty 2 to some degree, none reduced the LD<sub>50</sub> value to 10 organisms or less, as required by the USFDA for use in the typhoid vaccine mouse potency assay. However, because of its lower viscosity and the ease with which yeast suspensions can be prepared, handled and injected, attempts were made to achieve the desired virulence level by increasing the concentration of yeast employed.

In one experiment using the dried inactive baker's yeast product "Engevita", received from the Netherlands, four different concentrations were used. The results are recorded in Table XVI.

TABLE XVI

The Effect of Different Concentrations of Yeast on the Virulence of S. typhi, Strain Ty 2

<u>Yeast* Concentration</u>	<u>LD<sub>50</sub> Value</u>
5%	280 organisms
7%	12 organisms
8%	6.5 organisms
10%	2.1 organisms

\* "Engevita" (dried inactive baker's yeast) manufactured by Gist-Brocades N.V., Delft, The Netherlands.

The results demonstrate that increasing the concentration of yeast had a marked virulence-enhancing effect, and that LD<sub>50</sub> values of less than 10 organisms were attained with yeast suspensions of 8 and 10%.

Additional titrations of S. typhi, strain Ty 2 in several other yeast preparations, obtained from different manufacturers, all showed enhancement of virulence, but to varying degrees. Of the lots tested Squibb's #3M024, as well as the "Engevita", consistently gave LD<sub>50</sub> values of less than 10 organisms when used as 10% suspensions.

Further studies will be carried out to determine whether the iron content of the yeast preparation plays a role in the virulence-enhancing effect. In addition, the use of yeast as a substitute for mucin in the mouse protection tests for typhoid, cholera and meningococcal vaccines will be evaluated.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 171 Development of biological products

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL	
				DA OA 6449	75 07 01	DD-DRAE(AR)36	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>3</sup>	6. WORK SECURITY <sup>4</sup>	7. REGRADING <sup>5</sup>	8A. DISSEM INSTN <sup>6</sup>	8B. SPECIFIC DATA CONTRACTOR ACCESS	8. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
9. NO./CODES <sup>7</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3A161102B71Q	00	172			
b. CONTRIBUTING							
c. OBSERVING <sup>8</sup>	CARDS 114F						
11. TITLE (Provide w/3 Security Classification Code) <sup>9</sup>							
(U) Immunological Mechanisms in Microbial Infections							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>10</sup>							
010100 Microbiology 003400 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
62 08		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
EXPIRATION:				FISCAL YEAR		5.1	
b. NUMBER:				75		110	
c. TYPE:				CURRENT		4.0	
d. KIND OF AWARD:				76		110	
f. CUM. AMT.							
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21. GENERAL USE				ASSOCIATE INVESTIGATOR <sup>11</sup>			
Foreign intelligence not considered				NAME:			
				NAME:			
22. KEYWORDS (Provide each one Security Classification Code) <sup>12</sup> (U) Infectious Diseases; (U) Immunity; (U) Antibodies; (U) Complement Fixation; (U) Radioimmunoassay; (U) Serodiagnosis							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAMS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) The objective of this work unit is to elucidate the mechanisms operative in the natural and artificial induction of immunity to a variety of microbial infections of military importance. This includes the study of infections in model systems and the development of methodologies for the study of the immune reaction in humans for research as well as diagnostic evaluations.							
24 (U) The approaches used for these studies involve the measurement of various parameters of disease and of the immune response to disease both in in vivo and in vitro experiments. A variety of diseases are attacked with current emphasis on schistosomiasis and hepatitis. Immunological phenomena common to a variety of different diseases are also studied.							
25 (U) 74 07 - 75 06 Immunity to schistosomiasis in rats has been shown to be transferable by both antibody and T lymphocytes. A new method has been devised for the removal of inhibitors of rebella hemagglutination. The characteristics of erythrocyte membranes necessary for binding of arboviruses have been elucidated. Two new assays (immuno thin layer chromatography and liposome spin immunoassay) for lipid antigens have been developed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1974 to 30 June 1975.							

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1 MAR 66

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 172 Immunological mechanisms in microbial infections

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1. The removal of nonspecific inhibitors in the rubella hemagglutination test by means of colloidal silicic acid.

Description. The hemagglutination inhibition test (HAI), introduced by Stewart et al. (1) has provided the most important means for the diagnosis of rubella infections and the evaluation of the immune status of suspect populations. It is now well recognized that the accuracy of the HAI test depends upon the complete removal of nonspecific lipoprotein inhibitors without significant reduction of the specific antibody. These nonspecific inhibitors were first identified as beta-lipoproteins by Sedwick et. al. (2) Recently Blom et. al. (3) have found that all classes of serum lipoproteins act as inhibitors. Schmidt et.al. (4) have reported on the false positive and false negative reactions occurring with all three of the most commonly used methods for nonspecific inhibitor removal; kaolin, (5) heparin-MnCl<sub>2</sub> (6) and dextran sulfate - CaCl<sub>2</sub> (7).

This report introduces a new method for inhibitor removal based on the selective ability of an insolubilized matrix of colloidal silicic acid to absorb serum lipoproteins from solution (8). The details of the procedure for the removal of lipoprotein inhibitors and a comparative study using dextran sulfate - CaCl<sub>2</sub> and heparin - MnCl<sub>2</sub> inhibitor removal techniques are described.

The rubella antigen was a lyophilized antigen produced by Behring Diagnostics. Heparin-bovine serum albumin-gelatin buffer (HSAG) was used as diluent (Grand Island Biological Co.). The antigen and buffer were kindly supplied by Mr. Dan Cummings of National Health Laboratories.

The HAI test recommended by the Center for Disease Control was

followed (9). The removal of inhibitors by heparin -  $MnCl_2$  utilized the method of Cooper et al. (6). The dextran-sulfate -  $CaCl_2$  serum treatment method for inhibitor removal was that of Liebhaber (7). The only modification in both methods involved employing an initial serum dilution of 1:4. The natural agglutinins were removed from sera by adsorption with 50% chick erythrocytes which had been washed 3 times in dextrose-gelatin-veronal buffer. The adsorption time was 1 hour at  $4^{\circ}C$ . Chick erythrocytes for the HAI test were washed 3 times in dextrose-gelatin-veronal buffer and suspended to a concentration of 0.25% in HSAG. The microtiter system using disposable V plates was employed. All reagents were kept at  $4^{\circ}C$  except as specifically noted. Mixtures of sera and 4 U of antigen were incubated at  $4^{\circ}C$  for 1 hour before addition of erythrocytes. The completed tests were read after incubation at  $4^{\circ}C$  for  $1\frac{1}{2}$  hour or overnight. The reciprocal of the highest dilution of serum showing complete inhibition of rubella hemagglutination was taken as the HAI titer.

Two tenths of one milliliter of serum were added to individual glass test tubes containing 6 milligram aliquots of colloidal silicic acid (Aerosil Degussa Inc., New York, NY). The test tubes were mixed by a Vortex mixer and then shaken for 15 min at room temperature. This step removed the nonspecific lipoprotein inhibitory activity. The subsequent removal of natural agglutinins was accomplished by adding 0.5 ml of HSAG buffer to each tube alone with 0.2ml of a 50% chick red blood cell suspension. This natural agglutinin adsorption phase was done over a one hour period at  $4^{\circ}C$ . The tubes were then removed and centrifuged at room temperature for 5 min at 1500 rpm. The clear supernatant which is a 1:4 dilution of serum was used as a starting dilution for antibody titrations. The sera used were obtained from two sources, the 1st Army Medical Laboratory, Ft Meade, MD and the clinical desk, Dept of Communicable Diseases Walter Reed Army Institute of Research, Washington, DC.

Progress. Nonspecific inhibitors were demonstrated in all sera tested and some sera had inhibitors present at high levels. A study was conducted to determine the optimum concentration of colloidal silicic acid. Preliminary experiments had shown that serum inhibitors could be removed at room temperature ( $25^{\circ}C$ ) within the first 15 min of the adsorption procedure.

Table 1 demonstrates the relative efficiency of varying amounts of colloidal silicic acid to remove inhibitor. From the information shown in Table 1, 6 milligrams of CSA gave identical or equivalent ( $\pm 1$  dilution) titers to those obtained by the dextran sulfate -  $CaCl_2$  method in sera containing nonspecific inhibitors. All CSA adsorptions were done at  $25^{\circ}C$  (room temperature) for 15 minutes.

To test the possibility that 15 min might be a marginal time for

adsorption or that antibody titers would be affected by the longer exposure times required in adding diluent and erythrocytes to a large number of tubes, the effect of time of adsorption on the HAI titer was determined. Table 2 demonstrates the representative HAI titers following inhibitor adsorption with 6 milligram of CSA at 25°C from 15 min to 2 hours. The titers are identical or equivalent to those obtained by the dextran sulfate -  $\text{CaCl}_2$  method.

A study comparing colloidal silicic acid to heparin -  $\text{MnCl}_2$  and dextran sulfate -  $\text{CaCl}_2$  is shown in Table 3. The CSA procedure using 6 milligrams per 0.2 ml of serum to remove the inhibitor as previously described, gave equivalent titers to those obtained by using the dextran sulfate -  $\text{CaCl}_2$  or heparin -  $\text{MnCl}_2$  methods which require a longer time and must be carried out in the cold at all times.

Discussion. In previously described methods commonly used for the measurement of rubella antibody by HAI the removal of lipoprotein inhibitory activity must be done at 4°C during an adsorption period of from 15 minutes to one and a half hours. If they are not kept at 4°C the precipitated lipoproteins resolubilized and return to their functional role as inhibitors. In addition the dextran sulfate- $\text{CaCl}_2$  inhibitor removal method is technically unreadable or titers below 1:8 and the Heparin- $\text{MnCl}_2$  inhibitor removal procedure requires a special modification (10) to be read at 1:4 which increases the number of reagents required along with the time and expense of doing each test. The new procedure described in the paper permits the lipoprotein inhibitory to be removed from whole serum at room temperature by means of one stable reagent at a fraction of the present cost. CSA (Aerosil) can be purchased from Degussa Inc., at one dollar per pound and only 6 milligrams is required per test. In addition CSA can be readily suspended and aliquoted in a variety of standard buffers. It can also be stored at 4°C or 25°C for indefinite periods of time. Previously published data by Hedlund (11) has demonstrated the stability of the bond between CSA and lipoprotein like moieties. The data presented in table 3 shows that HAI antibody levels following inhibitor removal by CSA are identical or equivalent to those antibody titers obtained by either the heparin- $\text{MnCl}_2$  or dextran sulfate inhibitor removal methods. This demonstration that there is no reduction of antibody titers is supported by the work of Stephan (8) which shows no reduction in serum antibody titers to blood group antigen A, blood group antigen B, Herpes, measles, or polio type I following the adsorption of whole serum lipoproteins onto equivalent amounts of CSA.

It is presently our intent to apply this method to other arbor viral systems whose hemagglutinins are inhibited by serum lipoproteins and to investigate the nature of the interaction of the various subclasses of lipoproteins with these viruses.

Table I. Effect of Varying Concentrations of  
C.S.A. on H.A.I. Titers following 15 Minutes Adsorption at 25° C

Reciprocals of HAI Titers							
Specimens	No treatment	Dextran sulfate treatment	Colloidal silicic acid concentration (in milligrams)				
			2	4	6	8	10
1	123	64	64	64	64	64	32
2	1024	64	128	128	64	32	64
3	256	32	256	128	64	32	32
4	256	64	256	64	64	32	32
5	128	32	128	64	32	32	16
6	128	32	64	32	16	16	16

Table 2. Effect of time on HAI titer\*  
following CSA adsorption

Specimens	No treatment	Dextran sulfate treatment	CSA absorption times			
			15 min	30 min	1 hr	2 hr
11	256	0**	0	0	0	0
12	256	64	64	64	64	128
13	128	32	32	64	32	32
14	128	64	64	64	64	32
15	256	64	64	128	128	128
16	256	32	32	32	64	64
17	256	64	64	32	64	64
18	256	32	32	32	64	32
19	256	16	16	16	16	16
20	128	16	32	16	16	16

\*Titers expressed are the reciprocal of the dilution.

\*\*Serum was negative at 1:8. The 1:4 dilution was agglutinated.

Table 3. Comparative study of HAI  
using differing inhibitor removing techniques

Specimens	Reciprocal of HAI titers			
	Untreated	Dextran sulfate treatment	CSA treatment	Heparin treatment
21	128	16	64	32
22	128	32	32	64
23	256	64	64	64
24	256	64	64	128
25	512	32	32	32
26	256	64	64	32
27	128	64	64	64
28	128	64	64	64
28	128	32	16	32
29	128	32	32	32
30	128	16	16	16
31	128	0*	4	0*
32	256	128	64	64

\*Serums were negative at 1:8; 1:4 serum dilutions were agglutinated.

## 2. Influence of membrane composition on the interaction of a human monoclonal "Anti-Forssman" Immunoglobulin with Liposomes.

This study utilizes a unique "anti-Forssman" monoclonal immunoglobulin (Waldenstrom macroglobulin, or WM), which was isolated from the plasma of a patient (McG), to investigate the mechanisms of immune reactions with liposomes. The purposes were to (1) examine the reactivity of the WM as an "antibody" source against Forssman in liposomes and cells, (2) determine the relative degree of specificity of the interaction with a variety of highly purified glycosphingolipids in liposomes, and (3) utilize the homogeneous immunoglobulin to study the influence of membrane composition on the antigen-antibody binding characteristics. The interactions of the macroglobulin with Forssman hapten and complement were apparently analogous to those of anti-Forssman antibodies from hemolysin (rabbit anti-sheep erythrocyte serum). Hemolysin, anti-human erythrocyte serum, and anti-galactocerebroside serum were all compared with the macroglobulin for specificity with liposomes containing one of the following glycosphingolipids: glucocerebroside, galactocerebroside, lactocerebroside (cytolipin H), globoside I, or Forssman (Table 4). Each of the antisera reacted with two of the haptens, while WM reacted only with Forssman. These experiments demonstrated a marked similarity between the WM and the hemolysin anti-Forssman antibodies. The two could be distinguished, however, in the relative binding affinity to Forssman (Fig. 1). Under conditions in which the liposomal antigen concentration was limiting, the affinity of WM for Forssman, as reflected by relative glucose release from liposomes, was much less than those of hemolysin, or IgM, or IgG fractions from hemolysin. Glucose release due to WM was decreased still further in these latter experiments when a long chain phospholipid (sphingomyelin) was substituted for shorter chain phospholipids (e. g., dimyristoyllecithin) (Fig 2). By means of adsorption experiments it was demonstrated that sphingomyelin-containing liposomes bound fewer hemolytic or glucose-releasing anti-Forssman WM antibodies than did dimyristoyllecithin-containing liposomes. It was felt that the Forssman substance was probably partially "buried" in the sphingomyelin bilayer, thus accentuating the decreased affinity of the WM immunoglobulin. From this it was concluded that the liposomal phospholipid chain length may influence the binding infinity of an antibody to a membrane-associated antigen.

## 3. Interaction of sindbis virus with liposomal model membranes.

Radiolabeled Sindbis virus was found to bind to protein-free lipid model membranes (liposomes) derived from extracts of sheep erythrocytes (Fig. 3). The virus interaction was dependent on initial pH (Fig. 4) and the range of pH dependence (pH 6.0 to 6.8) was the same as that observed with virus-dependent hemagglutination. After the initial interaction, pH changes no longer influenced the virus binding to liposomes. Virus bound to liposomes prepared from a mixture of erythrocyte

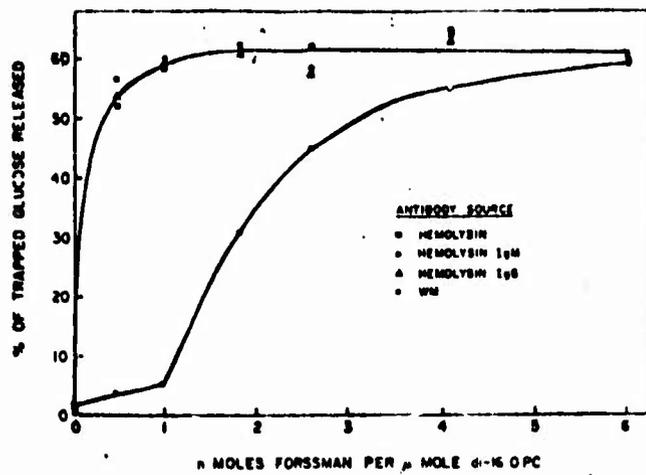


Figure 1: The effects of various antibodies on glucose release from liposomes containing different Forssman concentrations. Seven liposome preparations were made. In addition to di-16:0 PC, cholesterol, and di-cetyl phosphate, each preparation contained a concentration of Forssman which corresponded to an amount shown on the abscissa. The procedure was the same as in Figure 1, except that the liposomes were preincubated in either hemolysin (5 $\mu$ l), hemolysin IgM fraction (0.143 mg), hemolysin IgG fraction (0.610 mg), or WM (0.490 mg) and glucose release was measured 30 minutes after adding fresh GPS.

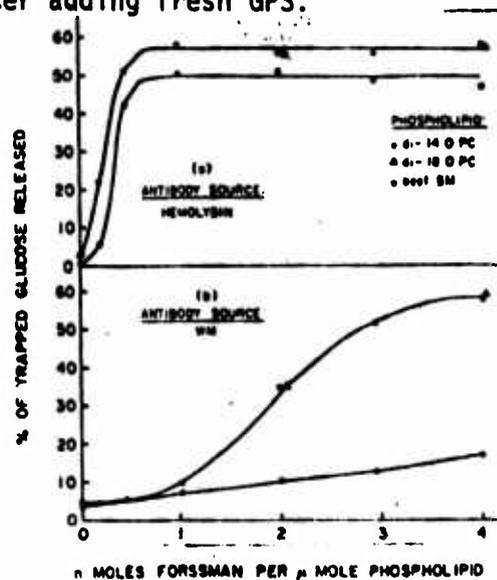


Figure 2: The effects of phospholipid composition on glucose release from liposomes containing varying concentrations of Forssman. The procedure was the same as in the legend of Figure 2, except that the phospholipid used to prepare the liposomes was, as indicated, either d14:0 PC, d1-18:0 PC, or beef SM. The liposomes in (a) were preincubated in 5  $\mu$ l of hemolysin, and in (b) were preincubated in 0.196 mg of WM.

TABLE 4. Specificities of the Interactions of Several Antisera and WM with Various Glycosphingolipids.<sup>a</sup>

Antibody Source	% of Trapped Glucose Released from Liposomes Containing One of the Following Antigens:					
	None (control)	Glu-cer	Gal-cer	Cytolipin H	Globoside I	Forssman
WM	5.8	2.6	7.5	4.6	4.3	56.0
Hemolysin	0.5	5.5	3.2	57.3	10.6	56.5
Anti-HRBC	0.5	3.6	5.9	67.6	59.0	4.0
Anti-Gal-cer	2.6	0	60.7	61.0	1.6	1.6

<sup>a</sup>Six liposome preparations were made which contained di-16:0 PC, cholesterol, dicetyl phosphate, and which also contained one (or none) of the glycolipids as indicated. Glucocerebroside, galactocerebroside, and cytolipin H were each in concentrations of 150  $\mu\text{mol}$  of PC (approximately 184, 184, and 154  $\text{nmol}/\mu\text{mol}$  of PC, respectively). Globoside I and Forssman were in concentrations of 100 and 6  $\text{nmol}$ , respectively, per  $\mu\text{mol}$  of PC. The liposomes were preincubated with either WM (0.196 mg), hemolysin (5  $\mu\text{l}$ ), anti-HRBC serum (25  $\mu\text{l}$ ), or anti-galactocerebroside serum (37  $\mu\text{l}$ ). Glucose release was measured 30 min after adding 120  $\mu\text{l}$  of fresh GPS.

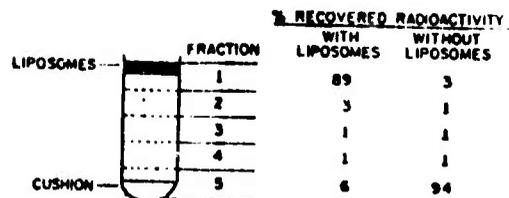


FIG. 3. Centrifugation characteristics of Sindbis virus and liposomes. Each tube contained 14,000 counts/min of radiolabeled virus, either with or without liposomes prepared from sheep erythrocyte extracts. 100  $\mu$ liters of liposomes was incubated with virus at 37 C, using borate-phosphate-saline buffer (2) at pH 5.8. The values shown are the means of either five experiments (with liposomes) or four experiments (without liposomes).

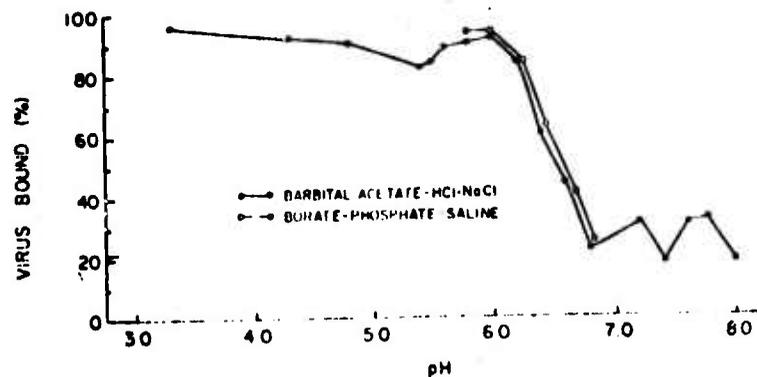


FIG. 4. pH dependence of Sindbis virus attachment to liposomes. Each point represents a separate liposome preparation. The isotonic barbital-acetate-HCl-NaCl buffers were prepared as follows: 10 ml of 0.16 M sodium barbital-0.143 M sodium acetate was mixed with 1.0 N HCl to achieve the desired pH. The solution was made isotonic by adding appropriate quantities of 1.54 M NaCl and water to a final volume of 50 ml. 100  $\mu$ liters of sheep erythrocyte liposomes, swollen in the indicated buffer at the appropriate pH, was incubated in the same buffer with radiolabeled Sindbis virus at 37 C.

phospholipids, but the binding was greatly diminished when either cholesterol or phosphatidylethanolamine (Fig 5) was omitted from the liposomal lipid mixture. It was concluded that phospholipids and cholesterol, in a bilayer configuration, may be sufficient for specific virus binding in the absence of membrane protein.

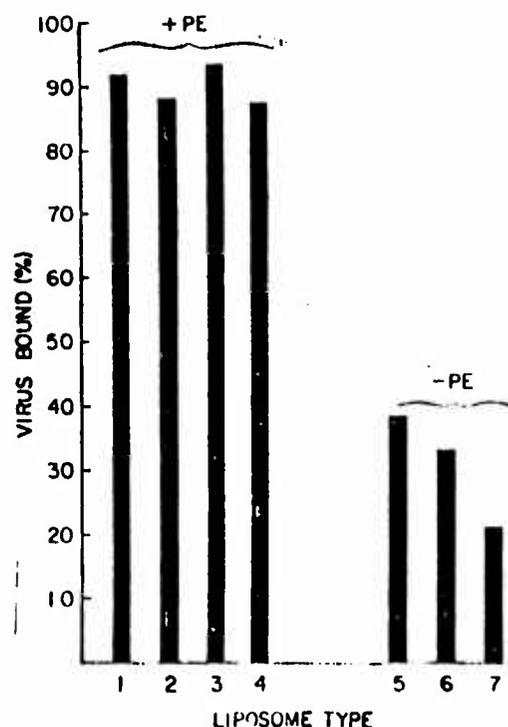


FIG. 5. Virus binding to liposomes containing mixtures of different phospholipids. Sindbis virus was incubated at 23 C with 20  $\mu$ liters of liposomes containing one of the following molar mixtures: (mixture 1) PL-cholesterol (2:1.5); (mixture 2) sheep erythrocyte phosphatidylethanolamine-sphingomyelin-phosphatidylserine-cholesterol (1:1:0.22:1.5); (mixture 3) sheep erythrocyte phosphatidylethanolamine-phosphatidylserine-cholesterol (2:0.22:1.5); (mixture 4) sheep erythrocyte phosphatidylethanolamine-sphingomyelin-cholesterol (1:1:1.5); (mixture 5) sphingomyelin-phosphatidylserine-cholesterol (2:0.22:1.5); (mixture 6) sphingomyelin-(1-stearoyl-2-oleoyl) phosphatidylcholine-phosphatidylserine-cholesterol (1:1:0.22:1.5); (mixture 7) (1-stearoyl-2-oleoyl) phosphatidylcholine-phosphatidylserine-cholesterol (2:0.22:1.5). Each bar represents the mean virus binding to 20- $\mu$ liter liposome samples observed in three assay tubes. The same peak fraction of radiolabeled virus was used with mixtures 1 through 3 and 5 through 7. Two other peak fractions of virus were used in the experiments with mixture 4.

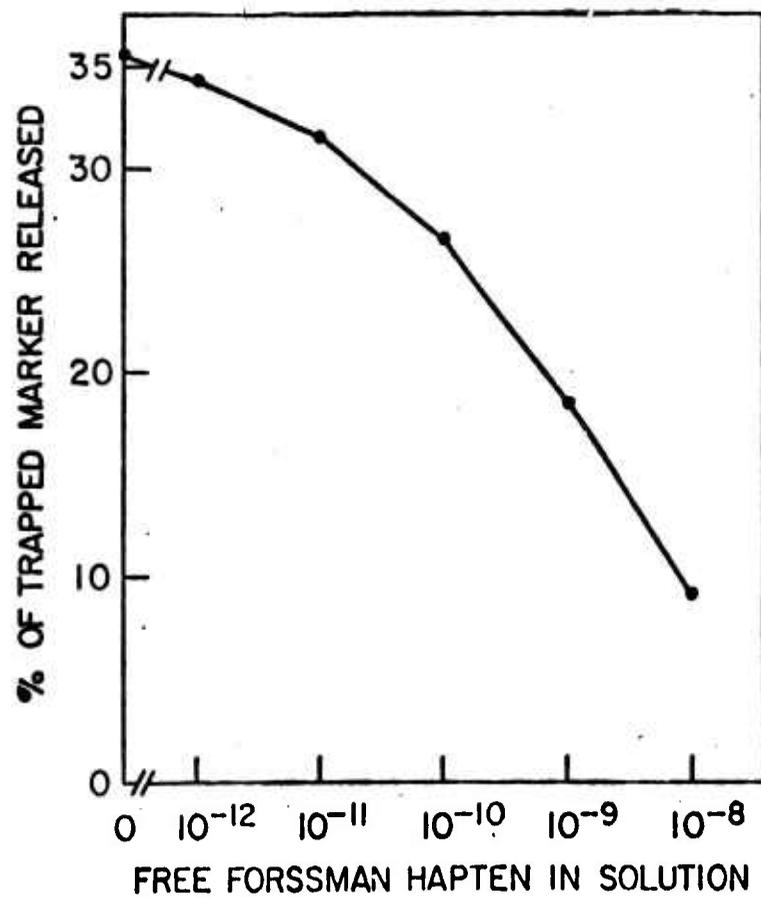


Fig. 6.

Inhibition by fluid phase hapten of spin label release. The data was corrected for baseline spin label observed in the absence of antibody or complement.

4. Immuno thin layer chromatography: A new specific method for detection of lipid antigens.

A simple method for detection of lipid antigens has been developed. The lipids or lipid mixtures are streaked on thin layer plates and are treated with antiserum either directly or after the plate has been developed. After nonimmune, or unreacted, protein has been washed away, the plates are sprayed with ninhydrin to detect protein bound to lipid antigen spots. This method provides a rapid and direct means for screening complex mixtures of lipids for antibody binding activity.

5. Liposome spin immunoassay: A new sensitive method for detecting lipid substances in aqueous media.

A new sensitive immunoassay procedure has been developed for the quantitative detection of glycolipids and other lipids in aqueous media. As with other immunoassays, specific antiserum is first reacted with the free lipid hapten. The amount of antibody activity remaining is measured by assaying the release, in the presence of complement, of spin label marker from liposomes containing the same lipid hapten. Using this method, 2.6 picomoles of aqueous Forssman hapten was detected (Fig 6), and the sensitivity could be increased further.

6. Abnormal lipid composition of the red cell membrane in congenital dyserythropoietic anemia type II (Hempas).

The lipids derived from the erythrocytes of two siblings with clinical congenital dyserythropoietic anemia Type II (CDA-II) and one sibling without clinical evidence of CDA-II were subjected to a detailed analysis. The total phospholipids, total cholesterol, and cholesterol: phosphate ratio were normal in all siblings. In the two clinically affected siblings there was a significant increase in the phosphatidyl choline with a concomitant decrease in the other phospholipids. The fatty acids of phosphatidyl choline from the CDA-II erythrocytes were normal. The total glycosphingolipids were increased in all siblings. The glycosphingolipids, di-, tri-, and tetrahexosyl ceramides were increased 1.5- to 10- fold over controls in the clinically affected siblings. The increases were present but less striking in the clinically unaffected sibling. Glucocerebroside was not significantly increased in any of the siblings.

7. Physical Characteristics of Microaggregates in Stored Blood

Alterations in the structure and function of human lungs have been known to result from infusion of amorphous material in stored human blood which is not removed by conventional 170  $\mu$  pore mesh clot mesh clot mesh clot filters (12,13). Studies of combat casualties in Viet Nam have implicated microembolization resulting from blood transfusion

in the pathogenesis of post-traumatic pulmonary insufficiency (14,15,16). As a result, blood filters consisting of Dacron wool, 40  $\mu$  pore mesh and polyurethane foam have been developed. Solis and Gibbs (17,18) found that these filters effectively remove the smaller particles from stored blood. In their evaluation, these authors measured the size distribution of microaggregates in fresh and stored human blood with a Model T Coulter electronic particles size analyzer. In the present study, this technique is evaluated and utilized to measure the extent of microaggregate formation in blood components stored under a variety of conditions.

**Description.** The principle of the Model T counter is similar to that of Model B and plotter except that measurements are made of the number of particles in 15 channels simultaneously rather than sequentially. Each channel of the instrument detects particles twice the size of those in the previous channel. The relationship of the channel to the geometric mean volume and the particle diameter size using a 400  $\mu$  aperture is shown in the first three columns of Table 5.

Microaggregates in stored blood were obtained from human blood drawn into plastic bags containing acid-dextrose-citrate (ACD) or citrate-phosphate-dextrose (CPD) and stored at 4 C for up to 31 days. Platelet aggregates were induced by adding adenosine diphosphate (ADP) to platelet-rich plasma (PRP) or whole blood (WB).

TABLE 5. Reproducibility of Eight Size Distribution Measurements of Microaggregates Made from a Single 1:200 Dilution of Stored Whole Blood

Channel	Instrument Calibration for 400 $\mu$ Aperture		Number of Microaggregates/20 ml Dilution		
	Mean Particle Volume ( $\mu^3$ )	Particle Size ( $\mu$ )	Mean	Range	S.E.*
0	$3.10 \times 10^6$	161	2.6	0.5	0.56
1	$1.555 \times 10^6$	128	8.5	2-13	1.6
2	$777.4 \times 10^3$	101	16.5	8-22	1.7
3	$388.7 \times 10^3$	80.6	22	14-34	2.1
4	$194.4 \times 10^3$	61.0	55	39-67	3.1
5	$97.2 \times 10^3$	50.8	126	112-133	2.6
6	$48.5 \times 10^3$	40.3	295	247-329	9.0
7	$24.3 \times 10^3$	32.0	661	552-757	21
8	$12.1 \times 10^3$	25.4	1,333	1,216-1,482	33
9	6,066	20.2	2,975	2,455-2,652	25
10	3,033	16.0	4,403	3,300-4,634	40
11	1,516	13.0	8,113	7,833-8,370	80
8-11	small size	15-25	16,424	16,082-16,982	132
3-7	medium size	32-80	1,160	996-1,285	29
0-2	large size	101-161	27.5	13-36	2.8

\* S.E. = standard error

**Progress.** The different methods of presenting particle size data are illustrated in Figure 7, where the size distribution of micro-aggregated in stored and fresh blood is shown. The cumulative and differential number of particles (left panel) and volume of particles in blood (right panel) are plotted on a log scale at each of 12 different diameter sizes. These data were obtained from eight units of fresh whole blood drawn into ACD plastic bags and analyzed after less than one hour at room temperature and from eight similar units after 14 days of storage at 4 to 6C. Statistical analysis of the data plotted in Figure 7 showed a significant increase in particles in the stored, as compared with the fresh blood, at each size from 13 to 101  $\mu$  ( $P < .001$  for particles measured in the following arbitrary size ranges: small (13-25  $\mu$ ), medium (32-80  $\mu$ ), large (101-161  $\mu$ ).

The mean, range, and standard error of counts of microaggregates recorded by the instrument after eight consecutive determinations from a single 300 ml preparation of hemolysed stored blood are presented in Table 5. As would be expected, the variation in the counts increased as

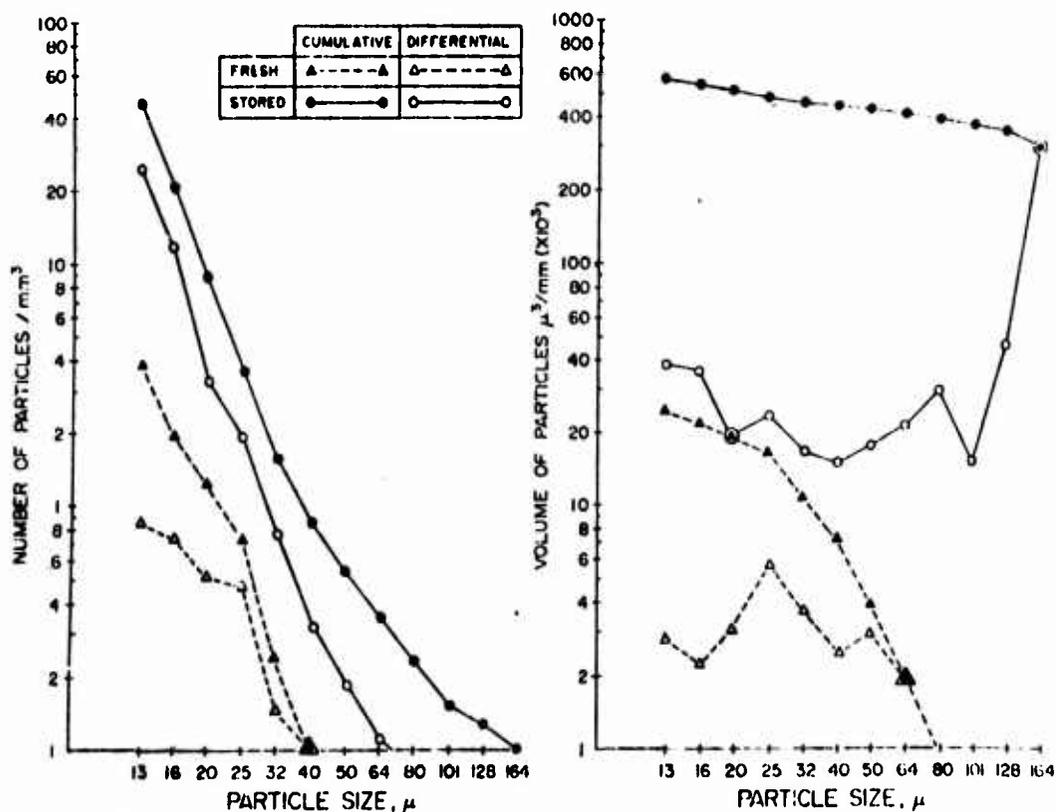


FIG. 7. Size distribution of particles in ACD human blood before (FRESH) and after (STORED) storage at 4 to 6C for 14 days (log scale). The measurements were performed two minutes after dilution (1:201) of blood in saline containing saponin (200 mg/100 ml).

the frequency of occurrence of particles of a given size decreased. The coefficient of variation (standard deviation/mean x 100) of the cumulative counts of microaggregates made of eight different test dilutions of a unit of stored blood was 7.8 per cent for the small (13-25  $\mu$ ), 7.2 per cent for the medium (32-80  $\mu$ ), and 30.9 per cent for the large (101-161  $\mu$ ) aggregates. The difference between these values and the coefficient of variation of the measurements of the small (1.9%), medium (6.9%), and large (28.6%) particles in Table 5 can be attributed to errors in the preparation of the former samples.

The loss of counts of particles as a result of coincidence, i.e., simultaneous passage of more than one particle through a critical part of the aperture, increases with the size of the aperture being used. Figure 8 the coincidence loss observed with the 200  $\mu$  aperture when identical suspensions of human cells were counted with this aperture on the Model T and the 100  $\mu$  aperture on the Model B Coulter Counter. The Model B counts were considered to be true counts since we had previously established their accuracy by hemocytometry (19). With the 200  $\mu$  aperture on the Model T there was little or no coincidence when less than 6,000 red blood cells per ml suspension were counted. A 10 per cent coincidence loss in counts was sustained when 11,000 cells per ml were counted. The coincidence loss with the 400  $\mu$  aperture has been found to be negligible when less than 1,500 particles per two ml

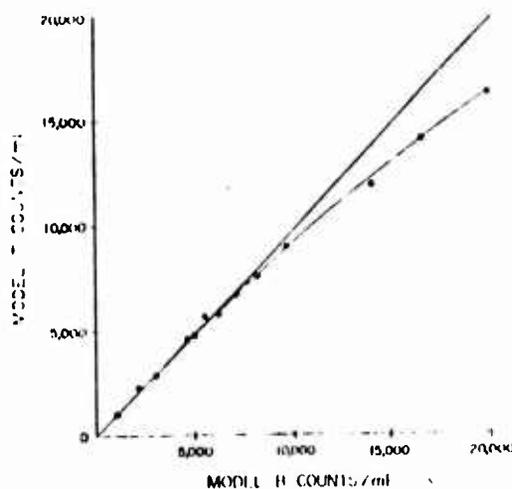


FIG. 8. Primary coincidence of human red blood cells observed with a 200  $\mu$  aperture on the Model T Coulter Counter. The line was drawn through the origin at 45°.

of saline are counted. Figure 9 shows that microaggregates  $32 \mu$  and larger can be measured in nonhemolyzed suspensions of blood. In the top panel of Figure 10, the dissociation of microaggregates in stored blood following dilution in saline is compared with that of ADP-induced platelet aggregates of the same size. Immediately after dilution (time 0), the volume of microaggregates in the stored blood was not significantly different from that of platelet aggregates. During the subsequent 120 seconds the microaggregates remained stable, while the platelet aggregates completely dissociated.

The rate of dissociation of the microaggregates in stored blood after dilution could be accelerated by altering the diluent. This is shown in the bottom panel of Figure 10 where measurements of the same microaggregates in saline are compared to those made in saline buffered at pH 7.9, and in Isoton, which is buffered at 7.4. There was a step-wise reduction in the volume of particles as the pH of the diluent was increased. In contrast to the acutely induced platelet aggregates, which completely dissociated, some of the microaggregates in stored blood remained stable after 120 seconds despite the differences in pH.

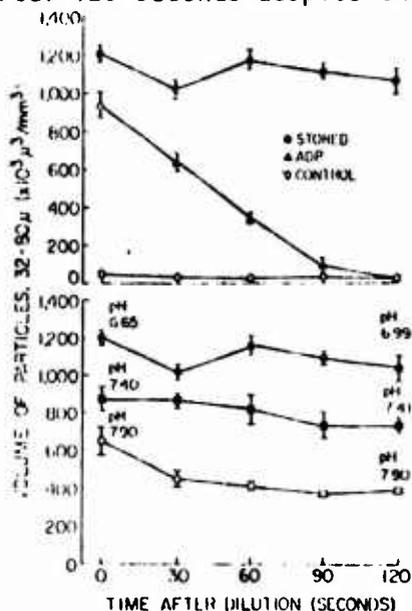


FIG 9. *In vitro* dissociation of microaggregates. In the top panel, the volume of particles  $32-80 \mu$  in size measured with a  $200 \mu$  aperture is plotted at various times after dilution in isotonic saline of stored human blood before (STORED), after Dacron wool filtration (CONTROL), and of blood containing ADP-induced platelet aggregates (ADP). In the bottom panel, the measurements of the unfiltered stored blood in saline (closed circles) are compared with similar measurements made after dilution of the stored blood in Isoton (closed squares) and in saline buffered to pH 7.9 with Thiam-buffer (open circles). The pH of the diluents immediately after and 120 seconds after dilution (1:101) are plotted near the respective particle measurements (Mean  $\pm$  S.E.).

MICROAGGREGATES IN STORED BLOOD

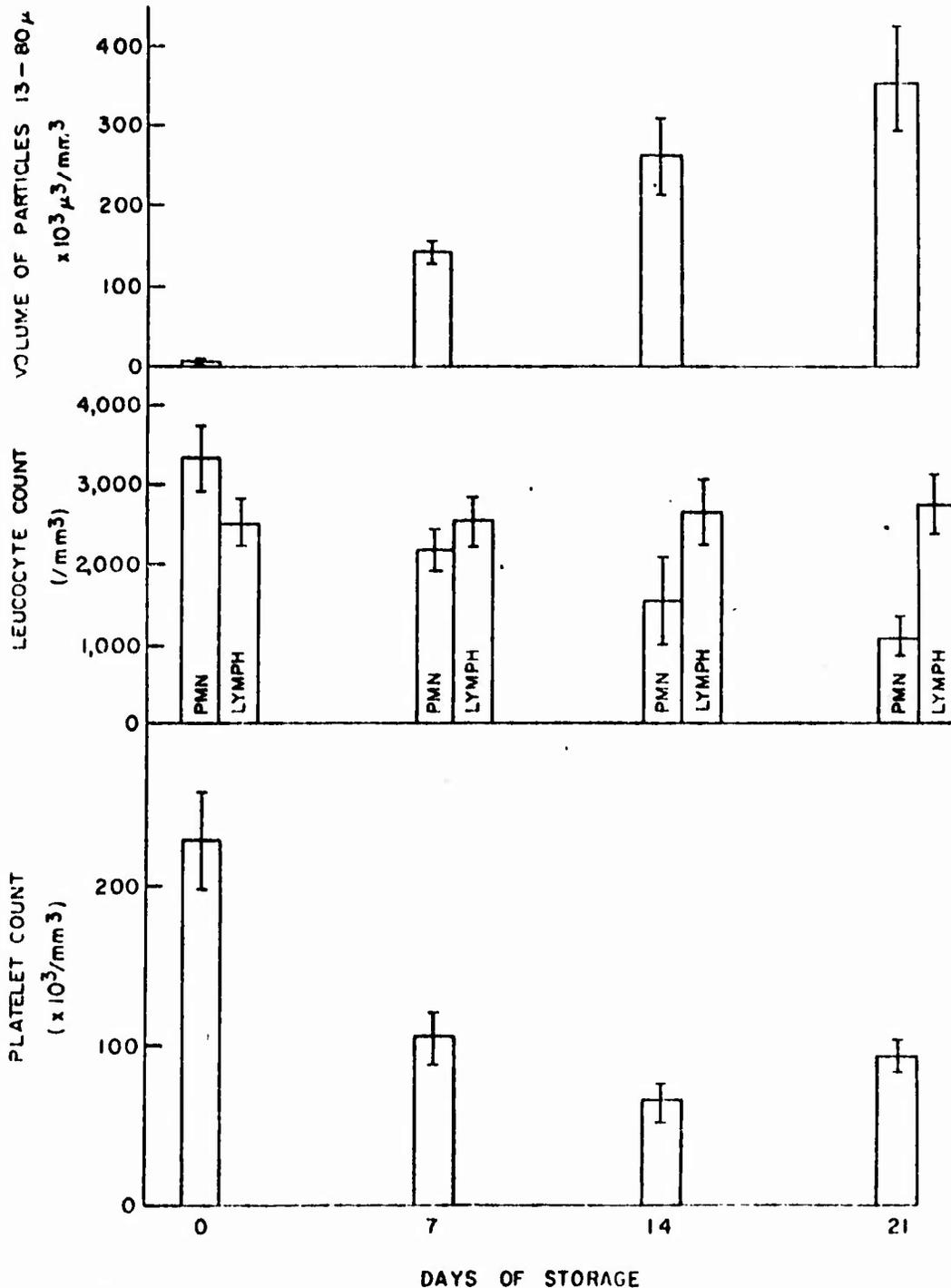
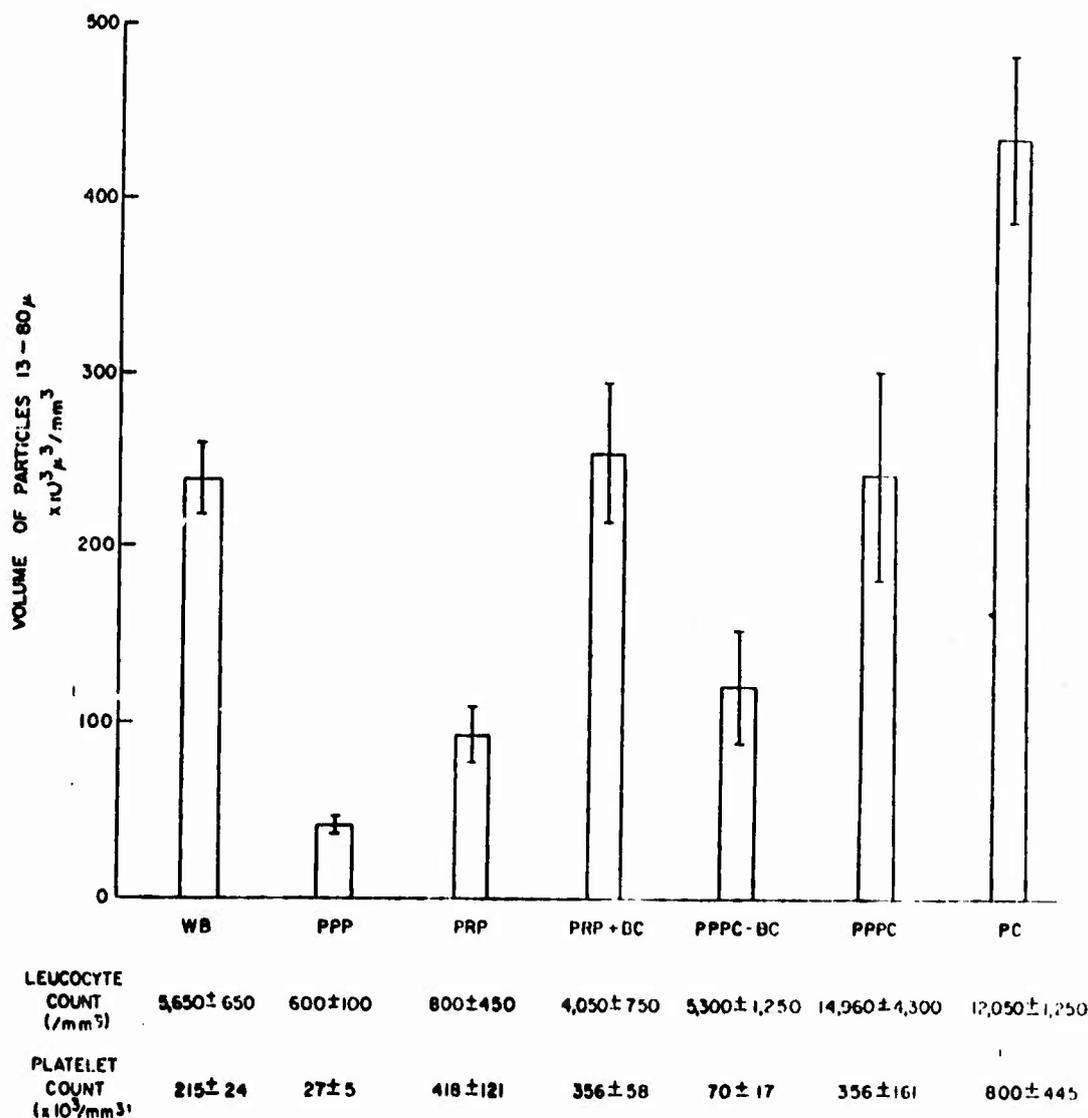


FIG. 10. Microaggregate formation during storage of ACD human blood at 4 to 6°C. The volume of particles 13 to 80  $\mu$  in size was measured two minutes after dilution of blood in saline containing saponin (200 mg/kg). The absolute polymorphonuclear (PMN), lymphocyte (LYMPH), and platelet counts were performed by phase hemocytometry and differential counting.

**Time Course of Development of Microaggregates During Storage:** Microaggregates were found to develop progressively during storage of blood in ACD plastic bags at 4 to 6C (Fig. 10). Coincident with the development of these particles there was a progressive reduction in the granulocytes, while the absolute lymphocyte counts remained constant (Fig. 10). The platelet counts fell only during the first week of storage. **Microaggregate Formation in Blood Components:** The extent of development of microaggregates in various blood components is shown in Figure 11.



**Fig 11.** Microaggregate formation in blood components after 21 days of storage at 4 to 6C. The volume of particles 13 to 80  $\mu$  in size was measured two minutes after dilution in saline containing saponin (200 mg/100 ml). Leukocyte and platelet counts were performed prior to storage. (Mean  $\pm$  S.E.)

Plasma (PPP) and platelet-rich plasma (PRP) did not develop the same volume of particles which was formed in whole blood, even though the initial concentration of platelets in the PRP was greater than in the whole blood. When buffy coat was added to platelet-rich plasma (PRP + BC) and the initial concentration of leukocytes was increased, the volumes of particles which formed after 21 days of storage was equal to that which developed in the whole blood. This along with the reduction in the volume of particles in the component which consisted of platelet-poor packed cells minus the buffy coat (PPC-BC) suggests that both platelets and leukocytes contribute to the formation of the microaggregates. In support of this is the finding that the microaggregates settle into the buffy coat and not to the plasma or to red blood cell layers following centrifugation of stored blood (Fig. 12), indicating that they have a density similar to that of platelets and leukocytes.

Effects of Anticoagulants, Temperature and Storage Containers on Microaggregates Formation: The extent of microaggregate formation was found to depend on the type of anticoagulant used and the temperature during the storage period (Table 6). Blood drawn into CPD and stored for 24 hours at 4° to 6°C developed a greater volume of microaggregates than did blood drawn into ACD (IB in Table 2,  $p < .001$ ). However, after seven and 23 days of storage under blood bank conditions in plastic bags, no significant difference was found in the volume of microaggregates formed in ACD or CPD blood. Microaggregate formation in ACD blood stored 24 hours at room temperature (23° to 25°C) was markedly reduced when compared with that in blood stored at 4° to 6°C (IBB in Table 2). However, blood drawn into ACD and stored in plastic bags developed a similar volume of microaggregates after 21 days of storage at 4° to 6°C as did blood drawn into ACD and stored in vacuum glass bottles (IIIA in Table 6).

Discussion. The present study has shown that electronic particle measurements can be used to quantitate the particulate material which develops in stored blood provided errors in coincidence are avoided. It also demonstrates that the cumulative volume of microaggregates increases progressively during blood storage. Simultaneous hemocytometry measurements also indicate that platelets contribute to their formation only during the first week, while granulocytes continue to clump through the 21 - day storage period. Moreover the extent of formation of the microaggregates is related to the concentration of leukocytes and platelets. The finding in Table 6 that a greater volume of microaggregates formed in blood stored at 4°C than at room temperature is consistent with the known effects of low temperature on platelet aggregation in plasma (20, 21). Similarly, the larger volume of microaggregates detected in CPD than in ACD anticoagulated blood after 24 hours of storage at 4°C agrees with other observations that platelets spontaneously clump more readily in plasma prepared from CPD blood (22). The observation that the same volume of particles was formed in blood stored in glass vacuum bottles as was formed in plastic bags suggests that the type of container does not significantly alter microaggregate formation during the 21-day storage period.

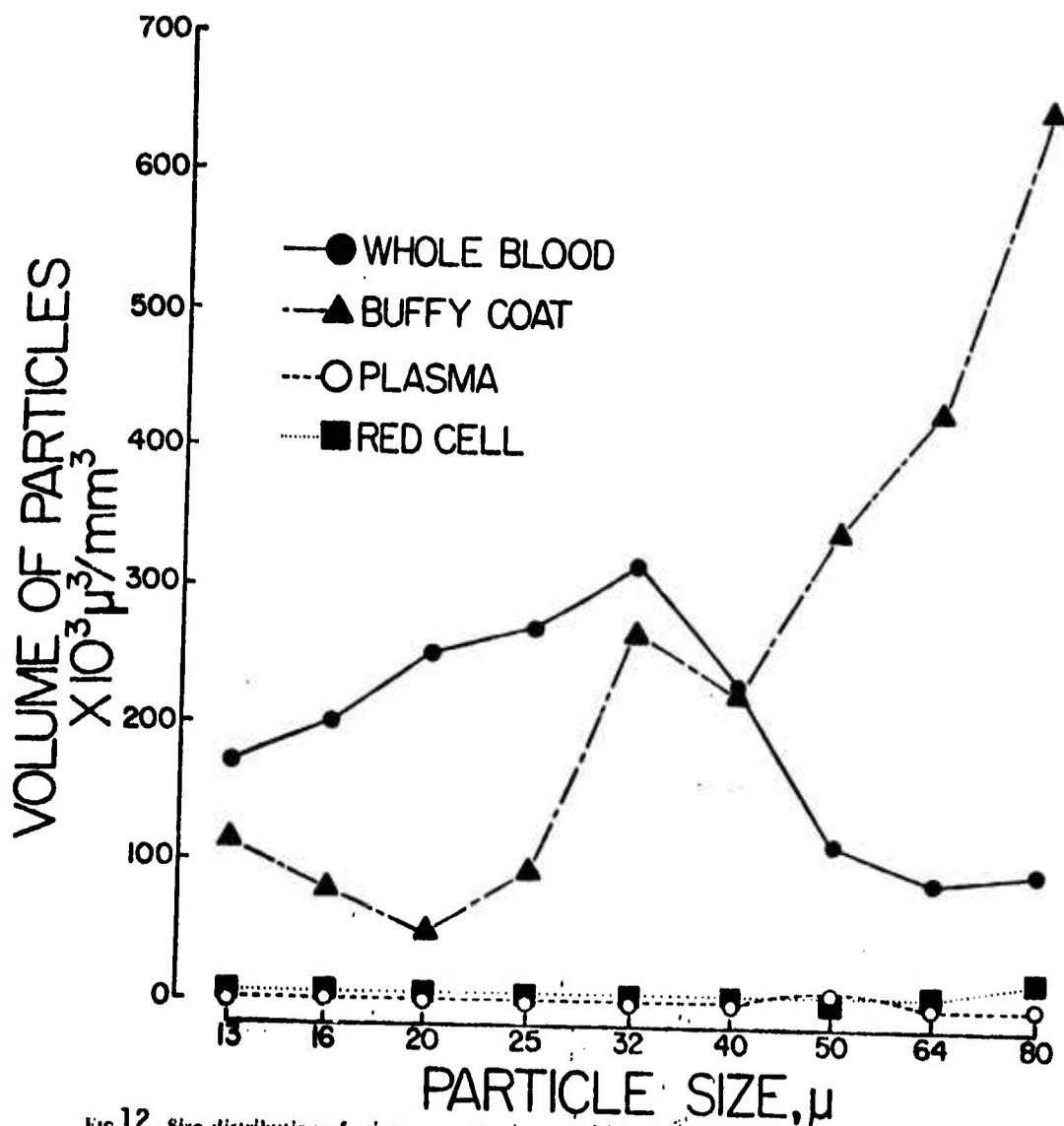


FIG. 12. Size distribution of microaggregates in stored blood before (WHOLE BLOOD) and in the plasma, buffy coat and red blood cell layers after centrifugation for five minutes at  $3,000 \times g$ . Particle measurements were made three to five seconds after dilution of blood in saline containing saponin (50 mg/100 ml). The blood had been stored for 21 days at  $4^\circ$  to  $6^\circ C$  with ACD anticoagulant.

The electronic measurements of the dissociation rates of microaggregates in stored blood in Figure 9 give some indication of the physical characteristics of these particles. Acutely induced platelet aggregates were completely dissociated within two minutes after dilution in saline-saponin while 40 per cent of the microaggregates in stored blood remained stable. Since the measurements shown in Figures 10 and 11 were made two minutes after dilution in saline-saopoin, they represent the volume of microaggregates which are most resistant to dissociation and would presumeably be most damaging to the tissue to which they embolize. The increase in dissociation rate of microaggregates in vitro as the pH was increased (Fig. 9) suggests that some dissociation would occur in vivo after transfusion has a result of the differences in pH of stored blood and that in the circulation of the recipient. Although the use of one of the more efficient blood filters may lower the incidence of pulmonary insufficiency in man after trauma and massive transfusion, the minimal amount of blood that can be safely administered using the standard mesh clot filter remains to be determined.

TABLE 6. Effects of Anticoagulant (I), Temperature (II), and Storage Containers (III) on the Volume of Particles 13-80  $\mu$  in Size Formed in Human Blood During Storage\*

Storage conditions	Mean Volume of Particles ( $\pm$ S.E.) $\times 10^3 \mu^3/\text{mm}^3$	
	ACD	CPD
I. Anticoagulant (n=8)		
A. Baseline, test tube, 23-25 C	80 $\pm$ 9	113 $\pm$ 9
B. 24 hours, test tube, 4-6 C	87 $\pm$ 10	392 $\pm$ 35
C. 7 days, plastic bag, 4-6 C	714 $\pm$ 69	713 $\pm$ 104
D. 23 days, plastic bag, 4-6 C	1,353 $\pm$ 215	1,470 $\pm$ 248
II. Temperature (n=6)	4-6 C	23-25 C
A. Baseline, ACD, plastic bag	-	100 $\pm$ 33
B. 24 hours, ACD plastic bag	327 $\pm$ 48	161 $\pm$ 46
III. Container (n=8)	Plastic bag	Glass bottle
A. 21 days, 4-6 C, ACD	987 $\pm$ 132	1,019 $\pm$ 169

\* The analysis was begun three to five seconds after dilution of the blood in saline saponin (50 mg/100ml).

## 8. Sequential appearance of guinea pig IgG1 homocytotropic antibody.

It was generally believed that the specificity of cell-mediated immune response requires both haptenic and carrier determinants whereas the specificity of humoral mediated response was directed primarily towards haptenic groups and to a lesser degree towards carrier protein. Gell and Silverstein (23,24) and others (25,26,27) have reported that the specificity of some circulating antibody, particularly early response, can be specific for the intact hapten-protein complex, that is the response requires a portion of the hapten as well as a portion of the carrier molecule. Borek and Silverstein (28) and later Henney (29) extended these observations and indicated that there was a temporal order of appearance of antibodies of differing specificities.

A previous investigation on the effects of adjuvant on the production of guinea pig IgG1 antibody suggested the possibility of detecting antibodies of differing specificities and their order of appearance (30). The results obtained demonstrate, particularly with certain antigen-adjuvant combinations, the sequential appearance of antibodies of differing specificities following immunization with hapten-conjugated homologous serum albumin.

Antigen. P-amino benzoate (PAB) and O-nitro anlyl (ONA) protein conjugates were prepared by a modification of the method of Nisonoff by coupling the diazonium salt derivatives to either guinea pig serum albumin (GPA) or bovine serum albumin (BSA). The amount of reactants used was calculated assuming an efficiency of 35% to obtain a yield of 8 hapten groups per molecule of serum albumin. P-amino benzoic acid and O-nitro aniline were purchased from Fisher Scientific Co. Guinea pig serum albumin and bovine serum albumin were obtained from Pentex, Inc. Protein was determined spectrophotometrically by the method of Lowry (32).

### Preparation of antigen for immunization:

Antigen without adjuvant (Ag). One ml of PAB-GPA at a concentration of 10  $\mu\text{g}/\text{ml}$  in 0.15M NaCl was given intraperitoneally for priming and booster injections.

Antigen emulsified with complete Freund's adjuvant (Ag-CFA). Equal quantities of PAB-GPA at 40  $\mu\text{g}/\text{ml}$  in 0.15M NaCl and Difco complete Freund's adjuvant were emulsified and a total of 0.5 ml per guinea pig distributed equally into all foot pads for priming. Booster injections were administered subcutaneously.

Alum adsorbed antigen (Ag-A). Equal quantities of PAB-GPA at 20  $\mu\text{g}/\text{ml}$  were mixed and allowed to stand for 30 minutes at room temperature. The precipitate obtained after centrifugation was then washed twice with 0.15 M NaCl and resuspended to the original volume. Each

guinea pig was injected with 1.0 ml intraperitoneally for priming and booster injections.

Immunization. Three major groups of 15 guinea pigs (400-500 gms) each were primed with 10  $\mu$ g of PAB-GPA<sup>2</sup>. The first group was primed with antigen emulsified in complete Freund's adjuvant (Ag-CFA), the second group with Ag adsorbed to alum (Ag-A) and the third group received Ag without adjuvant (Ag). Each group was subdivided into 3 additional subgroups of 5 guinea pigs each and boosted with either Ag-CFA, Ag-A or Ag. Each guinea pig in nine subgroups was injected every 14 days and bled 7 days post injection from the ophthalmic plexus and each individual serum obtained was frozen until assayed for skin sensitizing antibody. Antisera were routinely diluted in half with 0.15M NaCl and then heated at 56°C for 2 hours before assaying to eliminate possible interference by IgE antibody.

PCA reactions. The technique used was essentially the same as reported by Ovary (33), two-fold dilutions of antiserum were injected intradermally in the dorsal surface of shaved guinea pigs. After a sensitization period of 18 hours the animals were challenged with antigen contained in 0.35 ml of an antigen-dye mixture (1 part of antigen at 10 mg/ml plus 1 part of 6% pontamine sky blue). Thirty minutes after challenge the animals were sacrificed and the lesion diameters were measured and recorded. The estimation of the titer of antiserum was determined by plotting the diameter against the log of the antibody concentration injected. The titer of each antisera is expressed as the antibody dilution required to evoke a 5 mm reaction diameter as determined from the plotted values.

Double titrations: After sensitization, the guinea pigs were challenged first with one antigen-dye mixture, and thirty minutes later the results were measured and recorded. The same animals were then rechallenged with a different antigen-dye mixture and the reactions were measured thirty minutes later. The increase in titer by the second challenge antigen would reflect the specificity and potency of the antibody for that antigen. The limitation of this method for the demonstration of antibodies of different specificities in antisera are: 1) the second challenging antigen would not detect antibodies with a potency less than or equal to those demonstrated by the first challenging antigen; 2) the first challenge either inhibits or enhances the response towards the second challenging antigens.

Experiments comparing antisera either by single or by double titration showed no difference in titer suggesting that prior challenge did not inhibit or enhance the response towards the second challenging antigen.

The specificity of each antisera was determined by challenge with

each of the following antigens: 1) the homologous conjugate (PAB-GPA) used for immunization; 2) the homologous haptenic groups conjugated to a heterologous carrier (PAB-BSA); and 3) the homologous carrier (GPA). Table 7 lists the number of guinea pigs responding to challenge with the three test antigens as related to the adjuvant and sequence used for immunization. Twelve of the 44 guinea pigs did not respond to any of the three test antigens. Six guinea pigs produced antibody that reacted only to the homologous antigen throughout the course of immunization, while 24 animals reacted with both PAB-GPA and PAB-BSA. In the latter group 6 guinea pigs initially reacted to only PAB-GPA and then with further booster injections went on to produce antibodies that reacted to PAB-BSA. Also within this group there were three animals that first responded to PAB-GPA and PAB-BSA and with subsequent immunization went on to produce antibodies to GPA. In addition, there were 2 guinea pigs that produced antisera that reacted with all three test antigens at the first appearance of antibody activity.

The exclusive reaction of antisera from 6 animals with the homologous antigen suggests the production of antibodies specific for the intact hapten-protein complex requiring both a portion of the hapten and a portion of the carrier molecule-for reactivity. In addition to these 6 animals, there were 6 other guinea pigs that initially produced antisera reactive to PAB-GPA, the intact hapten-protein complex. The clear demonstration in 9 out of 12 guinea pigs producing antibodies specific for the immunizing antigen were obtained from animals primed with antigen adsorbed to alum and boosted with either Ag-A or antigen without adjuvant.

The 12 antisera that reacted exclusively with the homologous antigen used for immunization were tested to determine if reactivity was due to the intact hapten-protein conjugate or to newly created determinants on the GPA as a consequence of coupling. Each antisera diluted 1/10 were challenged with O-nitro-analyl-GPA (ONA-GPA); after 30 minutes the results were recorded. These same animals were then rechallenged with the homologous antigen (PAB-GPA) to establish that these sera had not lost activity. In addition, a control group of animals were challenged only with homologous antigen (PAB-GPA). Table 8 lists the results obtained. It is apparent that none of these antisera reacted with ONA-GPA, whereas a substantial number of antisera responded to the homologous antigen (PAB-GPA) on rechallenge. The data also shows that 3 antisera were unresponsive to the PAB-GPA although they had activity when tested months previously. These results indicate that the response obtained with the homologous antigen (PAB-GPA) is due to the presence of antibody with specificity directed towards the intact conjugate and not directed towards newly created determinants on the GPA carrier protein. The antibody specificity of the 24/44 guinea pig antisera that reacted to both PAB-GPA and PAB-BSA is difficult to interpret since the antigens possess overlapping antigenic determinants. A response with PAB-GPA, the immunizing antigen, could be attributed to antibodies directed toward either the intact conjugate or the modifica-

tion of the carrier by conjugation, or the haptenic determinant, or by any combination of these. The response with challenge by PAB-BSA is more restrictive in the detection of antibodies produced. With this antigen, the antibody specificities could be either towards the hapten determinant or the area modified by conjugation or both. Although the demonstration of antibodies of differing specificities with these challenging antigens is fraught with difficulties, it nevertheless is possible to discern antibodies directed toward specific determinants in many of the antisera. Within this group there are 18 sera in which the primary question is the demonstration of antibodies with specificities directed towards the intact conjugate. Ideally, the approach would be to demonstrate the presence of the antibody towards the intact hapten-protein complex after removal of all other antibodies by adsorption. Unfortunately, the limited quantity of antisera precluded any extensive adsorption studies. However, it was felt that discernment of antibodies could be accomplished by double titration of selected antisera.

Eleven of the 18 antisera were tested for the demonstration of antibodies specific for the intact conjugate by first challenging guinea pigs with ONA-GPA to demonstrate the presence of antibodies toward newly created determinants of the GPA and the second antigenic challenge was accomplished with PAB-BSA to demonstrate hapten specific antibodies. The same antisera were then tested first by challenge with PAB-BSA for haptenic determinants, followed by challenge with the immunizing antigen (PAB-GPA). By utilization of a common challenge antigen, namely PAB-BSA, in both groups it was possible to relate the relative potency of antibodies of differing specificities. The results obtained in Table 9, utilizing this method of analysis, clearly demonstrates that all the antisera tested possess antibodies with specificities directed toward the intact conjugate. In addition, the data also demonstrate that, although all the antisera possess haptenic antibody, not all possess antibody toward newly created determinants as a consequence of conjugation.

These results suggest that there is a definite appearance of antibodies of differing specificities to the PAB-GPA antigen used in this investigation. Table 9 lists the number of guinea pigs producing antibodies of differing specificities in their order of appearance. There were 6 guinea pigs that produced antibodies specific for the intact conjugate exclusively and an additional 6 guinea pigs that initially produced antibodies specific for the intact conjugate and then went on to produce hapten specific antibody. More importantly, none of the guinea pigs produced hapten or carrier specific antibodies either exclusively or prior to the appearance of antibodies directed toward the intact conjugate. The sequence of appearance indicates that antibody specific for the intact conjugate is the first to appear followed by hapten specific antibody, and finally, by antibody specific for the carrier protein. These data were statistically analyzed by the binominal probability model and the values obtained are highly significant ( $\alpha \leq .001$ ) so that one may conclude that this sequential order

Table 7

The number of guinea pig antisera responding to various challenging antigens.

Immunization Sequence		Challenging Antigens		
Priming Injections	Booster Injections	PAB-GPA	PAB-GPA or PAB-BSA	PAB-GPA or PAB-BSA or GPA
Ag	Ag-CFA	0/5*	4/5	0/5
	Ag-A	0/4	1/4	0/4
	Ag	0/5	0/5	0/5
Ag-CFA	Ag-CFA	0/5	4/5	1/5
	Ag-A	0/5	4/5	1/5
	Ag	0/5	5/5	0/5
Ag-A	Ag-CFA	0/5	4/5	0/5
	Ag-A	2/5	2/5	0/5
	Ag	4/5	0/5	0/5
Total		6/44	24/44	2/44

\*Number responding at 1/10 dilution

Table 8

The demonstration of antibody specific for the entire hapten-protein conjugate by double challenge

Antisera	Antigens			Titer* with PAB-GPA
	a	b	c	
	ONA-GPA	PAB-GPA	PAB-GPA	
F40 - 4B	0/7**	4/7	4/8	313
A10 - 1B	0/5	2/5	1/5	65
F100 - 2B	0/4	0/4	1/5	10
F50 - 2B	0/5	4/5	0/5	35
A300 - 2B	0/7	4/7	4/8	33
A100 - 4B	0/7	6/7	4/8	55
A400 - 5B	0/5	1/5	0/5	60
A500 - 5B	0/5	0/5	0/5	24
S100 - 3B	0/5	3/5	0/5	10
S300 - 4B	0/5	1/5	2/5	25
S200 - 5B	0/7	5/7	4/8	43
S400 - 5B	0/5	0/5	0/5	10

\* Reciprocal of dilution giving a 5mm reaction

\*\* Animals responding to total tested

a= Guinea pigs challenged with O-Nitro anlyl-GPA

b= Same guinea pigs as column a that were rechallenged with PAB-GPA

c= Guinea pigs challenged with PAB-GPA alone

Table 9

The relative PCA titers of antisera possessing antibodies of differing specificities.

Antisera	Titer*		
	Anti-new determinants	Anti-hapten	Anti-intact hapten-protein
F4	0	20	164
F5	74	124	878
F20	0	154	175
A20	0	1874	2295
A40	67	227	598
A50	0	329	477
S10	35	132	438
S20	0	4307	5094
S50	59	439	796
F200	210	210	427

\*Reciprocal of dilution giving a 5 mm reaction

Table 10

Number and order of appearance of antibodies of different specificities

Hapten-protein complex	Specificity of Antibody		Number of antisera possessing antibody
	Hapten	Carrier	
0	0	0	12
1*	0	0	6
0	1	0	0
0	0	1	0
1	1	0	15
1	1	1	2
1	2*	0	6
1	1	2	3

\*Order of appearance

of specificity is the more probable event.

The data from this investigation support two conclusions, namely, the broad area of recognition by circulating antibody, and the sequential appearance of antibodies of differing specificities. The results obtained may have been due to the particular antigen and the adjuvant or combination of adjuvants used for production of antibodies. The animals were immunized with minimally conjugated hapten to homologous serum albumin, an immunogen that has limited immunogenicity. The reports in the literature clearly demonstrate that the production of haptenic antibodies are related to the number of hapten conjugates (34,35). In addition, it has been repeatedly reported that the use of homologous serum protein as a conjugate is poor to anti-hapten antibody (35,36). The poor immunogenicity of this antigen and the adjuvant combinations has most likely accentuated the results obtained.

The inference drawn over the years has been that the determinants of specificity in the delayed reactions involved a larger portion of the antigen than the serum antibody in which the determinant was more restricted (34,35,37). Evidence has been obtained (38,39,40) particularly with low molecular weight hapten conjugates, that the specificity of such antibodies is directed also against the protein carrier most probably in the immediate vicinity or point of conjugation of simple haptens. The specificity of these antibodies are of two types, those directed toward determinants formed as a result of the conjugation of hapten to protein that are not apparent in the native protein carrier, and those antibodies specific for the intact hapten-protein complex in which both the hapten and a portion of the carrier comprise the determinant. The latter type of specificity was clearly evident with 12 antisera, where a positive PCA reaction was obtained only with the homologous immunogen. These antibodies appeared early, were of low potency, and persisted throughout the immunization schedule. The demonstration in some sera of antibodies specific for newly created determinants formed as a consequence of conjugation, can be seen in Table 9.

These results support the contention that determinants of serum antibody, thought to be rather restricted, are similar to the determinants of cell mediated reactions. Further, the results suggest that the early immune response is directed toward component parts of the immunogen.

The data show the sequential appearance of antibodies of differing specificities to the hapten-protein conjugate. The first antibody demonstrable in antisera from guinea pigs immunized with PAB-GPA was directed toward the intact hapten-protein conjugate. This response is followed by antibodies specific for the hapten determinant and/or newly created determinants and finally, the appearance, in a limited number of guinea pigs, of antibodies directed toward the carrier protein.

The demonstration of this sequential appearance of antibodies of differing specificities was facilitated by the use of a poor immunogen and the choice or combination of adjuvants used for immunization. The guinea pigs primed with antigen adsorbed to alum and boosted with antigen alone produced antibodies specific only for the intact hapten-protein conjugate throughout the course of immunization. This is contrasted with the group primed with antigen and complete Freund's adjuvant where the appearance of antibodies specific for intact hapten-protein complex and antibodies specific for haptenic determinants generally appeared simultaneously. In only two guinea pigs in this group was it possible to demonstrate the appearance of anti-intact conjugate before the appearance of anti-hapten antibody.

These findings are in agreement with Henney (29) who demonstrated a temporal appearance of antibodies of differing specificities to dinitrophenol conjugated to human gamma globulin. Although he used a heterologous carrier and a single antigen injection, his data is similar to the data obtained in this study. Henney demonstrated that intact conjugate and only late in the immune response does anti-hapten and anti-carrier antibodies appear. Although the results obtained indicate a sequential appearance of antibodies of differing specificities, it should be stressed that the results could have been influenced by the assay procedure, and/or the affinities of various antibodies.

#### 9. Investigations of basic immune mechanisms operative in *Schistosoma mansoni* infections.

Background: The DCD&I has conducted extensive studies on the basic immune mechanisms operative in experimental infection of rats with *S. mansoni*. Original studies under Dr. Elvio Sadun established the phenomenon of immunity in this animal. Subsequently, our laboratory has extended these observations. Fischer rats were infected with *Schistosoma mansoni* by exposure to cercariae. The effect of animal age at exposure and the size of that exposure were determined by quantitation of subsequent worm burdens. Optimal conditions for assay of protection mediated by cells or serum transferred from exposed donors were ascertained. The major initial protective immune response to infection was mediated by thymus-dependent lymphocytes as demonstrated by discrete subpopulation cell transfer experiments. Simultaneously harvested serum enhanced worm survival and obviated the protective effects of transferred cells when serum and cells were given concomitantly. Subsequently, host immunity became dependent on antibody formation. In the chronic phase of infection, a small number of surviving parasites coexisted with antibody which specifically interacted with schistosomal antigens but did not protect in transfer experiments. These studies suggest a form of immunologic enhancement with antibody modulation of cellular immunity as possible components of the immune response to both acute and chronic schistosomiasis.

Progress: These studies were then extended to include an examination of the immune response to Schistosoma mansoni infections during re-infection. Inbred rats were exposed to S. mansoni via cercarial penetration. The magnitude of infection was determined by perfusion and direct quantitation of worm burden. Following initial exposure, animals developed a population of thymic derived lymphocytes with specific protective capability as well as immunity mediated by serum factors. The present studies examine the responses of animals who were re-exposed to S. mansoni cercariae. Specific attention was paid to a description of the development of resistance and the optimal conditions for the development. These studies utilized the simultaneous analyses of multiple exposure subpopulations which were followed isotopically. Immunity developed within one week after initial exposure and was specifically directed against the most immature re-infecting schistosomule. This phenomenon, also known as concomitant immunity, was essentially complete by one week after exposure and was confirmed by discrete transfer of immune serum or cells. The optimal development of immunity require the establishment of an infection utilizing normal cercaria. The use of suboptimal numbers, drug cure, irradiation or disruption of cercariae resulted in a marked decrease in the level of immunity which was generated. Finally, although the mechanisms of immunity against re-infections are not clear, they appear to involve antibody and both qualitative and quantitative effects on cercarial development.

Discussion and Recommendations: These studies have now reached the stage whereby the basic immune response of the rat may be manipulated to alter his response to schistosomal antigens in a number of ways. The definition of specific immunogens which are related to protection ought be possible, and the optimal mode of presentation thereof ought be determinable. This, in turn, ought lead to the production of an effective immunoprophylactic schema.

10. Quantitative immunoglobulin class-specific antibody assay for use with soluble antigens.

Objective. Studies were continued to develop and evaluate the use of a competitive inhibition radioimmunoassay for quantitative of class-specific antibody against a soluble antigen.

Description. As discussed in the 1974 report cellulose acetate disks provide a solid matrix for attachment of BSA as antigen in the competitive inhibition radioimmunoassay. Non-antibody immunoglobulin is easily washed away. BSA bound to cellulose acetate disks was used to determine the amount of antibody found in the serum of Rhesus monkeys immunized with BSA.

Progress. The amount of antibody determined using the assay seemed consistently low considering the immunization schedule used. In using

this assay the theoretical assumption must be made that immunoglobulin bound to the BSA disk must compete as well as the radiolabeled immunoglobulin for the limited amount of anti-immunoglobulin available. If this assumption were not true, false low values of antibody might be obtained. In order to test this possibility, known amounts of immunoglobulin (IgG or IgM) were attached by adsorption to cellulose acetate disks to be used in place of soluble standards in the competitive inhibition standard curve. Figure 13 shows that IgM standards are not denatured by simple drying. IgM standards that were dried in test tubes at 37C or 4C before comparison to fresh standards in solution showed identical standard inhibition curves.

Standard curves were not identical however when comparing soluble standards to standards dried on cellulose acetate disks (Fig. 14). This was true for both IgG and IgM standards.

Discussion. These findings support the theoretical objection to using a bound unknown or standard as a competitor for an immunoglobulin in solution. This could be responsible for giving false low values for antibody concentration. The results however may only be a function of the matrix used to bind the immunoglobulin standards. Standards bound to a precipitable matrix such as sepharose particles may compete as well as soluble standards in the assay. Until alternatives to the cellulose acetate matrix are found, caution must be used when using a competitive inhibition radioimmunoassay to quantitate antibodies to soluble antigens.

### COMPARISON OF DRIED AND SOLUBLE IgM STANDARDS

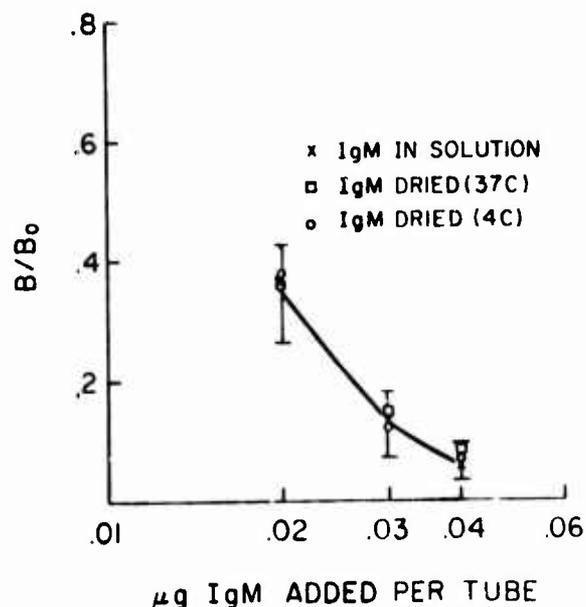


Figure 13. Comparison of inhibition curves using soluble or dried IGM as standards.

COMPETITIVE INHIBITION CURVES OF IgM IN SOLUTION  
OR BOUND TO CELLULOSE ACETATE DISKS

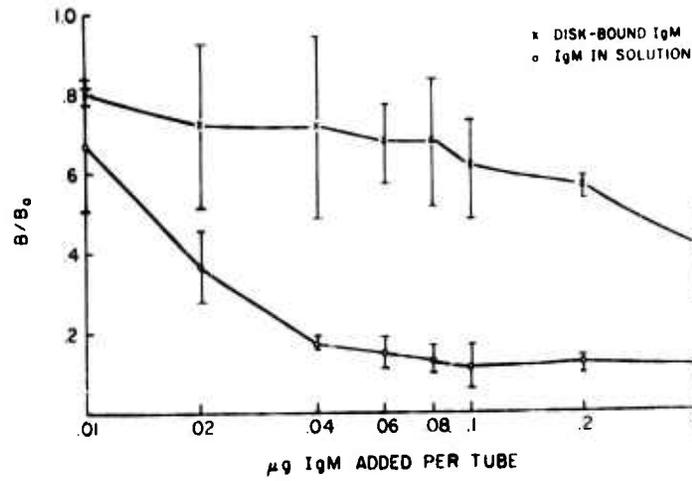


Figure 14. Comparison of inhibition curves using soluble or cellulose Acetate bound IgM standards.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 172 Immunological mechanisms in microbial infections

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DM&E (AR) 1616	
				DA OB 6530	75 07 01		
3. DATE PREVIOUS RT	4. KIND OF SUMMARY	5. SUMMARY ACT <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8. ORIGIN INST <sup>d</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>e</sup>	10. LEVEL OF SW <sup>f</sup>
74 07 01	D. Change	U	U	NA	NI	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES <sup>g</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	61102A	3A161102BT1Q		00	173		
B. CONTRIBUTING							
C. <del>CONTRIBUTING</del>	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Vaccine Development in Trypanosomiasis							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
002600 Biology 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
73 09		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: NA				PRECEDENCE		B. FUNDS (in thousands)	
B. NUMBER: NA				FISCAL YEAR		75	
C. TYPE:				CURRENCY		3.0	
D. KIND OF AWARD:				76		3.0	
E. AMOUNT:						95	
F. CUM. AMT.						150	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Kenya, Govt of Veterinary Service			
ADDRESS: Washington, DC 20012 (USAMRU-Kenya)				ADDRESS: Kabete, Kenya			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with U.S. Standard)			
NAME: Buescher, COL E. L.				NAME: Muriithi, I. Dr.			
TELEPHONE: 202-576-3551				TELEPHONE:			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Wykoff, D. E. COL			
				NAME:			
22. KEY WORDS (Precede each with Security Classification Code)							
(U) Kenya; (U) Trypanosomiasis; (U) Vaccine; (U) Africa; (U) Irradiation; (U) Immunity							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objective of the research program is to develop an effective and practical vaccine against African trypanosomiasis, useful to military and civilian agencies. Related benefits include acquisition of knowledge pertaining to trypanosome immunology, biology, host response and the pathology of the disease in natural and experimental hosts. There is a requirement for these well-conceived studies which should provide a basis for the rational development of a vaccine for the control of African trypanosomiasis, a disease which constitutes a serious hazard for military personnel operating in areas indigenous for this disease.</p> <p>24. (U) Experiments conducted at WRAIR and Kabete, Kenya, during the past several years have demonstrated that experimental animals can be successfully immunized with irradiated trypanosomes. Rodents, cattle, and monkeys could be rendered completely resistant to a challenging infection with T. rhodesiense by this method. Partial immunity was obtained in rodents and cattle to T. congolense. Immunization using this method, as well as passive protection of antibodies, will be studied with relation to field use.</p> <p>25. (U) Studies in Lambwe Valley, western Kenya, suggest that 1 of 15 human cases as well as 5 of 19 cases in cattle appear to be caused by the same strain of T. rhodesiense.</p> <p>This research is complementary to DAOB 7501, Work Unit 096, entitled Study of African Trypanosomiasis.</p> <p>For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74-30 Jun 75.</p>							

DD FORM 1498  
1 MAR 68

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PROJECT 3A161102B71Q COMMUNICABLE DISEASES & IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 173 Vaccine Development in Trypanosomiasis

Investigators:

Principal: Colonel Dale E. Wykoff, MSC

Associate: LTC Robert M. Kovatch, VC

Mr. Bruce T. Wellde, DAC

I. Antigenic Relationships Between Organisms of the Trypanosoma brucei Group in the Lambwe Valley, South Nyanza, Kenya.

A. PROBLEM: To determine the extent of antigenic variability in trypanosomes of the T. brucei group collected from man and animals in Lambwe Valley. T. rhodesiense is endemic in the Valley causing periodic disease in man. Domestic and game animals harbor the morphologically indistinguishable T. brucei. This project is designed to study reactions of various trypanosome isolates with various antisera to determine the variability of different antigenic types within the parasitic population. The findings will, in part, determine whether or not immunization could be a practical means of controlling the disease. Trypanosomes collected from adjacent countries will also be studied to determine the geographic extent of similar antigenic types. The antigenic relationship between the parasites of man and animals in these areas will also be examined.

B. BACKGROUND: Immunization against African trypanosomiasis appears to be dependent in large part on the number of antigenic types of the parasite found in a given area. Gray (1970) examined the same herd of cattle for five years in Nigeria and reported the presence of numerous different types of T. brucei. He postulated that this heterogeneity made immunization attempts impractical. It appears to us that the techniques employed were not specific enough to detect all variants of a given isolate thus giving an exaggerated number of basic antigenic types. In Lambwe Valley, human rhodesian sleeping sickness is endemic and domestic and game animals harbor T. brucei. Since the trypanosomes of man and animals are morphologically identical, their relationship to each other remains questionable. In near by Alego Station, T. brucei-like organisms were isolated from cattle and transmitted to human volunteers. These people developed typical T. rhodesiense-like infections (Onyango 1966).

C. PROGRESS: Isolates of T. rhodesiense were collected from patients at the Homa Bay Hospital on Lake Victoria, western Kenya, by members of the Kenya Medical Department. Blood was injected I.P. into rats which were then transported to us for study. Two strains of T. rhodesiense from Gambella, Ethiopia, were collected by the U.S. Navy Medical Research Unit Number 5, Addis Ababa, Ethiopia. Isolates of trypanosomes were tested by neutralization (Soltys 1957) with antiserum collected from bovines which had undergone long-term infections with various isolates. The first series of experiments was undertaken with antiserum against the initial strain of T. rhodesiense (LVH-1) collected in Lambwe Valley, August 1972. During the past year 12 new isolates from humans have been collected and tested, bringing the total to 16 (Table I). Three other strains of known T. rhodesiense were also tested, two from southern Ethiopia and a laboratory strain (Wellcome) which had been isolated in Tanzania and maintained in rodents for many years. These findings are shown in Table II. It is noteworthy that 12 of the 16 isolates from man were neutralized by the antiserum against LVH-1, indicating a persistence of these similar types since 1972.

When isolates of T. brucei from cattle were tested (Table III), the antiserum showed a strong effect on 5 of 19 isolates. No parasites appeared in mice given trypanosomes incubated with immune serum at any dilution. Two other isolates were neutralized at  $10^3$  and below, however, mice at  $10^4$  were positive. The nature of this partial reaction is unknown, but these isolates will be retested. It is reasonable to assume that these reacting parasites from cattle are T. rhodesiense since the neutralization test is species and even variant specific. The percentage of cattle parasites which react with the antibody is surprising and indicates that cattle may be a more important reservoir host than believed to now. While game animals have borne the onus of being the most important reservoirs, the close proximity of man and his domestic cattle in the Valley may pose a more important aspect in the cycle of the disease.

TABLE I

EFFECTS OF ANTISERUM TO T. RHODESIENSE (LVH-1) ON 16 ISOLATES OF T. RHODESIENSE  
FROM MAN

STRAIN	DATE ISOLATED	DONOR		SERUM	NO. TRYPANOSOMES PER MOUSE				
		AGE	SEX		10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>1</sup>	10 <sup>0</sup>
LVH 1	16-8-72	52	M	Immune	0*	0	0	0	0
				Normal	5	5	1	1	0
LVH 2	17-12-72	37	F	Immune	0	0	0	0	0
				Normal	5	5	2	0	0
LVH 3	73	?	?	Immune	0	0	0	0	0
				Normal	5	5	5	3	0
LVH 4	20-5-74	10	F	Immune	0	0	0	0	0
				Normal	5	5	5	5	5
LVH 5	18-7-74	55	M	Immune	0	0	0	0	0
				Normal	5	5	5	5	5
LVH 6	3-8-74	52	F	Immune	0	0	0	0	0
				Normal	4	5	2	2	0
LVH 7	3-8-74	22	F	Immune	0	0	0	0	0
				Normal	5	5	4	3	3
LVH 8	8-8-74	25	M	Immune	0	0	0	0	0
				Normal	5	5	5	5	5
LVH 9	28-8-74	41	M	Immune	5	5	4	5	5
				Normal	5	5	4	5	5
LVH 10	2-10-74	50	M	Immune	0	0	0	0	0
				Normal	3	4	4	4	0
LVH 11	28-10-74	28	F	Immune	4	3	4	3	1
				Normal	5	5	4	3	4
LVH 12	24-12-74	50	M	Immune	0	0	0	0	0
				Normal	5	4	5	4	4
LVH 13	22-1-75	30	F	Immune	0	0	0	0	0
				Normal	5	5	4	5	2
LVH 14	23-4-75	42	M	Immune	5	4	3	0	0
				Normal	4	4	5	3	1
LVH 15	26-6-75	60	M	Immune	0	0	0	0	0
				Normal	5	5	2	1	1
LVH 16	1-7-75	42	M	Immune	5	**	**	**	**
				Normal	5	**	**	**	**

\* Number of mice (out of 5) developing patent infection

\*\* Not tested

TABLE II

EFFECTS OF ANTISERUM ON T. RHODESIENSE PARASITES ISOLATED IN DIFFERENT  
GEOGRAPHICAL AREAS

STRAIN	DATE ISOLATED	DONOR		SERUM	NO. TRYPANOSOMES PER MOUSE				
		AGE	SEX		$10^4$	$10^3$	$10^2$	$10^1$	$10^0$
ETH-1	30-10-73	30	F	Immune	5*	5	5	1	0
				Normal	4	5	5	5	3
ETH-2	26-11-74	?	M	Immune	5	5	4	5	4
				Normal	5	5	5	5	5
WELLCOME	over 20 years ago	?	?	Immune	5	4	4	2	0
				Normal	5	5	4	5	1

\* Number of mice (out of 5) developing patent infections

TABLE III

EFFECTS OF ANTISERUM ON T. BRUCEI-GROUP PARASITES ISOLATED FROM CATTLE  
IN LAMBWE VALLEY

ISOLATE	SERUM	NO. OF TRYPANOSOMES PER MOUSE				
		10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>1</sup>	10 <sup>0</sup>
LVB 15A	Immune	0*	0	0	0	0
	Normal	5	5	5	5	5
LVB 16A	Immune	0	0	0	0	0
	Normal	5	5	2	1	0
LVB 18A	Immune	5	1	1	0	0
	Normal	4	3	5	0	1
LVB 3B	Immune	3	3	2	0	0
	Normal	4	2	3	2	0
LVB 24B	Immune	5	5	5	3	0
	Normal	5	5	5	5	5
LVB 2B	Immune	5	0	0	0	0
	Normal	5	5	4	5	3
LVB 4B	Immune	5	5	5	3	3
	Normal	5	5	5	5	5
LVB 10B	Immune	5	5	4	1	0
	Normal	5	5	5	5	3
LVB 36B	Immune	5	5	5	0	2
	Normal	5	5	5	2	5
LVB 16B	Immune	0	0	0	0	0
	Normal	5	5	5	4	0
LVB 12C	Immune	0	0	0	0	0
	Normal	5	5	4	5	5
LVB 36C	Immune	4	3	0	0	0
	Normal	5	4	1	1	0
LVB 42C	Immune	5	0	0	0	0
	Normal	5	5	4	4	**
LVB 43C	Immune	5	4	4	5	5
	Normal	5	5	4	5	4
LVB 65C	Immune	0	**	**	**	**
	Normal	5	**	**	**	**
LVB 66C	Immune	3	**	**	**	**
	Normal	5	**	**	**	**
LVB 72C	Immune	5	2	0	0	0
	Normal	5	5	2	3	0
LVB 77C	Immune	5	5	3	1	0
	Normal	5	5	5	5	4
LVB 78C	Immune	4	4	2	1	0
	Normal	4	5	5	5	1

## II. Pathophysiology of Trypanosoma rhodesiense in Bovines

A. PROBLEM: To attempt documentation of the pathophysiological process in a systemic study of bovines inoculated with T. rhodesiense recently isolated from man.

B. BACKGROUND: During our early studies of the immunogenicity of irradiated trypanosomes in cattle, we noted that control animals challenged with viable Trypanosoma rhodesiense (Wellcome strain) underwent a mildly pathogenic course of infection which resulted in self cure. Fever and leukopenia were noted early in the course of infection but after a brief period the animals appeared to suffer no untoward reaction to the infection. However, in a later experiment one animal (268) which was infected with this strain suffered a severe form of disease characterized by a lack of growth and terminally by a rapid weight loss and a severe episode typical of central nervous system disorder. Since these symptoms occurred in only one of approximately 10 animals infected with this strain, we were not convinced that these symptoms were entirely due to the trypanosome infection although we had not observed this behavior in any other animal on our farm.

Subsequently, 4 of 5 animals which were infected with recently isolated strains of T. rhodesiense from Lambwe Valley experienced a disease pattern similar to that described above. To date, two (243 and 6882) of the four diseased animals have been killed and autopsied while the remaining two are still under observation. Cerebrospinal fluid obtained from three of these animals showed a pleocytosis ranging from 330 to 512 WBC per mm<sup>3</sup>. Trypanosomes have also been isolated from the CSF by subinoculation IP in mice. Bacterial cultures of the CSF were negative.

The extent of the nervous signs seen terminally varied between animals. Animal 6882 exhibited only uncoordinated leg movements. Animal 268 had uncoordinated leg movements, circling to the right and opisthotonos. Animal 243 had uncoordinated leg movements and a continuous unilateral periorbital and muzzle tremor.

Gross alterations in the brain were limited to thickened dull gray meninges in all animals. In addition, the brain of 243 had exaggerated sulci, submeningeal scars and small cystic cavitations near the external capsule. The salient histological feature was a moderate to severe meningoencephalitis. It was least severe in animal 6882 where the perivascular and meningeal infiltrates were usually 1 to 2 cells thick and were comprised mainly of lymphocytes admixed with a few plasma cells. Focal areas of gliosis were randomly distributed in the brain.

A severe generalized meningoencephalitis was found in the brain of animals 268 and 243. In animal 268 wide cuffs of an infiltrate-proliferate of lymphocytes and plasma cells were randomly disseminated in the brain. Meningeal involvement with the same inflammatory cells was moderately extensive. Diffuse and focal areas of gliosis were most prominent in the gray matter. Lesions in animal 243 were more extensive with the perivascular and meningeal infiltrate-proliferate primarily plasmocytic. Large numbers of Mott's cells or mature plasma cells were a prominent component of the inflammatory reaction and were found in all levels of the brain, brain stem and spinal cord sections that were examined. In addition, periarterial edema, cystic cavitations and extensive glial scars were found.

The Wellcome strain of T. rhodesiense has been maintained in the laboratory by syringe passage in rodents for many years. It would be reasonable to assume that its pathogenicity for cattle would be reduced as a result of its laboratory history. However, the T. rhodesiense of Lambwe Valley origin has been shown to be a parasite of cattle in the Valley and would presumably, as we have found, be more pathogenic for these animals.

The lesions described are commonly found in chronic trypanosomiasis in man but have not been described in the CNS of bovines. Therefore, the following study was initiated.

In this laboratory bovines were inoculated with certain strains of T. rhodesiense isolated from humans as controls for other studies or in attempts to obtain large amounts of convalescent serum. This immune serum was used in the definition of antigenic relationships between morphologically identical T. rhodesiense collected from humans and T. brucei isolated from animals in the Lambwe Valley (details elsewhere in this report). Some of the experimentally infected bovines showed an unexpectedly severe chronic disease process late in the course of infection. Three of the animals were killed in extremis. The common and salient histological finding was a moderate to severe non-purulent meningoencephalomyelitis. In two additional experimentally infected bovines that remain alive, examination of cerebral spinal fluid revealed a marked pleocytosis, 254 and 512 WBC/mm<sup>3</sup>, respectively.

Of 19 T. brucei isolates from the Lambwe Valley, 5 (26%) were neutralized by the antibody obtained from a T. rhodesiense (LVH-1) experimentally infected bovine. This indicates that an agent the same, or very similar antigenically to T. rhodesiense, is present in the cattle in Lambwe Valley. The bovine is therefore likely to be highly important in the epidemiology of the natural disease occurring in humans as well as being important in harboring the cattle parasite.

There is considerable controversy in the literature on the significance of T. rhodesiense infection in bovines. Some report patent infections that induce a mild clinical disease which eventually self-cure. Others report more virulent infections in experimentally infected cattle. In either case, pathophysiological aspects of infection have not been described.

To systematically study these aspects, 10 bovines 5-8 months of age comprised of 5 Herefords and 5 Aryshires, weighing 220-400 pounds were selected from the Veterinary Department herd at Kabete, Kenya. They were inoculated with the LVH-12 strain,  $1 \times 10^4$  parasites per 200 pounds. Four young bovines, 2 Herefords and 2 Aryshires, of similar size and age, were maintained as controls. This report describes the first 23 weeks of a study designed to define the clinical, hematological, serum chemical, serological and histopathological alterations.

C. RESULTS: In one of the ten infected animals a single parasite was found in a thick blood film on day 3 post-exposure (PE). The remaining 9 infected animals were positive on thick films examined on day 4 PE. Peak fevers occurred on day 5 PE in two animals, day 6 PE in five animals and day 7 PE in three animals. Peak parasitemias calculated from the number of parasites per 100 WBC varied between 21,700 and 434/mm<sup>3</sup>. The mean of the peak parasitemias was 9,756/mm<sup>3</sup>.

Temperatures were taken daily during the first 2 weeks PE, and weekly thereafter. The average control animal temperature was 101.6°F while the infected animals were approximately 102.3. Other clinical observations include a mild depression that occurred during the period of peak parasitemia and fever. Lymphadenopathy (3 to 4 times normal) was noted 30 days PE and persisted during the remainder of the 23 weeks of infection.

Hematological findings include transient leukopenia at the time of peak parasitemia followed by leukocytosis. Differential WBC counts done on jugular blood indicate a transient absolute neutropenia and lymphopenia on day 8 PE. The average high WBC occurred during the 4th, 5th, and 6th week PE. During this period, an absolute lymphocytosis and mild absolute neutrophilia were observed. The packed cell volume (PCV) results showed a mild depression in RBC values accompanied the depressed PCV's. Mean corpuscular hemoglobin concentrations, mean corpuscular volume and mean corpuscular hemoglobin indices have remained within normal limits to date. A mild transient depression in platelet counts was noted on the 8th and 12th day post exposure in infected animals.

During the first seven weeks PE Giemsa-stained thick films prepared from tail blood were positive in 70 to 100% of the infected animals when 200 oil

immersion fields were examined. Subsequently, a progressive diminution of trypanosome positive smears occurred with no positives detected after day 135 PE.

Subinoculation of blood into rats was initiated during the fourth month of infection. The sub-inoculation of lymph node aspirate was initiated during the fifth month of infection. The results are summarized in Table I. These initial data indicate that the subinoculation of a small amount of lymph node aspirate (usually less than 0.1 cc expanded to 0.5cc in 10% fetal calf serum) injected IP into rats appear to be an effective method of isolating the parasite late in the course of infection.

Serum preserved at  $-20^{\circ}\text{C}$  for serological, serum chemical, and immunoglobulin analysis has not yet been examined.

TABLE I

THE RELATIVE EFFECTIVENESS OF DIRECT SMEAR EXAMINATION COMPARED TO IP INOCULATIONS, USING BLOOD AND LYMPH NODE ASPIRATES FOR THE DETECTION OF CATTLE HARBORING T. RHODESIENSE-

	Duration of Infection in Cattle			
	4 months		5 months	
	Infected	Control	Infected	Control
Blood (5ml) IP to rats	8/10*	0/4	2/10	0/4
Blood, thick smears	0/10	0/4	0/10	0/4
Lymph node aspirate IP to rats	**	**	10/10	**
Lymph node smears	5/10	0/4	5/10	0/4

\* 8 of 10 rats showed patent infection by day 30

\*\* Not done

### III. Pathology of Trypanosoma congolense in Bovines (Anemia)

A. PROBLEM: To define the etiology of the anemia which develops in cattle infected with Trypanosoma congolense.

B. BACKGROUND: Anemia of undetermined nature is a characteristic of most infections caused by pathogenic trypanosomes. Previous work by Wellde, et al. (1973) indicated that T. congolense produces a rapid and severe anemia in Hereford cattle, these animals therefore being good study models. Baseline factors including erythrocyte indices, thrombocyte, reticulocyte, and leukocyte levels have been determined. Bone marrow biopsies of infected and control animals have also been done in an effort to judge erythrocyte potential. Radioisotopic labels have recently been employed to determine erythrocyte survival and sites of sequestration. Iron kinetics are also being studied to determine plasma iron clearance rates and utilization in the production of new erythrocytes.

C. RESULTS: There was an increase in both mean corpuscular volume and mean corpuscular hemoglobin in the five infected animals. Mean corpuscular hemoglobin concentration did not differ from control values. A limited reticulocyte response was evident beginning about the 30th day after infection, and persisted through the 100th day. However, only one animal had a reticulocytosis greater than 1%. After this period of limited erythroid response the mean corpuscular volume and the mean corpuscular hemoglobin levels appeared to return to lower values although the hematocrit remained around 20%. A striking thrombocytopenia occurred with the onset of parasitemia. Levels of thrombocytes were reduced to as low as 30,000/mm<sup>3</sup> from the normal mean of 492,000/mm<sup>3</sup>. We have found the thrombocytopenia to be dependent on the numbers of trypanosomes in the peripheral blood.

Leukocyte levels are generally depressed throughout the infection and they appear to be also dependent on the trypanosome levels. When infected animals were treated with Berenil thrombocyte levels rapidly became elevated over pre-infection values. Leukocyte levels also returned to normal (or somewhat higher than normal levels) shortly after therapy. These results strongly suggest that when trypanosomes are present in the blood, there is an increased consumption of thrombocytes. This phenomenon is consistent with diffuse intravascular coagulation although grossly infected animals show minimal signs of hemorrhage. Histopathological examination of tissues indicates that some vessels appear to contain leukocytes, trypanosomes and other material in plug-like formations. Erythrocytes, however, do not appear to be a prominent component of these formations (Kliner in press, Kovatch unpubl). Thrombocytopenia has been described in man and animals infected with T. rhodesiense and in these infections

the detection of fibrin split products as well as decreased levels of fibrinogen tend to indicate a coagulopathy. It has also been shown that trypanosomes in the presence of specific antibody and complement attract platelets which adhere to them. Whether or not this process of immune adherence can occur in vivo as well as in vitro has not been established, but could well be involved in the initial clumping of thrombocytes and their subsequent removal by the reticuloendothelial system. After curative therapy packed cell volumes return only slowly to normal. The erythroid response after therapy is also not characterized by a strong reticulocyte response although the packed cell volumes gradually return to normal levels.

Bone marrow biopsies taken early in the course of infection showed a normal or somewhat hyperactive appearance. Normoblasts were plentiful, as were cells of the leucocytic series. These observations confirm our previous findings in bone marrow obtained early during the course of infection.

In order to study erythrocyte survival in infected animals, four male Hereford-Boran calves weighing approximately 175 pounds each were used in an experiment. Two animals were infected with  $1 \times 10^4$  T. congolense (Trans Mara) while the other two served as controls. Prior to infection, all animals had received transfusions of homologous Cr<sub>51</sub>-labelled erythrocytes. The calves were housed in metabolism cages and blood, urine and fecal samples were collected daily, measured and aliquoted for radioactivity determinations. The animals were also followed by routine hematology, levels of parasitemia and temperature. Since these experiments have only been recently initiated, only preliminary data are available. These do indicate a trend toward increased plasma volumes, decreased erythrocyte survival times and increased splenic sequestration of labeled erythrocytes in the infected calves. It appears that a dilution of RBC's occurred early in the infection as a result of an expanded plasma volume. Later (day 26), however, there is an actual decrease in the number of circulating erythrocytes in the infected animal.

Surface counting over selected body sites indicated that the spleen of infected animals was sequestering labelled erythrocytes to a greater degree than the spleens of control animals (Table I). All animals were bled on day 33 and their erythrocytes again labelled with Cr<sub>51</sub> and retransfused. Results from this second transfusion have not yet been calculated.

In order to determine the plasma clearance rate and subsequent incorporation of Fe<sub>59</sub> into erythrocytes, 4 infected cattle and 2 controls were injected with Fe<sub>59</sub>. Plasma half time clearance rates for infected animals were 65 minutes and for controls were 119 minutes.

These studies have been restricted because of limited available space for radioactivity experiments. We are now in the process of renovating a two-room structure which will greatly add to our existing facilities.

TABLE I

RESULTS OF THE IN VIVO COUNTING (COUNTS PER MINUTE) OVER SELECTED BODY SITES (DAY 32)

Animal	Group	Sternum	Heart	Liver	Spleen	Spleen/ Liver
5F	Control	3018	3498	1551	1515	0.98
6F	Control	3696	3013	3140	3067	0.98
7F	Infected	2248	3618	1666	4121	2.47
8F	Infected	2074	2517	1675	4607	2.75

#### IV. The Feasibility of Artificial Immunization by the Use of Irradiated Trypanosomes.

A. **PROBLEM:** To study possible immunization of animals against infection with T. rhodesiense by use of a gamma-irradiated vaccine.

B. **BACKGROUND:** Our initial results with T. rhodesiense showed that bovines could be immunized with irradiated trypanosomes (Wellde, et al. 1974). If the immunizing inocula contained sufficient irradiated trypanosomes, the recipients became refractive to challenge with non-irradiated parasites of the same strain. Similar experiments with T. congolense did not yield as great an immunity as that produced by T. rhodesiense, but prepatent periods of immunized animals were extended and antibodies developed after immunization (Lötzsch and Diendl 1974). Mice immunized with irradiated T. congolense were highly immune to a viable challenge of organisms. Recently, we have found that immunization with irradiated trypanosomes is specific for the antigenic type of the immunogen.

The antigenic typing of trypanosomes transmitted by tsetse flies has been little studied. Gray (1970) reported that antigenic variants of a given isolate return to a basic antigenic composition in the tsetse fly. Workers in Nigeria (personal communication) believe that the tsetse transmits the antigenic type which is ingested.

To study this problem (which seems to be of great importance in terms of practical immunization) we have proposed the establishment of a small colony of tsetse flies here in Kabete. Initially, this was to have been undertaken during the early part of 1975, but budget limitations imposed by DA and USAMKDC have postponed the initiation. We are hopeful that some progress can be made during 1976.

Our experiments involving irradiated trypanosomes have also been hampered by the breakdown of the only Cobalt-60 source available to us. The maintenance is the responsibility of the Atomic Energy Commission and although they have been notified that it is out of order, repairs have not been made. The new International Laboratory for Research on Animal Diseases (ILRAD) will have a Cobalt source installed soon.

C. **RESULTS:** Serum from cattle chronically infected with T. congolense conferred protection to mice infected with the homologous parasite. In a preliminary experiment, 30 mice were injected with  $2 \times 10^6$  T. congolense IP. Subsequently, 10 of these mice (experimental group) received 1 ml of serum from a chronically infected animal. As controls, 10 were injected with normal serum

and 10 were not treated. Fifty percent of the mice receiving serum from the infected animal did not become patent and survived for at least 25 weeks after challenge, whereas 100% of the control animals became patent and 10% survived. An extension of the prepatent period was also observed in the experimental group. In a second experiment, globulins of both the immune and normal serum were collected by salt precipitation at 50% saturation, dialyzed, and reconstituted to 1/3 the original serum volume. These globulins were then tested in mice, using the above procedure. Only 10% of the mice treated with immune globulins developed patent infection, whereas all the controls became positive. Nine weeks after challenge, 90% of the experimental group and 40% of the control group survived. Column fractionation of immune globulins on the Sephadex G-200 and subsequent testing in mice indicated that the protection activity is associated with the 7S gamma globulins.

Cattle which had been infected with T. congolense (Trans Mara) and cured with Berenil were rechallenged at various times after treatment with the homologous strain of parasite. Four animals undergoing infection of from 36 to 77 days did not show appreciable resistance when rechallenged with the stock strain. Prepatent periods for previously infected animals ranged from 6 to 8 days, whereas five control steers were patent on the 5th day. Parasitemia levels appeared to be lower as the infection progressed, although packed cell volumes in both groups were similar. One animal which had undergone two long periods of infection (total 10 months) remained refractive when challenged for the third time. These results indicate that infection and cure can be a method of immunizing cattle against T. congolense. It appears, however, that long-term infections are needed in order to stimulate a significant degree of immunity.

PROJECT 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 173 Vaccine Development in Trypanosomiasis

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	3. REPORT CONTROL SYMBOL <sup>c</sup>	
				DA OB 6531	75 07 01	DD DR&E(AR)6.16	
4. DATE PREV SUMMARY	5. KIND OF SUMMARY	6. SUMMARY SCTY <sup>d</sup>	7. WORK SECURITY <sup>e</sup>	8. REGRADING <sup>f</sup>	9. DRG'S INKYP <sup>g</sup>	10. SPECIFIC DATA CONTRACTOR ACCESS	
74 07 01	D. Change	U	U	NA	NI.	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO A. WORK UNIT	
10. NO CODES <sup>h</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
		61102A		3A161102H71Q		00	
11. PRIMARY		61102A		3A161102H71Q		174	
12. CONTRIBUTING		CARDS 114F					
13. TITLE (Provide with Security Classification Code) <sup>i</sup>							
(U) Ecology of Plague							
14. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>j</sup>							
010100 Microbiology							
15. START DATE		16. ESTIMATED COMPLETION DATE		17. FUNDING AGENCY		18. PERFORMANCE METHOD	
73 07		CONT		DA		C. In-House	
19. CONTRACT GRANT				20. RESOURCES ESTIMATE		21. PROFESSIONAL MAN YRS	
22. DATES EFFECTIVE: NA				23. EXPIRATION		24. FUNDS (in thousands)	
25. NUMBER *				26. TYPE		27. FISCAL YEAR	
28. KIND OF AWARD				29. CUM. AMT.		30. CURRENT YEAR	
						75 2 80	
						76 2 175	
31. RESPONSIBLE DOD ORGANIZATION				32. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution)			
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33. GENERAL USE				34. ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered.				NAME: Williams, James E., CPT, MSC			
				NAME: Harrison, Daniel N.			
35. KEYWORDS (Provide each with Security Classification Code) (U) Yersinia pestis; (U) Plague; (U) Vaccines; (U) Immunization; (U) Serological tests; (U) Genetics							
36. TECHNICAL OBJECTIVE, 37. APPROACH, 38. PROGRESS (Provide individual paragraphs identified by number. Provide rest of each with Security Classification Code)							
23. (U) Determine the factors influencing outbreaks of plague infection and the most appropriate methods to prevent the infection of troops engaged in field operation.							
24. (U) Using standard methods, sera from humans and animals are tested for the presence of F-1 antibody to Y. pestis.							
25. (U) 74 07 - 75 06 Virulent variants of Y. pestis isolated from rats dying many months after experimental challenge demonstrate varying phenotype. The phenotype, Fraction 1 negative: VW positive: Pigment positive: Pesticin-Coagulase-Fibrinolytic factor complex positive, however, is apparently recovered with great frequency from animals with appreciable F1 antibody titer. Clonal selection and animal passage resulted in the isolation of one strain approaching classical virulence. When incubated at 37C, only 6 of the aberrant plague bacilli were required to induce fatal infection in experimental mice. Vaccination with F1 antigen and plague vaccine USP did not prevent fatal plague infection of rats and mice challenged with these variants. Potential presence of such strains in nature may require supplementary diagnostic serological tests employing specific antigens of Y. pestis other than F1. For technical report see: Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE DD FORMS 1498A 1 NOV 65 AND 1498 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 174 Ecology of Plague

Investigators.

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Description.

To determine the factors influencing outbreaks of plague infection and the most appropriate methods to prevent the infection of troops engaged in field operation.

Progress.

I. Variant strains of Yersinia pestis isolated from laboratory rats at the WRAIR.

In WRAIR Annual Report for 1 July 73 - 30 June 74, we reported upon a series of investigations that demonstrated a positive relationship between Fraction 1 capsular antibody (IHA) titer and protection against both bubonic and pneumonic plague, using the laboratory rat as experimental model. In the course of those experiments, two startling isolations of Y. pestis were made from buboes of vaccinated rats that died 424 and 541 days after challenge with virulent Y. pestis. The implications of such latent infections was recognized (Williams, Harrison, and Cavanaugh, 1975), not only for vaccine development and for vaccine prophylaxis, but for disease detection and surveillance. Consequently, additional studies were focused on cryptic plague in laboratory rats during this report period.

A. Virulence determinants.

The two cryptic plague strains isolated were examined for:

- (1) production of capsular or Fraction 1 (F1) antigen, by serum agar assay and by stimulation of antibody in laboratory animals;
- (2) production of VW antigen, by magnesium oxalate agar assay;
- (3) pigmentation on Congo Red agar; and (4) production of pestin I, by pestin agar assay. Results of tests are given in Table 1.

The cryptic plague strains (i.e., CPS-1 and CPS-2) were found to be capsule-deficient. By magnesium oxalate agar assay, 12% of CPS-1 colonies isolated from an abdominal bubo were calcium dependent (indicating presence of VW antigen and virulence), while 92% of CPS-2 colonies were calcium dependent. In strain CPS-1, 24% of original colonies were calcium independent (indicating lack of VW

Table 1. Virulence determinants in variant Y. pestis compared with classical-type strain 195P

Strain	Virulence determinant			
	F1 antigen	VW antigen	Pigmentation	Pesticin I
Virulent strain 195P	+	+	+	+
Strain CPS-1, isolated from a rat at the WRAIR 424 days after infection with 195P	-	Variable	+	+
Strain CPS-2, isolated from a rat at the WRAIR 5 1/2 days after infection with 195P	-	Variable	+	+
Strain M23, isolated by cultural methods in England	-	+	+	+
Strain "Bryans", isolated from a fatal human case in the southwestern U.S., 1957	+/-	+	+	+

antigen and avirulence), while only 4% of CPS-2 colonies were independent. The remainder of colonies (64% of CPS-1 and 4% of CPS-2) gave intermediate test results. Judging by the prevalence of VW antigen, which is required to establish infection, the original bubo population of strain CPS-2 possessed a considerably higher potential for virulence than that for strain CPS-1.

Those colonies of CPS-1 and CPS-2 producing VW antigen appeared to be similar to Y. pestis variant strain M23 (Table 1). The strains were also reminiscent of the virulent human plague strain "Bryans" that, although giving positive results in serum agar assay, does not illicit antibody responses in laboratory animals of form capsular substance detectable by ordinary and fluorescent microscopy. We have found that "Bryans" strain has the disturbing effect of being of higher virulence in mice vaccinated with F1 antigen than in unvaccinated mice (Table 2).

Table 2. Mouse LD<sub>50</sub> values for "Bryans" strain of Y. pestis isolated from a fatal human case.

Group	LD <sub>50</sub> (2 weeks after IP challenge)
Unvaccinated (control) mice	4,500
Mice vaccinated with two 50% protective doses of F1 antigen	40-400

#### B. Pathogenicity in laboratory mice.

Experiments were undertaken in mice with the WRAIR variants to provide a basis of comparison. Y. pestis strain M23 is known to possess high virulence in laboratory mice, while "Bryans" strain is of moderate virulence.

Titration in mice were done for the original CPS-1 isolate, using cultures incubated at 25°C and 37°C, simultaneously with cultures obtained from first-passage mice (Table 3). Mice were inoculated subcutaneously to mimic transmission by fleabite. Mouse LD<sub>50</sub> calculations were based only on mice that died with typical bacteremia or mice from which Y. pestis was recovered from autopsy material. Of 62 mice that yielded Y. pestis at autopsy, 97% had bacteremia at the time of death.

Table 3. Mouse LD<sub>50</sub> values for Y. pestis strain CPS-1

Source of inoculum (temperature of incubation)	LD <sub>50</sub> (in thousands of bacilli)				Notes on confirmed deaths by small inocula
	2 weeks	4 weeks	6 weeks	9 weeks	
Original bubo culture (25°C) (37°C)	> 500	71.0	61.8	22.8	Deaths on days 25 and 50 after 50 bacilli
	> 121	121	54.7	43.0	Death on day 19 after 121 bacilli
First-mouse-passage spleens:					
A: Culture (25°C) from mouse dead 2 days after inoculation	> 484	102	28.7	15.8	Death on day 12 after 6 bacilli
B: Culture (25°C) from mouse dead 7 days after inoculation	> 606	252	152	100	Death on day 6 after 60 bacilli
C: Culture (25°C) from mouse dead 25 days after inoculation	> 111	7.3	4.5	3.5	Deaths on days 31, 52, and 55 after 6 bacilli

At 24-48 hours after inoculations, most mice looked sick (i.e., "ruffled"), especially those in dilutions  $10^{-1}$  to  $10^{-5}$ . Although deaths commenced the following day, most mice initially recovered to a normal looking condition. However, mice in all titrations subsequently died of plague in substantial numbers over a period of 9 weeks. The cumulative effect of progressive plague mortality was a steady decrease in LD<sub>50</sub> values (Table 3). The pattern was quite unlike that seen in titrations with classical encapsulated virulent strains of Y. pestis, where deaths rarely occur after 3 weeks.

Little difference was noted in results from titrations done with the original CPS-1 isolate incubated at 25°C and at 37°C (Table 3). LD<sub>50</sub> values were somewhat less for the 25°C culture, but the difference may not be significant. Seemingly significant differences were noted among titrations of first-passage cultures. One culture (A, Table 3) produced LD<sub>50</sub> values similar to those seen in the original 25°C titration; one (B) with high LD<sub>50</sub> values appeared to be less virulent than other cultures; and one (C) produced considerably lower LD<sub>50</sub> values. This last, most virulent culture came from the spleen of a first-passage mouse that died 25 days after infection. In effect, late death was not associated with a reduction in virulence in this non-encapsulated variant of Y. pestis. Although LD<sub>50</sub> values were useful as comparative estimates of virulence, some mice died as a result of the inoculation of very small numbers of capsule-deficient bacilli.

At 75 days after inoculation, 72 mice in cages distributed among the various titrations were exsanguinated for sera. The mice bled came from groups that experienced 10-50% plague mortality. None of the 72 sera possessed detectable titers of IHA antibody to the specific F1 antigen of Y. pestis.

In a subsequent experiment, a calcium dependent (VW<sup>+</sup>) clone of strain CPS-2 was established from a single colony derived from the abdominal bubo. We, hereafter, refer to this clone as CPS-2a. A titration in normal mice with strain CPS-2a gave a 2 week LD<sub>50</sub> of 60 bacilli and a 4 week LD<sub>50</sub> of 34 bacilli. Consequently, this isolate demonstrated a virulence in mice at least 100X greater than any LD<sub>50</sub> observed for strain CPS-1. Furthermore, strain CPS-2a appeared to be 35X more virulent than Y. pestis strain M23, which gave LD<sub>50</sub> values around 2,100 bacilli in mouse titrations, and 75X more virulent than "Bryans" strain (Table 2) in normal mice.

Further titrations of strain CPS-2a were performed simultaneously in normal and vaccinated mice, concurrently with similar titrations of encapsulated strain 195P (Table 4). Mice were vaccinated with a single injection of 100 µg specific F1 antigen. The 50% protection

Table 4. Mouse LD<sub>50</sub> values for Y. pestis strains 195P and CPS-2a in unvaccinated and F1 vaccinated mice

Strain (incubation)	LD <sub>50</sub>		Ratio: LD <sub>50</sub> vaccinated/ LD <sub>50</sub> unvaccinated
	Unvaccinated	F1-vaccinated <sup>a</sup>	
195P (25°C)	91	>16,000	
(37°C)	16	> 3,300	
CPS-2a (25°C)	60	1,075	18
(37°C)	6	860	143

<sup>a</sup> A single dose of 4 PD<sub>50</sub>; see text.

dose (PD<sub>50</sub>) of the F1 utilized was shown to be  $\pm$  25  $\mu$ g in classical vaccine titrations done according to the USPHS protocol for testing plague vaccine. Thus, vaccinated mice received 4 PD<sub>50</sub> of F1 antigen. The titrations with strain 195P produced the expected results. The virulence of 195P was greater in culture prepared at 37°C than in that incubated at 25°C. LD<sub>50</sub> values could not be calculated for strain 195P in F1-vaccinated mice; most mice were protected. In contrast, LD<sub>50</sub> values of 860-1,075 were obtained for strain CPS-2a in F1-vaccinated mice. Although the virulence of CPS-2a increased approximately 10X in normal mice when incubated at 37°C, the virulence for 25°C and 37°C cultures of this variant did not differ appreciably in vaccinated mice. Strain CPS-2a appeared to be somewhat (2-10X) less virulent than "Bryans" strain for vaccinated mice. The behavior of strain Bryans and CPS-2a in mice vaccinated with plague vaccine USP is presently under investigation.

#### C. Pathogenicity in laboratory rats.

Although non-encapsulated variants, as M23 and "Bryans" strains, have generally been found to possess some degree of virulence for mice, such strains have not been studied in rats. Because rats were the original source of the CPS-1 and CPS-2a isolates, and in view of the fact that rats are the most important vehicles for infecting and transporting fleas to man in most parts of the world, the pathogenicity of non-encapsulated variants in rats was investigated.

Varying quantities of Y. pestis strain CPS-1, ranging from 250-600,000 bacilli, were inoculated into 30 rats, and only one rat (3%) died of plague (Table 5). Ten rats were inoculated with 190-1,900

Table 5. Experiments with Y. pestis strains CPS-1 and CPS-2a in laboratory rats

Initial inoculum	Number of rats		Challenge inoculum	Number of rats	
	Inoculated	Survived		Inoculated	Survived
<u>Y. pestis</u> strain CPS-1:					
250	3	3	1,450 <u>Y. pestis</u> 195P (25°C culture)	3	0
850	3	3		3	2
550	8	8	13,800 <u>Y. pestis</u> 195P (25°C culture)	8	0
1,200	4	4		3	0
50,000	4	4		2	1 <sup>c</sup>
120,000	4	3 <sup>a</sup>		2	2 <sup>c</sup>
600,00	4	4		4	3 <sup>c</sup>
<u>Y. pestis</u> strain CPS-2a:					
190	5	4 <sup>a</sup>	830,000 <u>Y. pestis</u> 195P (prepared in mouse peritoneum)	4	3
1,900	5	4 <sup>b</sup>		4	3

a One rat died 6 days post inoculation; Y. pestis recovered from spleen.

b One rat died 4 days post inoculation; Y. pestis recovered from spleen.

c From 219-341 days after 195P challenge, 4/6 rats in indicated groups expired with confirmed Y. pestis infections in buboes.

Y. pestis strain CPS-2a, and 2 rats (20%) succumbed to plague. Thus, the greater virulence of CPS-2a in mice seemed to apply equally well to rats. The 27 rats surviving inoculations with these strains were bled at one month. IHA tests with the sera did not detect F1 antibody. The rats were then challenged with Y. pestis 195P. Results demonstrated that previous exposure to the non-encapsulated variants could protect rats against virulent challenge with encapsulated Y. pestis (Table 5). Small inocula of the non-encapsulated strain CPS-1 could provide protection against subsequent challenge with small (1,450), but not large (13,800), challenge doses of encapsulated strain 195P. However, a large inoculum of strain CPS-1 protected most rats against acute, but not chronic disease arising from 13,800 Y. pestis 195P. Seventy-five percent of the rats surviving relatively small inocula of variant strain CPS-2a were protected against acute infection initiated with 830,000 Y. pestis 195P prepared in vivo, and therefore, in the most virulent physiological state possible. However, these animals remain under observation to detect cryptic disease.

Second-rat-passages were made (Table 6) using Y. pestis CPS-2a obtained from the spleens of two rats (Table 5). Results indicated that virulence of strain CPS-2a had increased for rats through passage, but LD<sub>50</sub> values were still 45-383X higher than those for strain CPS-2a in mice.

Table 6. Second rat passage of Y. pestis strain CPS-2a

Source of inoculum	Number of rats inoculated	LD <sub>50</sub> (in thousands of bacilli)	
		2 weeks	4 weeks
Spleen of rat dead 6 days after inoculation of 190 <u>Y. pestis</u> strain CPS-2a (25°C culture)	20	2.7	2.0
Spleen of rat dead 4 days after inoculation of 1,900 <u>Y. pestis</u> strain CPS-2a (25°C culture)	20	23.0	23.0

D. Additional cryptic plague infections in laboratory rats.

To date, only two isolates from rats with long-term infections have been studied in detail. However, a considerable number of cryptic plague strains have been isolated (Table 7). Studies of virulence determinants in the various strains are underway but are insufficiently complete for inclusion in this report.

Cryptic plague has now been detected in rats infected with classical Y. pestis 195P after vaccination with F1 antigen, as well as living plague vaccine, and in a rat that recovered by virtue of antiserum therapy. Also, the non-encapsulated strain M23 has been shown capable of cryptic infections of 212 days duration in an unvaccinated rat and up to 277 days duration in rats vaccinated with plague vaccine USP.

Results from the group of rats vaccinated with F1 antigen are particularly noteworthy. Prior to 195P challenge, these rats received subcutaneous injections, at weekly intervals, of 500 µg, 200 µg, and 200 µg of F1 antigen in adjuvant. Rats dying spontaneously on days 161, 166, 242, and 280 after 195P challenge demonstrated intense bacteremias, as well as involvement of the spleen and bubo. The rat that died on day 242 had an IHA titer of 2,048 at death, while the rat that died on day 280 had a titer of 512. At autopsy of the latter rat, the abdominal viscera were found to be covered with small yellow abscesses. Y. pestis was recovered from all tissues taken from the abdominal cavity, including liver, spleen, bubo, and peritoneal fluid. The pathology observed in this rat was very similar to that of latent plague reported in 1911 for the ground squirrels of California.

E. Rats and primates vaccinated with plague vaccine USP.

Additional studies are being directed toward determining if long-term cryptic plague infections occur following infection with encapsulated Y. pestis in animals vaccinated with plague vaccine USP. If non-encapsulated strains can be transmitted directly, such as by fleabite (a possibility yet to be investigated), our present data (see M23 challenges, Table 7) suggest that cryptic infections could occur with some frequency. However, if non-encapsulated strains arise through strong in vivo selection from an initial Y. pestis population consisting of capsule-forming bacilli, plague vaccine USP might possibly possess a capacity to

Table 7. Cryptic plague infections observed in laboratory rats

Vaccination and challenge	Number of rats dying with <u>Y. pestis</u> infection *	Range in day of death post challenge	Sources of <u>Y. pestis</u> at autopsy (number of rats infected in lesion indicated)
<u>Vaccination:</u> 17,800 <u>Y. pestis</u> of vaccine strain EV76(51f) <u>Challenge:</u> 497,000 <u>Y. pestis</u> 195P	2 (CPS-1;CPS-2)	424 & 541	abdominal bubo (2)
<u>Vaccination:</u> None <u>Challenge:</u> 48 <u>Y. pestis</u> 195P; Lederle anti-plague serum administered after 48 hrs	1	342	abdominal bubo
<u>Vaccination:</u> 900 µg F1 antigen <u>Challenge:</u> 3,500 <u>Y. pestis</u> 195P	14	110-280	pleural bubo (12) abdominal bubo (3) peritoneal fluid (1) liver (1) spleen (5) blood (4)
<u>Vaccination:</u> None <u>Challenge:</u> 33,400 <u>Y. pestis</u> M23	1	212	pleural bubo
<u>Vaccination:</u> Plague vaccine USP <u>Challenge:</u> 33,400 <u>v. pestis</u> M23	6	96-277	pleural bubo (5) abdominal bubo (4)

\* Experiments are in progress; rates of cryptic infection for each vaccine group remain to be determined.

prevent chronic infections from becoming established. At the present time (July, 1975), we are investigating these possibilities in laboratory rats and in Rhesus monkeys. Thirty-four rats that were vaccinated with plague vaccine USP are now 3 months post-challenge with 60,700 Y. pestis 195P. Rhesus monkeys in the second study are still undergoing vaccination with plague vaccine USP. When vaccination has been completed, the Rhesus will be challenged and thereafter observed for the occurrence of plague.

F. Implications of non-encapsulated variants of Y. pestis for the epidemiology and control of plague.

In many animals infected with non-encapsulated strains of Y. pestis, death is delayed for weeks or months. Animals infected with capsule-deficient Y. pestis do not produce antibody to the F1 antigen of the plague bacillus. Thus, the transmission of capsule deficient strains in a natural environment would be extremely difficult to detect. Mortality distributed throughout a lengthy time frame would present a pattern different from the onset of high morbidity and mortality observed in typical human epidemics and rodent epizootics. Presently employed serological tests would be inefficient, if at all capable, of identifying infected individuals. At the present time, the only index that could be obtained for the level of transmission is the rate of Y. pestis isolation from field specimens, a very expensive, inefficient and often impossible methodology to employ due to lack of facilities. It is obvious that new serological procedures will have to be applied in any situation where capsule-deficient variants of the plague bacillus are involved or suspected.

In most mice and some rats infected with non-encapsulated Y. pestis, death is accompanied by intense bacteremia, and strains recovered after a period of latency may be of the same or greater virulence than the strain that originally initiated infection. Experimental data suggest that all the non-encapsulated strains discussed in this report could be transmitted by fleas. Certainly, a flea would imbibe enough plague bacilli from a septicemic animal to become infected, and experiments reported herein, most of which employed subcutaneous inoculation to mimic fleabite, have demonstrated that rodents inoculated with very small numbers of non-encapsulated bacilli died of plague. Furthermore, many LD<sub>50</sub> values found in experiments were well within the range of bacilli injected via fleabite, and one non-encapsulated strain of Y. pestis was demonstrated to possess a mouse LD<sub>50</sub> approximately equal to the LD<sub>50</sub> for the highly virulent, classical encapsulated

strain 195P. The possibility that fleas might transmit such virulent capsule-deficient forms of Y. pestis hinges on the ability of fleas to become "blocked" with non-encapsulated Y. pestis and therefore infective. Experiments with fleas are clearly indicated.

The most important aspect of infection with non-encapsulated variants of Y. pestis is, perhaps the fact that vaccines in present use, both killed and living, do not always provide protection against disease. The specific capsular F1 antigen of Y. pestis elicits a potent antibody response that, in short term experiments, correlates well with resistance to lethal infection. However, vaccination with F1 does not prevent the selection of non-encapsulated variants in vivo and these eventually kill the animal protected against acute disease (Table 7). Likewise, plague vaccine USP, does not prevent chronic infection with non-encapsulated variants which progress to a fatal conclusion (Table 7). Hopefully, present studies may indicate some solution to immunological problems associated with these interesting, and disturbing, variants of the plague bacillus.

## II. Plague surveillance on Fort Ord and Hunter Liggett Military Reservation (HLMR), California.

A plague research and surveillance program was established, in cooperation with investigators at the Letterman Army Institute of Research (LAIR), to evaluate the threat of disease that might arise from an alarming population explosion of rodents on Fort Ord and the HLMR. Progressive increases in rodents have occurred over several years since the Environmental Protection Agency prohibited the application of 1080 rodenticide on federal lands. Cause for concern arose because rodent plague has occurred on both military reservations in the past and was rather prevalent in the 1940's. The state of California records indicate that plague still persists in the immediate area. A region bordering the northwest portion of the HLMR recently was quarantined due to plague in ground squirrels. The potential for an outbreak of plague under present circumstances is largely unknown, and important base-line data required to make an assessment does not exist.

A research protocol was developed jointly with investigators at the LAIR that could generate much of the data needed to make an assessment. While research investigations are conducted, it was deemed prudent to maintain a program of plague surveillance in the northwest region of the HLMR, near the bordering area recently quarantined. In the surveillance effort, this Department is examining rodents and fleas for infection with the plague bacillus and is performing serological tests on rodent sera collected by LAIR personnel.

To date, 206 wild-rodent sera, most from the California ground squirrel, have been tested for antibody, and all sera were negative. A few rodent carcasses and 68 pools, containing 3,196 fleas (Table 8), have been examined, and no isolations of Yersinia pestis have been made.

Table 8. Fleas of the California ground squirrel (Spermophilus beecheyi) collected on the Hunter Liggett Military Reservation, California, January-March 1975, examined for infection with Y. pestis

Species	Number of pools processed	Number of fleas	Isolations of <u>Y. pestis</u> <sup>a</sup>
<u>Diamesus montanus</u>	59	2,994	0
<u>Hoplopsylla anomalus</u>	<u>7</u>	<u>202</u>	<u>0</u>
	68	3,196	0

<sup>a</sup> Work on some pools is in progress; results suggesting plague infection have not been observed.

### III. Plague in Central Java, Indonesia.

The Boyalali district of Central Java has been recognized as a plague focus since 1957. In 1968, the focus displayed renewed activity. At this time, the plague bacillus was isolated from rats, fleas, and humans. Sera collected from rodents were occasionally positive for F1 antibody, although positivity rates were quite low. Following the 1968 episode, it was not until 1972 that a surveillance program could be initiated in the area. Although it is presently impossible to evaluate the focus from the standpoint of human disease (a vigorous surveillance is maintained for fever, and people with fever are treated undiagnosed, with antibiotics administered immediately), several isolations of the plague bacillus have been made from rats and fleas. Of interest, however, is the fact that several thousands of rat sera collected in the area were tested for F1 antibody utilizing reagents provided by the USSR with entirely negative results. It was possible, utilizing coded sera to compare the Soviet reagents with those employed by this Department (See Figure 1). A straight-line regression for the data of Figure 1 gave a coefficient of correlation equal to 88%.

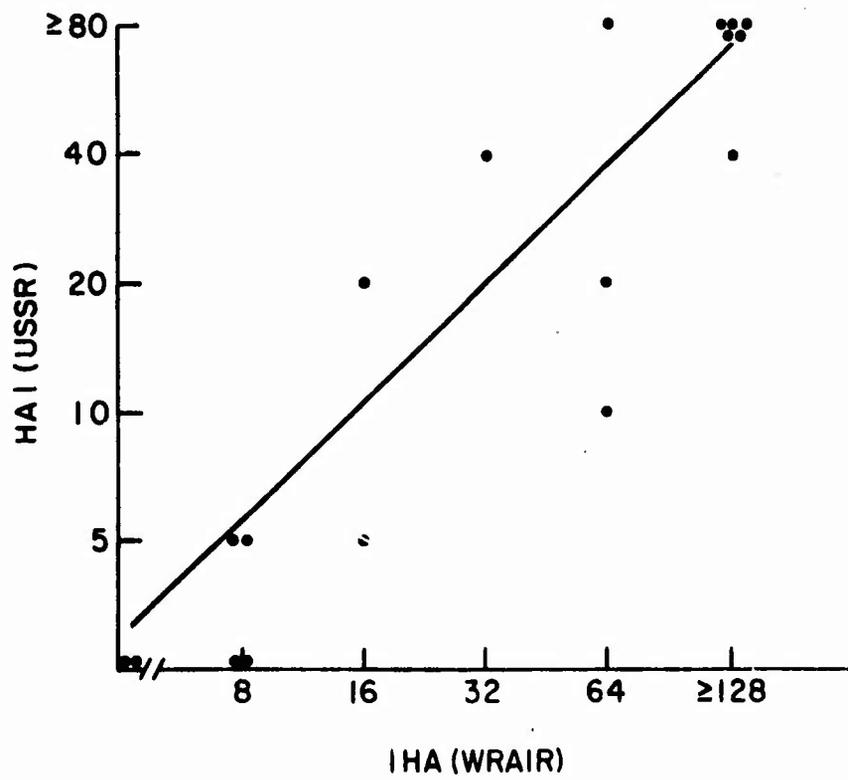


Fig. 1 Tests on coded laboratory sera with HAI reagents prepared in the USSR and with IHA reagents prepared at the WRAIR.

Some 1,500 wild-rodent sera, collected in this region were tested in this Department. F1 antibody was not detected in any of these specimens, confirming the earlier observations of the Indonesian workers. The complementary results obtained in two independent laboratories indicate several possibilities, any or all of which may be present in the focus:

1. The incidence of infection has diminished to an extremely low level.

2. The collecting program for rodent sera involves the wrong areas or time frames, although the large number of sera tested argue against this possibility.

3. Y. pestis deficient in F1 antigen have been selected in the area and are the cause of rodent, flea, and possibly human infections.

Future studies should elucidate the mechanisms involved. However, the possibility that mutant plague bacilli involved in endemic foci is intriguing.

IV. Electrophoretic techniques to identify geotypes of Y. pestis.

An experimental program was initiated to establish and further perfect electrophoretic techniques capable of identifying different geographic types of Y. pestis. Only such techniques were capable of demonstrating that recent isolates of Y. pestis in Tacoma, Washington, and in Indonesia possessed considerably different characteristics compared with Y. pestis strains from Vietnam. The only previously established capability (not DOD) for doing this type of analysis has now been disbanded.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 174 Ecology of Plague

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL <sup>a</sup>	
				DA OB 6537	75 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8. DRG/INSTR <sup>d</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS	
74 07 01	D.Change	U	U	NA		<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO / CODES <sup>e</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		61102A	3A16110:671Q	00	175		
B. CONTRIBUTING							
C. XEROX/PHOTODUPLICATION		CARDS 114F					
11. TITLE (Precede with Security Classification Code) <sup>f</sup>							
(U) Histopathologic Manifestations of Zoonotic Diseases of Military Importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>g</sup>							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 02		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: NA				B. PRECEDING		C. FUNDS (In thousands)	
B. NUMBER: NA				FISCAL YEAR		2	
C. TYPE:				75		50	
D. AMOUNT:				CURRENCY		76	
E. KIND OF AWARD:				76		1	
F. CUM. AMT.				55			
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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Foreign Intelligence not considered				ASSOCIATE INVESTIGATORS			
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23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Enterocolitica; (U) Yersinia; (U) Peritonitis; (U) Bronchopneumonia							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23(U) This study is being conducted to determine the pathogenesis of Yersinia enterocolitica infection in laboratory animals and determine the zoonotic implications of this bacterium, a current problem in military working animals.</p> <p>24 (U) Pilot studies in rats inoculated with Yersinia enterocolitica via several parenteral and oral routes demonstrated systemic bacterial lesions characterized by pyogranulomas in visceral organs. Young dogs were inoculated by intraperitoneal subcutaneous and oral routes to determine pathogenicity and capability of shedding the organism in feces.</p> <p>25 (U) 74 07 - 75 06 Seven of ten experimentally inoculated puppies shed organisms in their feces for 3-11 weeks. All dogs remained healthy during the experiment. Morphologic pathology results were limited to mild lymphadenopathy of the mesenteric lymph nodes. Tissues from dogs that were fecal culture negative were occasionally positive at the time of necropsy. Results reveal that puppies can serve as a reservoir for Yersinia enterocolitica and be chronic, healthy carriers for man. This portion of the project has been completed. No additional studies with this organism are anticipated. For technical reports, see Walter Reed Army Institute of Research Annual Report, 1 Jul 74 - 30 Jun 75.</p>							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

689

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 175 Histopathologic Manifestations of Zoonotic Diseases  
of Military Importance

Investigators.

Principal: MAJ Charles A. Montgomery, VC

Associates: MAJ Michael J. Reardon, VC; MAJ Bryce C. Redington, MSC; MAJ James H. Thrall, MC; CPT Wayne T. Hockmeyer, MSC; MAJ Michael G. Groves, VC; LTC Kenneth E. Kinnamon, VC; LTC Paul K. Hildebrandt, VC; MAJ Anthony J. Johnson, VC; Ronald Fayer, Ph.D.

Background

To define, study, diagnose and control known and potential bacterial and parasitic diseases common to both man and animals of potentially military significance. The major effort is directed toward defining the pathogenesis of these diseases utilizing gross pathology, microscopic pathology, histochemical and ultrastructural methods.

During the reporting period, research activities have been concerned with: (1) Pathophysiologic studies in Patas monkeys exposed to multiple, unilateral infections of Brugia malayi; (2) Experimental Yersinia enterocolitica infections in dogs; (3) Clinical disease and pathology of experimental Leishmania donovani infection in cynomolgus monkeys; (4) Pathology of experimental Sarcosporidiosis in animals.

Results and Discussion

Pathophysiologic Studies in Patas Monkeys Exposed to Multiple Infections of Brugia malayi. Lymphatic dynamics of Patas monkeys with unilateral infections of Brugia malayi were monitored and compared to gross and microscopic histopathologic observations at necropsy two years later. Each animal had been exposed to 75 infective stage larvae in one hind foot and to 50 larvae in the same foot each month for the next 9 months. The contralateral leg of each monkey, never exposed to larvae,

served as an autocontrol. All monkeys developed microfilaremias by 9 to 17 weeks after initial exposure and remained patent throughout the study. Lymphatic dynamics studies using the radionuclide  $^{99m}\text{Tc}$ -sulfur colloid (Tc-SC) were performed on both legs of each monkey at various intervals throughout the course of the disease. Lymphangiograms using the radio-opaque contrast medium Ethiodol<sup>R</sup> were performed on the legs of some animals prior to necropsy. Evans blue dye was injected subcutaneously in the pedal area 2-4 hours prior to necropsy to highlight lymphatic channels and nodes. In general, the radionuclide studies indicated a progressive alteration of lymph flow patterns and in some cases, a significant reduction in flow rates from the pedal injection site to the abdomino-pelvic nodes on the infected side of the host. A significant change noted was blockage of the afferent lymphatic to the popliteal node and the appearance of a collateral channel which was less efficient in its ability to transport the radiolabeled lymph. Also, pooling of the radionuclide was noted in the lower leg area. Gross and microscopic histopathologic observations at necropsy correlated markedly with both radionuclide flow and the lymphangiographic studies. Lymphatic channels distal to the popliteal node in infected legs appeared dilated and tortuous compared to the unaltered vessels in the control legs. Microscopic examination revealed that lesions were limited almost entirely to the areas distal to the popliteal node on the infected side only. Three basic types of lesions were observed: 1) dilated lymphatics associated with live worms; 2) partially obstructive lesions associated with degenerating worms with villous endolymphangitis and chronic inflammatory cells and detritus within the lumen; and 3) obliterative necrotizing lymphangitis with foreign body granulomatous perilymphangitis associated with dead, partially calcified worms. In spite of these pathophysiologic manifestations, only a slight transient edema was ever observed in the infected legs of these monkeys throughout the entire experiment.

Experimental Yersinia enterocolitica Infections in Dogs. Yersinia enterocolitica infections may range from asymptomatic to septicemic, but most commonly present as a non-bloody diarrhea or mesenteric lymphadenitis. Large outbreaks of diarrheal disease among school children in Japan have been attributed to Y. enterocolitica (1), and clusters of cases within families have also been reported. The organism has been recovered from a variety of wild and domestic animals.

Human infections have, in at least one instance (2) and probably a second (3), been associated with Y. enterocolitica infections in young puppies.

Because the dog may be a potentially important transmitter of Y. enterocolitica infections to man, a cooperative study with the Division of Veterinary Medicine defining the study in young dogs was done. A previous study using rats indicated that the organism could cause a severe pyogranulomatous reaction when administered parenterally. The microbiological, pathological and serological findings are reported here.

Ten 6 to 8 week old puppies of mixed breeding were infected with  $10^{12}$  Y. enterocolitica organisms each. Three puppies each were inoculated subcutaneously and intratracheally, and 4 puppies were infected per os. Feces were cultured twice weekly beginning 2 weeks prior to infection.

Results of the cultures are summarized in Table 1. Seven of the puppies had intermittently positive cultures through the 27th to the 59th day post-infection. One puppy had a single positive on day 6 and two puppies remained negative throughout the entire experiment. Only 8% of the fecal cultures from the subcutaneously infected dogs yielded Y. enterocolitica compared to 31% of the dogs infected orally and 42% of the dogs infected intratracheally. Synovial fluid, brain, mesenteric and prescapular lymph nodes, thyroid gland, duodenum and ileum were cultured during postmortem examination. One puppy (#9) had positive cultures from both sets of lymph nodes, the duodenum, and the ileum. Three puppies (#2, #4, and #7) had Y. enterocolitica cultured from only the ileum, and 6 puppies had no positive postmortem cultures.

Serum samples were collected at weekly intervals and titered for Y. enterocolitica by the Widal agglutination method using homologous antigen prepared from the same organism ("Brewer" Yersinia enterocolitica serotype 8) as that used to infect the puppies. This work was done by Dr. Thomas J. Quan, Plague Branch, Center for Disease Control, Fort Collins, Colorado. The serological results are presented as Graph 1.

It is readily apparent that significant circulating titers are attained only in individuals inoculated or exposed by a route other than oral.

Histopathologic changes were minimal and consisted primarily of a mild reactive lymphadenitis of mesenteric lymph nodes. There was no evidence of pyogranulomatous reaction in the tissues examined.

The intermittent isolation of Y. enterocolitica from the feces of experimentally infected puppies for varying periods up to 59 days post-infection affirms that dogs can be infected, remain clinically healthy, and serve as potential reservoirs of the agent. Fecal cultures should be attempted on several occasions in efforts to identify carrier individuals due to this intermittent shedding of the organism. Infections via oral or respiratory routes appears to be more efficient in establishing the carrier state than subcutaneous inoculation. It is of particular significance to note that serological results probably only indicate degree of systemic or septicemic disease and will not accurately identify a carrier.

This portion of the work unit is complete and no further studies are anticipated.

Clinical Disease and Pathology of Experimental Leishmania donovani Infections in Cynomolgus Monkeys. Four cynomolgus monkeys (Macaca fascicularis) were given the Khartoum strain of Leishmania donovani intravenously. Clinical signs in the host consisted of weight loss, splenomegaly, and pancytopenia. One animal recovered from the infection. Others developed a progressive wasting disease and severe hemolytic anemia. They were euthanized in extremis 80-98 days post-exposure. Gross lesions consisted of severe hepatosplenomegaly and hemorrhage in the brain. Spleen weights increased 10 times over normal. Salient histopathologic lesions were severe reticuloendothelial hyperplasia of the spleen and lymph nodes, nonsuppurative periportal hepatitis, interstitial pneumonia, nonsuppurative interstitial epididymitis and submeningeal hemorrhages. LD bodies were demonstrated throughout the clinical disease in bone marrow biopsies and in many of the tissues examined postmortem. Data show this animal to be an excellent host for experimental visceral leishmaniasis. Chemotherapeutic studies and more definitive pathologic studies are in progress in a second group of six cynomolgus monkeys.

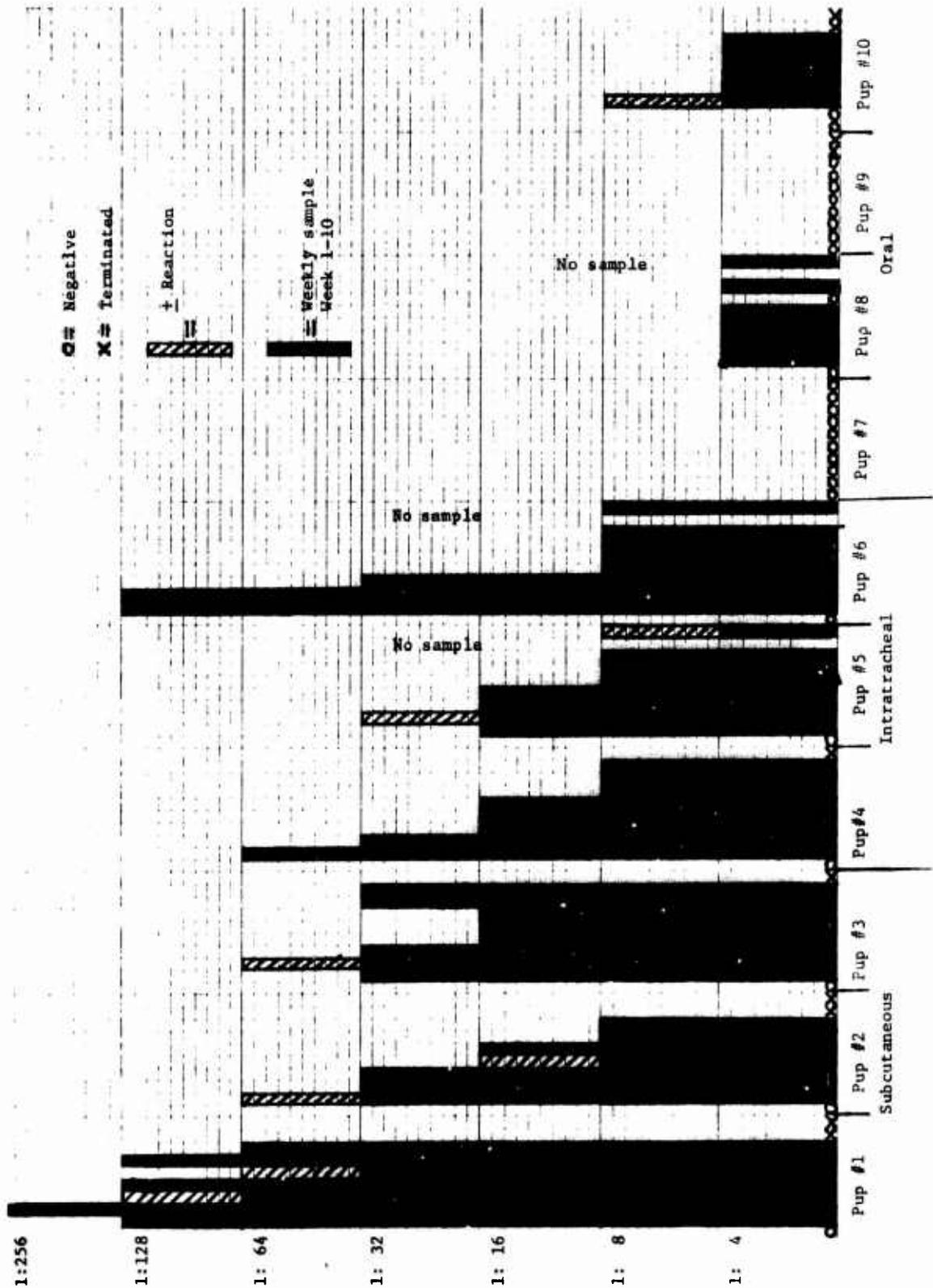
The Pathology of Experimental Sarcocystis in Animals. The pathology of experimental sarcocystis has been studied in bovine, ovine, porcine, canine, feline and rodents. Calves fed sporocysts of Sarcocystis isolated from the feces of dogs and coyotes revealed the most significant pathological changes. They became anorectic, lost weight, anemic, prostrate and died. Necropsy revealed lymphadenopathy, paleness of the mucous membranes and visceral organs, ascites, hydrothorax, hydropericardium, and serous atrophy of fat. Microscopic changes observed in tissues of nearly all calves consisted of hemorrhage, mononuclear cell (primarily lymphocytic) infiltration, and edema in heart, brain, liver, lung, kidney and striated muscle. Necrotizing myocarditis with dystrophic calcification of striated muscle and fat, as well as nonsuppurative inflammation of meninges and glial nodules in the brain, were observed in several calves. Schizonts of Sarcocystis were located throughout the body between 26 and 33 days post-inoculation. After 33 days post-inoculation, only cyst stages of Sarcocystis were observed in skeletal and cardiac muscle.

The infection in dogs and coyotes was restricted to the mucosal epithelium of the intestine. Cytolysis of infected cells was the only change associated with the intestinal infection. No evidence of infection of this species of Sarcocystis was detected in the other animals inoculated.

TABLE I. RESULTS OF FECAL CULTURES FOR YERSINIA ENTEROCOLITICA

Day Post Infection	Route of Infection									
	Subcutaneous			Intratracheal			Per Os			
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
3				+				+		
6		+	+	↓	+		+	↓		+
10			↓	↓	↓	+	↓			
13				↓	↓	↓				+
17				↓						↓
20					+		+			
24			+				↓			
27			↓	+	+	+		↓		↓
31							↓			
34							↓			
38										
41										
45					+		+			
48				+	↓		↓			
52	PM				↓				PM	PM
55		PM	PM	PM	↓					
59					PM	PM		PM		
62										
66										
69							PM			

PM - Postmortem examination performed and organs cultured.



Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 175 Histopathologic Manifestations of Zoonotic Diseases  
of Military Importance

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA QB 6513	75 07 01	DD FORM 1 (AR) 75	
3. DATE PREVIOUS SUMMARY <sup>a</sup>	4. KIND OF SUMMARY <sup>a</sup>	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DRG/N INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUMMARY WORK UNIT <sup>a</sup>
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
11. NO. / CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	6T102A	3A161102B71Q		00	176		
B. CONTRIBUTING							
C. <del>XXXXXXXXXX</del>	CARDS TT4F						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Mechanisms of Transmission of Hepatitis Viruses							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002600 Biology 010100 Microbiology 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		FUND (in thousands)	
B. NUMBER <sup>a</sup>				FISCAL YEAR		75	
C. TYPE				CURRENT		2.5	
D. KIND OF AWARD:				76		125	
E. AMOUNT:							
F. CUM. AMT							
18. RESPONSIBLE DOD ORGANIZATION				19. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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22. KEYWORDS (Precede EACH with Security Classification Code) (U) viruses; (U) Hepatitis; (U) Antigen; (U) Immunology.							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23 (U) To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses - antigens and antibodies to determine host factors important in resistance to disease and infection in military personnel.</p> <p>24 (U) New methods for identification and antigenic analysis of hepatitis viruses are under development. The immune response of patients infected with hepatitis viruses is studied to define sensitive parameters of infection and to define factors critical in immunity. The epidemiology of Hepatitis B in military populations is defined.</p> <p>25(U) 74 07 - 75 06 A new radioimmunoassay for Hepatitis B surface antigen (HBsAg) has retained the sensitivity and increased the specificity of HBsAg detection in serum. Hepatitis B core antigen (HBcAg) was obtained from liver of infected chimpanzees which were treated with the immunosuppressive drug cyclophosphamide. A recently developed serologic test for antibodies to HBcAg has proven to be highly efficient in the diagnosis of Hepatitis B; in a series of military cases of hepatitis, Hepatitis B was confirmed by detection of HBsAg in serum of 50 percent of cases and by detection of anti-HBc in acute serum in 95 percent of cases. The prevalence of antibody to HBsAg in antibody positive soldiers identified in an epidemic of Hepatitis B at Fort Hood declined significantly over a year's period; only 84 percent of those with antibody reacting only to the subtype of virus causing hepatitis on post had detectable antibody in serum one year later. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 74 - 30 June 75.</p>							

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Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable disease and Immunology

Work Unit 176 Mechanisms of transmission of hepatitis viruses

Investigators.

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Associate: LTC Herbert E. Segal; MSG Milton Willhight; SSG Michael  
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SP4 Robert Putnak; Hubert G. Cannon.

### Description

To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses/antigens and antibody to determine host factors important in resistance to disease and infection.

### Progress

#### I. Epidemiology of Hepatitis B infection in military populations

##### A. A study of the incidence of Hepatitis B and Hepatitis B virus infection in soldiers at Fort Hood, Texas.

Following an extensive serologic prevalence survey conducted in February - April 1973 during the hepatitis B outbreak at Fort Hood, Texas, a longitudinal survey of troops entering the First Cavalry was initiated in February 1974. Men were bled on arrival at Fort Hood and at 4 month intervals over the course of one year. All entering personnel regardless of rank overseas tour or duration of active duty filled out an initial questionnaire and donated a sample of blood. The initial starting cohort numbered 2333 men. Although preliminary information suggested that the majority of these troops would enter a newly formed brigade, they in fact were assigned to units throughout the First Cavalry Division. There was an appreciable decrease in the number of men studied; 1916, 1216 and 950 men were bled at 4, 8, and 12 months respectively. Failure to obtain follow-up questionnaires and blood samples was linked to transfer out of Ft. Hood, ETS, DFR, AWOL, or unwillingness to volunteer for subsequent blood samples. Table 1 lists reasons for

non compliance with requests for follow-up blood specimen.

Table 1 - Reasons for failure to obtain blood at 4 month intervals in 1st Cavalry Division study.

Blood Specimens obtained	4 month 1916	8 month 1216
Not obtained because of:		
ETS	19	164
DFR	13	33
AWOL	21	18
PCS	14	92
DEATH	0	1
OTHER*	350	392

\*Includes TDY, Special Assignment, no record of presence at Fort Hood or unwillingness to volunteer or report for blood drawing.

The serologic analysis of analysis of the initial bleed is listed in Table 2. Data on the 12 month serum samples is incomplete. During the period of observation from February 1974 through March 1975, acute hepatitis B infection rates at Fort Hood fluctuated between 5-10/1000/year which was substantially lower than the peak clinical infection rate of 19/1000/year which occurred in November, 1972. Among the 1216 patients followed for 8 months, there were 5 cases of hepatitis and 41 cases of HBV infection.

The objective of this study, to determine the incidence of hepatitis B and subclinical HBV infection in troops entering a post with endemic drug associated hepatitis B, was not fully realized. Rates of hepatitis declined to low levels during the study so that only 5 of the 1216 soldiers studied for 8 months developed disease with an additional 6 having subclinical antigenemia. A second problem was the unexpectedly high rate of loss of soldiers from the sample studied. Only half donated blood at 8 months, and only one third of the original cohort

volunteered blood at the 12 month bleeding. A significant proportion of this loss was due to unanticipated transfer of troops from the first Cavalry Division but a large proportion were unwilling to volunteer blood samples.

Table 2 - Development or loss of HB<sub>s</sub>Ag or Anti-HB<sub>s</sub> in soldiers of First Cavalry Division, Fort Hood, Texas

	<u>Initial Bleed</u>	<u>4 Months</u>	<u>8 Months</u>
<b>Total</b>	2333	1916	1216
No. Positive for anti-HB <sub>s</sub>	142	130	74
HB <sub>s</sub> Ag	24	16	10
No. who acquired anti-HB <sub>s</sub>	-	60	36
HB <sub>s</sub> Ag	-	5	2
No. who lost anti-HB <sub>s</sub>	-	34	21
HB <sub>s</sub> Ag	-	3	4

Tests of the prevalence of HB<sub>s</sub>Ag and anti-HB<sub>s</sub> in the 950 soldiers sampled at 12 months are in progress. In addition, the prevalence of anti-HB<sub>c</sub> and the incidence of HBV infection determined by seroconversion of anti-HB<sub>c</sub> will be determined in soldiers followed for 8 or 12 months. Thus, a comparison of the diagnostic efficiency of anti-HB<sub>s</sub> and anti-HB<sub>c</sub> in asymptomatic HBV infection will be made.

#### B. Persistence of Hepatitis B surface antibody.

Two major antibody systems comprise the spectrum of antibodies induced by hepatitis B virus. The first group of antibodies is to hepatitis B surface antigen (anti-HB<sub>s</sub>) which include: The common determinant A and the usually mutually exclusive determinants Y or D, (Le Bouvier, 1971) and W or R (Bancroft *et al*, 1972) respectively. A more recently described antibody response to the core antigen of the Dane particle (anti-HB<sub>c</sub>) comprises the second antibody system (Hoofnagle *et al*, 1973).

Various studies have determined the prevalence of anti-HB<sub>s</sub> in different populations. In general, the prevalence of anti-HB<sub>s</sub> is higher in lower economic groups (Cherubin *et al*, 1972) institutionalized populations, (Szmunn W. *et al*, 1972) or health personnel in the USA and in underdeveloped countries where HB<sub>s</sub>Ag prevalence is high (Punyaguptas *et al*, 1973). There is evidence that anti-HB<sub>s</sub> correlates with resistance to hepatitis B. Persistence of anti-HB<sub>s</sub> after HBV infection has not been well studied but has significant implications in evaluation of the immunogenicity of wild HBV strains and of candidate hepatitis B vaccines. In this study, the persistence of anti-HB<sub>s</sub> with specificities for a, y, and d antigens of HB<sub>s</sub>Ag over a one year period of observation was examined in individuals determined to have anti-HB<sub>s</sub> during investigation of a military hepatitis B outbreak.

Initial sera were derived from a prevalence survey of Army personnel at Fort Hood, Texas during a hepatitis B outbreak in February and March of 1973. Previously reported investigations indicated that nearly all (95%) of Hepatitis B cases at Fort Hood were of subtypes ayw. Approximately 523 individuals out of 3335 tested were found to have anti-HB<sub>s</sub>. One year later, a second sample of serum was obtained on 217 of the anti-HB<sub>s</sub> positive individuals. Paired sera were tested together for anti-HB<sub>s</sub> by (PHA) passive hemmagglutination as described below.

The passive hemmagglutination test was performed according to the method of Vyas *et al* (1970). Sera were tested with red blood cells coated with HB<sub>s</sub>Ag of subtype ayw and with subtype adw (obtained from Electronucleonics Lab Inc., Bethesda, MD). Each serum (0.025ml) was diluted in "V" bottom plastic plates (Linbro Co) using 0.05M sodium phosphate buffer in 0.85% NaCl, pH 7.3 containing 0.5% bovine serum albumin, 0.0025% polyvinyl pyrrolidone and 1:20,000 dilution of Tween 80. An equal volume of a 40% suspension human type O erythrocytes coated with purified HB<sub>s</sub>Ag of subtype adw or of subtype ayw was added to the serum dilutions. The mixture was incubated at 27°C for 30 minutes, then centrifuged at 1200 rpm for 1 minute. The plates were allowed to rest at a 60° angle for 20-30 minutes before reading. Hemagglutination was indicated by the failure of the erythrocyte button to stream across the bottom of the plate. All positive PHA results were repeated for confirmation; a reproducible titer of 1:8 or greater was considered a positive test for anti-HB<sub>s</sub>.

Four patterns of anti-HB<sub>s</sub> were observed using PHA: (1) serum reactive to both HB<sub>s</sub>Ag/adw and ayw subtypes with a titer  $\geq$  1:32 (2) sera reactive to both subtypes but with a titer of 1:8 or 1:16 (3) sera reacting only with HB<sub>s</sub>Ag/adw subtype (anti-d) (4) sera that react only with HB<sub>s</sub>Ag/ayw (anti-y).

In order to confirm the specificity of sera reacting with cells coated with HB<sub>s</sub>Ag of only one of the 2 subtypes ayw or adw, several

sera in each of the four anti-HB<sub>s</sub> categories were tested by a radio-immune inhibition assay. Block titrations were prepared using 3 different HB<sub>s</sub>Ag subtypes (ayw, adw, adr) against human serum as well as specific rabbit anti-HB<sub>s</sub>. A standard dilution of each HB<sub>s</sub>Ag subtype was made in saline (1:100 dilution of adw and ayw and 1:1000 of adr). Antibody was diluted serially from 1:8 to 1:4096. One tenth ml each of antigen and antibody was mixed in a 75mm glass tube and allowed to incubate for 2 hours. Following incubation 0.1 ml of the mixture was transferred to an Ausria (Abbott Labs) tube and tested for the presence of HB<sub>s</sub>Ag (Ling *et al* 1972, Irwin *et al* 1974). Dilution controls for antigen and antibody alone were included in each test. In addition a serum without anti-HB<sub>s</sub> was diluted 1:2 in saline and dispensed into 10 Ausria tubes in replicate. A 25% or more reduction in counts per minute of the test as compared to the mean of these 10 controls was considered to be significant inhibition.

In the initial 1973 prevalence study at Fort Hood, Texas, 523 of the 3355 personnel tested (15.6%) had anti-HB<sub>s</sub>. One year later, serum was obtained on 217 of the 523 (41.5%) soldiers originally positive for anti-HB<sub>s</sub>. As shown in Table 3, there was no essential difference in the distribution of anti-HB<sub>s</sub> specificity in original serum between those personnel bled one year later and the total group originally determined to have anti-HB<sub>s</sub>.

Table 3 - Distribution of anti-HB<sub>s</sub> specificity in personnel in Fort Hood prevalence survey February and March 1973.

	All Personnel with anti-HB <sub>s</sub>	Personnel with anti-HB <sub>s</sub> with serum obtained 1 year later
Total Anti-HB <sub>s</sub> Positive	524	217
Anti-HB <sub>s</sub> to both adw and ayw coated RBC	346 (66%)	138 (63.6%)
Anti-HB <sub>s</sub> to adw coated RBC <u>ONLY</u>	89 (17%)	41 (18.9%)
Anti-HB <sub>s</sub> to ayw coated RBC <u>ONLY</u>	89 (17%)	38 (17.5%)

The persistence of anti-HB<sub>s</sub> over one year for the 4 serologic categories previously described is shown in Table 4. Of persons with sera reactive to both subtypes at titers  $\geq 1:32$ , 92% had anti-HB<sub>s</sub> persistent at one year; a slightly lower proportion (74%) of those with PHA titers of 1:8 or 1:16 to both subtypes had persistent anti-HB<sub>s</sub>. Seventy-nine soldiers had anti-HB<sub>s</sub> reactive to but one subtype: 41 reactive to erythrocytes coated with HB<sub>s</sub>Ag/adw and 38 reactive to erythrocytes coated with HB<sub>s</sub>Ag/ayw. Almost 60% of those with adw reactivity had persistent anti-HB<sub>s</sub> while a much smaller proportion of personnel with ayw reactivity (16%) had anti-HB<sub>s</sub> one year later.

Table 4 - Persistence of anti-HB<sub>s</sub> after one year according to specificity of initial sera for adw or ayw HB<sub>s</sub>Ag coated red cells. Sera initially positive to one or both types of cells, if positive one year later reacted with the same specificity.

Anti-HB <sub>s</sub> status of initial serum		Number with anti-HB <sub>s</sub> persistent at one year (%)
$\geq 1:32$ titers to both adw and ayw coated RBC	99 (45.6)	91 (91.9)
Titers 1:8 - 1:16 to both adw and ayw coated RBC	39 (18.0)	29 (74.4)
Titer $\geq 1:8$ to adw coated red cells ONLY	41 (18.9)	24 (58.5)
Titer $\geq 1:8$ to ayw coated red cells ONLY	38 (17.5)	6 (15.8)
TOTAL	217 (100%)	150 (69.1%)

Human sera of only D or only Y activity in original sera maintained this specificity over a one year period; none became reactive to the heterologous subtype. In order to test the specificity of sera with monotypic PHA reactivity, radioimmune inhibition assays against HB<sub>s</sub>Ag of the 3 subtypes ayw, adw and adr were undertaken with representative human serum and with hyperimmune rabbit anti-sera raised against purified antigen of each of these 3 subtypes. Hyperimmune sera produced precipitating antibody to the immunizing subtype. Considerable specificity for the anti-D and anti-Y was evident by the RIAI technique with the specific hyperimmune rabbit sera. (Table 5) Also the RIAI test confirmed the specificity of the human sera of D or Y activity determined by the PHA test.

Table 5 - Inhibition titers of control hepatitis B antigen subtypes by hyperimmune subtype antisera and human sera positive for anti-HB<sub>s</sub> by PHA.

Types of PHA Reaction	Serum #	Radioimmune Assay Inhibition Titer		
		HB <sub>s</sub> Ag/ayw	HB <sub>s</sub> Ag/adw	HB <sub>s</sub> Ag/adr
Anti-HB <sub>s</sub> titer $\geq$ 1:32 to both adw and ayw coated RBC	M 938	1:8	1:8	1:8
Anti-HB <sub>s</sub> titer $\geq$ 1:8 to adw coated RBC <u>ONLY</u>	E 1205	< 1:8	1:8	1:8
Anti-HB <sub>s</sub> titer $\geq$ 1:8 to ayw coated RBC <u>ONLY</u>	M 307	1:8	< 1:8	< 1:8
Rabbit Hyper-immune anti-HB <sub>s</sub> /ayw	R 228	1:1024	1:128	1:64
*Rabbit hyper-immune anti-HB <sub>s</sub> /adw	R 181	1:128	1:2048	1:2048
Rabbit hyper-immune anti-HB <sub>s</sub> /adr	R 560	1:64	1:512	1:512

\* Rabbit hyperimmune sera contained precipitating antibody

In Table 6 is compared the proportion of soldiers in each of the 4 anti-HB<sub>s</sub> classifications who had previous overseas tour, a past history of hepatitis, contact with a jaundiced person, a history of previous blood transfusion. Also the mean age of soldiers in each antibody group is shown. A larger proportion of soldiers with monotypic reactivity to adw or ayw had no previous overseas tour and had recent contact with jaundiced persons than the groups reacting to both HB<sub>s</sub>Ag subtypes. Also the median age of soldiers with only Y reactivity was significantly lower (23.7) than the other 3 groups.

Using the PHA test, sera found to be positive for anti-HB<sub>s</sub> (titer  $\geq$  1:8) were arbitrarily divided into 4 groups based on observed differences in the titer and specificity of the PHA reaction. The first 2 groups represent anti-HB<sub>s</sub> that react with red cells coated with both HB<sub>s</sub>Ag subtypes adw and ayw with a titer of  $\geq$  1:32 and 1:8 or 1:16 respectively. The remaining two groups of serological reactions included sera that agglutinated either adw or ayw HB<sub>s</sub> coated red cells.

Table 6 - Comparison of epidemiological parameters of individuals surveyed with initial anti-HBs serology.

	Overseas Tour			Clinical Hepatitis History			
	No.	Asia*	Europe	None**	Clinical Hepatitis	Contact with Jaundiced Person	Blood Transfusion
Anti-HBs both adw and ayw coated RBC titer $\geq$ 1:32	99	54(54.6%)	14(14.1%)	31(31.3%)	2(2.0%)	18(18.2%)	4(4.0%)
Anti-HBs both adw and ayw coated RBC titer 1:8 or 1:16	39	27(69.2%)	-	12(30.8%)	3(7.7%)	5(12.8%)	1(2.6%)
Anti-HBs to adw coated red cells ONLY: titer 1:8 or greater	41	13(31.7%)	3(7.3%)	25(61%)	2(4.9%)	13(31.7%)	3(7.3%)
Anti-HBs to ayw coated red cells ONLY: titer 1:8 or greater	38	12(31.6%)	2(5.3%)	24(63.1%)		8(21%)	1(2.6%)
TOTAL	217	106(48.8%)	19(8.8%)	92(42.4)	7(3.2%)	44(20.3%)	9(4.1%)

\* Vietnam Primarily

\*\* Mostly new recruits - USA only

Our interpretation of these serological reactions is that sera reacting to both types of coated red cells contains at least anti-HB<sub>s</sub> to the "a" antigenic determinant of HB<sub>s</sub>Ag in varying quantities; while those sera reacting to only adw or ayw coated red cells have subtype specific anti-HB<sub>s</sub> to "d or y" antigenic determinants.

Using a second serological test (RIAI) of inhibiting standard dilutions of HB<sub>s</sub>Ag of known subtypes adr, adw and ayw, confirmation of this distinction was obtained. (Table 5) Whether the anti-HB<sub>s</sub> reacted with both adw and ayw HB<sub>s</sub>Ag coated red cells also contained subtype specific anti-HB<sub>s</sub> was not however examined because of the limited quantities of sera available and the multitude of homologous, and heterologous absorption steps required to examine this less significant point.

In 3 of the 4 categories of anti-HB<sub>s</sub> response, the prevalence of antibody decreased significantly over one year. Only in the soldiers with relatively high titers to both subtypes of HB<sub>s</sub>Ag did prevalence of antibody remain relatively constant. In the other 3 groups, anti-HB<sub>s</sub> was lost in 84% of those with anti-Y, 41% of those with anti-D, and 25% of those with low titered anti-A specificity. The decrease in antibody prevalence in these 3 groups did not appear to be related to PHA titer since monotypic antibody was rarely greater than 1:32.

The rapid rate of loss of anti-Y (84%) over anti-D (41%) may possibly reflect a lesser immunogenicity of the Y determinant in comparison with the D determinant. In guinea pigs immunized with HB<sub>s</sub>Ag, ayw or adw subtypes, antibody to Y is more difficult to raise than antibody to D; (personal observation); however the persistence of specific antibody raised has not been determined. A more likely possibility is that individuals with specific anti-Y had more recent infections than individuals with anti-D. Consistent with this hypothesis is the almost exclusive detection of HB<sub>s</sub>Ag/ayw among hepatitis cases at Fort Hood during the epidemic and the younger median age of individuals with anti-HB<sub>s</sub> of Y reactivity (hepatitis was limited nearly exclusively to young enlisted personnel!) Although the time of acquisition of anti-HB<sub>s</sub> is unknown for individuals studied, it is likely that older soldiers with anti-D were exposed to HBV at some more remote time than those with anti-Y. Thus the more rapid decay in anti-HB<sub>s</sub> of Y rather than D specificity may be a reflection of more recent infection in the former.

Regardless of the subspecificity of antibody involved, a larger proportion of soldiers (31%) lost anti-HB<sub>s</sub> over a one year period. In young soldiers with presumably recent infection with HB<sub>s</sub>Ag/ayw, 84% of those who developed only antibody to the Y specificity no longer had detectable anti-HB<sub>s</sub> one year later. Thus a significant decrease in prevalence of anti-HB<sub>s</sub> in adults acquiring antibody by subclinical HBV infection was found in the study.

This finding is not without precedent in the literature. In a study of HBV infection in residents of Bangkok, Thailand, Grossman et al (1975) noted that 13% of the urban population studied acquired evidence of HBV infection over a 9 month period. During the same time 7% of individuals lost detectable evidence of HBV infection. In persons under 14 years of age (in whom HBV infection was more likely primary than in persons of greater age), 10% lost anti-HB<sub>s</sub>. This 10% decrease in anti-HB<sub>s</sub> prevalence per year in the Thai urban population was less than that seen in soldiers at Fort Hood, but differences in the route, dose and frequency of transmission between the 2 populations may account for the slower decay of antibody in the Thai population. Clearly, further studies (ideally prospective) of the decay of anti-HB<sub>s</sub> after clinical and subclinical infection are required.

The significance of this diminishing anti-HB<sub>s</sub> response after subclinical HBV infection is unclear, since it remains to be determined whether loss of detectable anti-HB<sub>s</sub> is equivalent to loss of immunity to HBV infection or disease. Several different lines of evidence suggest the importance of anti-HB<sub>s</sub> in immunity, however. The fall of anti-HB<sub>s</sub> titers to undetectable levels in a significant proportion of persons with nationally acquired HBV suggests that they may be susceptible to reinfection if not hepatitis B. The ephemeral nature of anti-HB<sub>s</sub> induced by HBV infection with the ayw strain may prove an obstacle in development of inactivated HB<sub>s</sub>Ag vaccines for humans.

C. Follow-up of Fort Hood hepatitis patients for chronic antigenemia and efficiency of serologic diagnosis of hepatitis B.

In March 1975, a roster of soldiers with hepatitis who had been admitted to Darnall Army Hospital at Fort Hood, Texas from January 1973 through February 1975 was assembled. Approximately 600, such patients were sent a letter requesting them to report for further study at Fort Hood or if no longer there, to forward a sample of serum to the Department of Virus Diseases, WRAIR. About 60% of the letters were returned undelivered from Fort Hood. However sera were received from 50 patients from 47 of whom sera had been obtained at time of hospitalization. Three acute sera were negative for HB<sub>s</sub>Ag by counter-electrophoresis but insufficient quantities were available for additional tests. Paired sera of 29 patients were run for anti-HB<sub>c</sub>, anti-HB<sub>s</sub> and HB<sub>s</sub>Ag. Results are shown in Table 7.

As noted in the annual report from last year, anti-HB<sub>c</sub> has proved valuable in the serologic confirmation of hepatitis B infection in individuals who are HB<sub>s</sub>Ag negative. Although this series of patients is small and is biased in the sense that these individuals responded to a written request for participation the available data has much speculative potential.

Table 7 - Follow-up of 29 Acute Hepatitis B cases at Fort Hood, Texas

Initial Hepatitis B Cases		Acute Sera Anti-HB <sub>c</sub>	Convalescent Sera*		
			HB <sub>s</sub> Ag+	Anti-HB <sub>s</sub> +	Anti-HB <sub>c</sub>
HB <sub>s</sub> Ag+	13	13	4*	6	10
HB <sub>s</sub> Ag-	16	13	0	3	13

\* Obtained 11 to 26 months after clinical hepatitis

Anti-HB<sub>c</sub> was found in all sera of the 13 hepatitis patients with antigenemia tested and in 81% (13/16) of HB<sub>s</sub>Ag negative hepatitis cases tested. Although incomplete and perhaps due to poor quality of PHA of cells, anti-HB<sub>s</sub> was found in 6 individuals lacking HB<sub>s</sub>Ag 2-28 months following acute HB<sub>s</sub>Ag+ hepatitis. In those patients without HB<sub>s</sub>Ag only 3/21 had detectable anti-HB<sub>s</sub> 2-27 months following the acute episode. Clearly anti-HB<sub>c</sub> is a more sensitive indicator of symptomatic HBV infection than anti-HB<sub>s</sub>.

In the 15 hepatitis patients in whom HB<sub>s</sub>Ag was initially detected, antigen persisted beyond 3 months in 4, this represents 8% of the 50 HBV patients which were followed. This relatively high proportion of persistent antigen carriers is disturbing in view of long term consequences of HB<sub>s</sub>Ag persistence. Further prospective studies of long term drug induced Hepatitis B and HBV infection in military personnel are indicated. This study confirms the hypothesis that nearly all hepatitis cases hospitalized at Fort Hood, were Hepatitis B.

#### D. Prevalence of HB<sub>s</sub>Ag and anti-HB<sub>s</sub> in military health personnel.

1. Hepatitis B infection is considered an occupational hazard of health personnel. For the past 2 years, a prevalence survey of anti-HB<sub>s</sub> and HB<sub>s</sub>Ag in officer and enlisted personnel entering the Health Service Academy, Fort Sam Houston, Texas in collaboration with the Division of Preventive Medicine, WRAIR has been conducted. In addition an attempt was made to obtain a 2 year follow-up specimen on the initial cohort of people who participated in the study. Analysis of approximately 10,000 serum samples for HB<sub>s</sub>Ag and anti-HB<sub>s</sub> has been completed and the results correlated with demographic data. This study is reported in detail in the section of the Division of Preventive Medicine in this Annual Report.

2. Laboratory support of Hepatitis outbreaks in military personnel or dependents.

The hepatitis section tested sera for HB<sub>s</sub>Ag, anti-HB<sub>s</sub> and performed subtyping of HB<sub>s</sub>Ag from 2 military outbreaks of hepatitis at Camp Zama, Japan and Fort Riley, Kansas. These results are included within detailed descriptions of the outbreaks in the Annual Report of the Division of Preventive Medicine.

## II. Basic studies on Hepatitis viruses

### A. Production of hepatitis B core antigen (HB<sub>c</sub>Ag)

The Dane particle is felt to be the complete Hepatitis B virion. With the aid of the electron microscope (Almieda, (1972)) reported a second antigen antibody reaction distinct from the surface antigen determinants a, d, y, w, and r. Complement-fixing antibody to this core antigen (anti-HB<sub>c</sub>) was found to be uniformly present in asymptomatic hepatitis B carriers, in most cases of acute hepatitis cases with HB<sub>s</sub>Ag and in 50-80% of acute hepatitis cases without antigenemia. The presence of anti-HB<sub>c</sub> was found to be transient by Hoofnagle (1974). An antibody such as anti-HB<sub>c</sub> which rises early in the course of infection or disease and disappears after infection would have significant diagnostic advantages over the current serologic test for anti-HB<sub>s</sub> and should permit a firmer laboratory base for epidemiologic studies of HBV.

The limiting factor for further investigation in this area of HBV was the inability to produce or harvest core antigen. Original studies by Almeida were fortuitous in obtaining plasma from an asymptomatic antigen carrier rich in Dane particles. Core antigen was extracted from the Dane rich plasma by detergent treatment with Tween 80. Few carriers however have large amounts of circulating Dane particles. Hoofnagle *et al* (1973) at the BOB found core antigen in a chimpanzee that had been treated with cyclophosphamide and died of pneumonia. Core antigen derived from this chimp liver and from another one of approximately 10 other chimps similarly treated at the BOB have been the main source of core antigen for the last 1½ years.

Because of the growing importance of core antigen and anti-HB<sub>c</sub>, a project to produce core antigen in immunosuppressed chimps was undertaken. Two large chimps (50-65 lbs) were bled 3 times a week and immunosuppressed with Cytoxan (cyclophosphamide) initial dose 15mg/kgm, for several weeks prior to inoculation intravenously with infectious plasma from HB<sub>s</sub>Ag subtypes adw and adr. (Kindly supplied by Dr. Hoofnagle of the Bureau of Biologics, FDA). Each animal was bled 2-3 times/week and samples obtained for CBC, liver function tests (SGOT, LDH, Bilirubin etc), HB<sub>s</sub>Ag, anti-HB<sub>s</sub>, anti-HB<sub>c</sub> and DNA polymerase (this test was kindly performed by Drs Kaplan and Gerin of the NIAID, Rockville Lab). After the development of detectable HB<sub>s</sub>Ag, liver biopsies were performed every 10-14 days and homogenates of the liver tested for the



In addition to liver, sections of salivary glands, pancreas, intestine, kidney and spleen of chimp 683 were sent to Dr. Thomas Edgington of Scripps Clinic and Research Foundation for detection of HB<sub>C</sub>Ag by fluorescent antibody techniques to determine if these tissues might support replication of HBV. Core antigen was found in the liver parenchymal cells of chimp 683 but was not detected within cells of the other organ. Thus the mechanism by which HB<sub>S</sub>Ag to present in saliva in the absence of bleeding could not be related to replication of HBV in the salivary glands.

#### B. Attempts to purify Hepatitis B core antigen

Hepatitis B core antigen was produced in two chimpanzee livers as described. Serologic antigen for complement fixation was initially produced by the method of Barker et al. Liver tissue was homogenized in hypotonic (0.45%) saline. The 20% (wt/vol) homogenate was clarified at 2,000 rpm for 30 min. The supernatant fluid was then pelleted at 25,000 rpm for 2 hrs in a number 30 rotor. After 2 pelleting cycles, the final pellet was dissolved in distilled water and used as a serological antigen.

This procedure has now been modified to include 0.05% sodium azide in the liver homogenate to inhibit proteolytic enzyme action. The complement fixing titer of this preparation after clarification at 10,000 rpm for 10 minutes is 1:16 or 1:32 when tested with human or chimpanzee anti-HB<sub>C</sub>. These serologic antigen preparations are now being used to test sera from previous epidemiologic studies for the presence of anti-HB<sub>C</sub>.

Attempts have been made to purify the HB<sub>C</sub>Ag from the serologic antigen preparation by layering onto a continuous CsCl gradient. The gradient mixture was centrifuged at 75,000g for 16 hours in a Spinco model L ultracentrifuge. Fractions were collected from the bottom of the tube in 1ml amounts and dialyzed against phosphate buffered saline and examined for core antigen by complement fixation. Core antigen was detected at a density of 1.30g/ml but the yield was in an attempt to find a gentle means of purification.

Sucrose gradients (5-25% w/w) centrifuged at 24,000 rpm for 4 hours appears to protect the core antigen but did not provide adequate separation of HB<sub>C</sub>Ag from HB<sub>S</sub>Ag or contaminating liver proteins. To separate the large amount of contaminating liver proteins that interfere with both isopycnic and rate zonal attempts to purify the hepatitis B core antigen, the liver homogenate is being passed through a G-200 sephadex column. These studies are currently in progress. The ultimate goal of purification of HB<sub>C</sub>Ag is to permit development of a sensitive assay for HB<sub>C</sub>Ag and anti-HB<sub>C</sub>Ag which maximally conserves this precious antigen.

Work is currently under way to develop a microtiter solid-phase radioimmunoassay for hepatitis B core antigen and antibody.

Plasma units from several chronic hepatitis B carriers have been identified as containing core antibody. Briefly the antibody is precipitated by ammonium sulfate and partially purified on G-200 Sephadex chromatography. The IgG is then concentrated to its original volume and used to coat the microtiter plates. A second aliquot is labeled with radioactive iodine; 500 uCi of  $I^{125}$  is used to label 1-10ug of protein by the chloramine-T method at room temperature for 15 seconds. The labelled antibody is separated from the reaction mixture on a Sephadex column. This assay when perfected will be used to test for HB<sub>C</sub>Ag and anti-HB<sub>C</sub>.

#### C. Immune complexes in the blood of patients with Hepatitis B infection

The pathogenesis of hepatitis and of extrahepatic syndromes due to Hepatitis B virus is incompletely understood. The simultaneous occurrence of antigens and antibodies to hepatitis B virus in serum of some patients suggests a possible immunopathogenetic role of immune complexes. In the past year, in collaboration with Dr. Argyrios Theofilopoulos of the Scripps Clinic and Research Foundation, La Jolla, California, a coded series of serum samples from hepatitis B cases and asymptomatic carriers have been analyzed by means of his newly developed assay, for the detection of immune complexes employing the Raji cell line. Patients and carriers were derived from surveys conducted at Ft. Hood, Texas. Results from this serum panel are recorded in Table 8. Immune complexes were found mainly in hepatitis patients with HB<sub>S</sub>Ag. In acute hepatitis B patients with HB<sub>S</sub>Ag initially detected in their serum 12/17 had immune complexes during acute illness. Approximately 3 months later however when HB<sub>S</sub>Ag was no longer detectable by radioimmune assay (RIA) 7/9 individuals still had circulating immune complexes. Of interest was the much lower prevalence of immune complexes in asymptomatic HB<sub>S</sub>Ag carriers (10%).

Individuals who had acute hepatitis B infection as confirmed by epidemiological association with antigen positive cases, and the presence of anti-HB<sub>C</sub> but who lacked demonstrable HB<sub>S</sub>Ag by RIA, also had evidence of immune complex formation in 9/15 acute phase sera tested.

The preliminary studies suggests that hepatitis B infection resulting in hepatitis is associated with circulating immune complexes whether or not HB<sub>S</sub>Ag is detected in serum. In contrast, only a small portion of individuals with chronic antigenemia had circulating immune complexes. Most surprising was the persistence of circulating immune complexes after hepatitis; nine of 12 patients with immune complexes in acute sera had immune complexes in convalescent sera obtained 3 to 4 months later. This would suggest that hepatitis antigen(s)

Table 8 - Immune Complexes and Hepatitis B Infection

Case#	HB <sub>s</sub> Ag+		HB <sub>s</sub> Ag-				Asymptomatic HB <sub>s</sub> Ag Carriers		
	Acute Serum	Case#	Convaless** cent	Case#	Acute Serum	Case#	Convaless** cert	Case#	Serum
69	24*	A83	84	46	15	A103	Neg	A-7	Neg
452	100	A88	102	499	Neg	A108	8	A-8	Neg
480	51	A94	Neg	130	Neg	A109	Neg	A-12	Neg
172	80	A102	114	66	42	A110	24	A-13	Neg
145	24	A106	20	179	12	A114	50	A-16	Neg
60	27	A107	6	2311	Neg	- CNA -		A-22	50
44	50	A113	45	2316	Neg	- CNA -		A- 31	Neg
155	65	A115	50	2318	200	- CNA -		A-34	Neg
491	Neg	A124	Neg	2324	24	- CNA -		A-42	Neg
2205	7	- CNA -		2328	6	- CNA -		A-52	Neg
2206	Neg	- CNA -		2330	Neg	- CNA -		A-58	Neg
2209	Neg	- CNA -		2333	Neg	- CNA -		A-72	Neg
2214	7.5	- CNA -		2334	8	- CNA -		A-78	Neg
2223	Neg	- CNA -		2335	6	- CNA -		A-80	Neg
2228	50	- CNA -		2336	5	- CNA -		A-81	Neg
2231	3	- CNA -						A-89	Neg
2242	Neg	- CNA -						A-91	24

CNA - Convalescent not available

\*mgm of immune complexes

\*\* Convalescent sera obtained 3-4 months after clinical disease

continue to be produced or persists for long periods after infection in the face of circulating antibody.

D. The clinical consequence of hepatitis B infection in relationship to histocompatibility antigens.

The reason for the observed host response to hepatitis B infection varying from asymptomatic HB<sub>s</sub>Ag carrier state to acute infection and chronic aggressive hepatitis in different individuals is unknown but likely related to variables in the immune response. A number of diseases such as ankylosing spondylitis and ulcerative colitis have been associated with a high frequency of specific histocompatibility antigens (HLA) types (Hart, 1973). In mice, the gene controlling HLA-type has been demonstrated to be close to the locus concerned with the immune response to hapten-protein conjugates. (McDevitt, 1972)

At present a survey is being conducted amongst asymptomatic HB<sub>s</sub>Ag carriers, acute hepatitis B cases, and negative controls in order to identify the role of any of HLA types as a marker of types of disease.

Approximately 30 individuals in each group have been identified and adequate preparations of leukocytes obtained for tissue typing (Tissue typing has been done in collaboration with COL Everett Spees of the Transplant Service, Walter Reed Army Hospital Center). Because of the effect of race on HLA gene frequency and our lack of patients, and carriers, insufficient numbers are present for conclusive statistical significance. However it is contemplated based on statistical prediction that about 20 more individuals will have to be obtained in the carrier and acute hepatitis groups to provide sufficient data to complete the study. Hopefully this goal will be achieved in the next year.

E. Basic studies on a virus isolated during an institutional outbreak of Hepatitis A

A virus labeled ELNI, #108, P<sub>4</sub> and P<sub>5</sub> was received from Dr. Larker of Electronucleonics, Bethesda, Maryland. This virus had been isolated from a stool culture of a child during an institutional outbreak of hepatitis A by Mr. Frank Mundon of Electronucleonics laboratory and was forwarded for characterization. The virus was received in infected Wi-38 tissue culture. Initial tissue culture studies indicated the virus would grow in a wide variety of common cultured cell types e.g. HEK, Hela, Wi-38 and HR-6.

As shown in table 9, the virus was chloroform resistant, resistant to pH3.0 and infectivity was stable at 50°C for 1 hour with MgCl<sub>2</sub>. These findings suggest that preliminary the strain is an enterovirus. These studies were hindered by the relatively low virus titers achieved.

Table 9 - Virus titer (TCID<sub>50</sub>/0.2ml)

Test	ELNI 108		POLIO 2		HERPES SIMPLEX	
	Control	Treated	Control	Treated	Control	Treated
Chloroform Sensitivity	10 <sup>4</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>5</sup>	10 <sup>3</sup>	0
pH 3.0	10 <sup>3</sup>	10 <sup>4</sup>	not tested		not tested	
50°C w/o MgCl <sub>2</sub>	10 <sup>3</sup>	0	not tested		not tested	
50°C w MgCl <sub>2</sub>	10 <sup>3</sup>	10 <sup>4</sup>	not tested		not tested	

To determine whether the low titer represented a discrepancy between titer measured by cytopathic effect or by interference, Polio 2 was used to challenge Wi-38 cells previously infected with ELNI #108 which did not manifest CPE. No viral interference effect was observed.

Acute and convalescent sera from 4 patients from Fort Riley, Kansas considered to have had hepatitis A were used for a tube neutralization test. None of the sera tested had neutralizing activity against the unknown virus. In addition convalescent serum from Joliet volunteer Miles, infected with MS-1, failed to neutralize the virus in plaque reduction neutralization tests.

Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY  
Task 00 Communicable Disease and Immunology  
Work Unit 176 Mechanisms of Transmission of Hepatitis Viruses  
Literature Cited.

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**PROJECT 3A161102B71R**  
**RESEARCH IN BIOMEDICAL SCIENCES**

**Task 02**  
**Internal Medicine**

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OA 6451	75 07 01	DD-DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. DESIG. INSTR <sup>a</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES <sup>a</sup>		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY		61102A	3A161102B71R	02		085	
B. CONTRIBUTING							
C. <del>NON-PERFORMING</del>		CARDS 114F					
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Circulatory Responses to Disease and Injury							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
012900 Physiology 002400 Biengineering 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
63 08		CONT		DA 1		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				B. FISCAL YEAR		C. FUNDS (in thousands)	
B. NUMBER <sup>a</sup>				CURRENT			
C. TYPE				75		7	
D. KIND OF AWARD:				76		8	
E. CUM. AMT.						435	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursue BEAR if U.S. Academic Institution)			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Khouri, E.M.			
				NAME: Elliot, Dr. E.C. DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Blood; (U) Coronary vessels; (U) Myocardium; (U) Oxygen							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pursue individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. (U) Research is devoted to studies of the hemodynamic and biochemical controls of the heart and circulation particularly during stresses of military importance, such as exercise and shock.</p> <p>24. (U) This program integrates studies of subcellular metabolism with physiological studies in anesthetized and also conscious trained animals. While standard preparations are used, new ones are developed to meet specific program requirements.</p> <p>25. (U) 74 07 - 75 06 Long-term dietary depletion of potassium does not impair skeletal muscle carbohydrate metabolism or the oxygen cost of physical work. The adverse effects of potassium depletion with deoxycorticosterone appear to be due to the steroid itself, which may cause a myopathy. Cardiac 5'-nucleotidase is an ectoenzyme whose in vivo activity is substrate-limited. Adenosine exerts its coronary effects by binding to a specific receptor on the surface of the vascular smooth muscle cell. Coronary occlusion causes a rise in the oxygen tension of the blood draining from the ischemic area. There are at least 2 protein kinases involved in the stimulation of cardiac calcium ATPase by cyclic AMP. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.</p>							

PII Redacted

DD FORM 1498  
1 MAR 68

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Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 085 Circulatory responses to disease and injury

Investigators.

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Description.

The department is responsible for the development and application of standardized biological preparations to long-term biophysical and biochemical studies of the controls of the circulation in the normal state and under the influences of physiological and pathological stresses.

Progress and Results.

The department's work in FY 75 continues and/or completes projects begun in earlier years, and is focused on the circulatory response to physiological stresses and disease. Animal studies have defined the impact of the potassium deficiency which occurs in soldiers during basic training in hot climates in terms of their ability to exercise and on the activity of skeletal muscle glycolytic enzymes. The examination of the response to coronary constriction and occlusion has been extended to studies of cardiac oxygen metabolism. Work on the adenosine hypothesis of coronary flow regulation consisted of the development of methods for purifying 5'-nucleotidase, the enzyme mediating adenosine production in the heart, chemical studies of its carbohydrate composition, and animal studies of its orientation in the cardiac cell membrane and regulation in the beating heart. Adenosine and theophylline were covalently linked to oligosaccharides and polypeptides to produce soluble derivatives unable to penetrate cell membranes, and these compounds were used to demonstrate the existence of an adenosine receptor on the surface of the coronary vascular smooth muscle cell. Work in the role of cyclic AMP in regulating the activity of the  $Ca^{++}$ -sensitive ATPase of the cardiac sarcoplasmic reticulum, a major determinant of cardiac contractility, showed that an endogenous cAMP-dependent protein kinase stimulates ATPase activity by phosphorylating a subunit of the ATPase which has a molecular weight of 20,000 daltons.

## 1. Effect of Potassium Depletion on Exercise Capability.

The studies of the effect of dietary potassium depletion on the oxygen cost of exercise which were briefly mentioned in the FY 74 annual report are completed and data analysis is underway. This investigation is an outgrowth of the observation of Knochel et. al. (1) That soldiers undergoing basic training in a hot climate may have a reduction in body exchangeable potassium of up to 20 per cent. Although none of the subjects in that study experienced any ill effects, the possibility remained that potassium depletion might impair exercise performance. Subsequently, Knochel examined this question in dogs depleted of potassium by the administration of a high-salt diet and deoxycorticosterone acetate (DOCA) which is a standard laboratory technique for producing potassium depletion. He found severe impairment of performance (2) and overt destruction of skeletal muscle with exercise (3). Because these results are so different from the apparent lack of effect in humans, we decided to investigate this problem further.

Five dogs trained to run on a treadmill were fitted with aortic electromagnetic flowmeters and blood sampling catheters in the aorta and main pulmonary artery. These dogs had previously been splenectomized in order to ameliorate the marked changes in hematocrit which occur during heavy exercise in this species. During convalescence from surgery the animals were fed an artificial diet containing vitamins and a salt mixture including 60 mEq sodium 40 mEq potassium per day, and were given deionized water to drink. They were exercised 7 days/week for 20 minutes on a treadmill at 9km/h and 11% grade.

Cardiac output and aortic blood pressure recorded continuously before and during exercise and samples of aortic and pulmonary artery blood were obtained prior to exercise and at 2 and 18 min after the beginning of exercise. Samples of semitendinosus muscle were obtained under local anesthesia and sterile precautions daily (4-5 days) during the control period and at weekly intervals thereafter. The oxygen cost of performing this exercise was calculated at the product of total cardiac output during the exercise period times the average whole body arteriovenous oxygen difference. Further, pre- and post-exercise plasma samples were assayed for creatine phosphokinase activity,  $K^+$ ,  $Na^+$  and renin. Each dog showed a training effect, i.e. a decrease in the oxygen cost of the work to a stable level with 7-10 days of beginning the exercise program. When 3-5 studies showed a stable oxygen consumption, the diet was changed by the deletion of potassium from the diet and the exercise and skeletal muscle biopsy schedule was continued for 2 to 10 weeks.

None of the dogs had any change in heart rate cardiac output, blood pressure or oxygen consumption responses to exercise despite decreases

in muscle potassium which ultimately fell by 20 - 30 percent. None had evidence of rhabdomyolysis or hypokalemic paralysis. Because these results were negative, one dog was repleted by adding potassium to his diet and when muscle potassium levels were again normal 2 weeks later he was placed on a diet containing an electrolyte mixture which included 205 mEq of sodium/day, no potassium, and was given mg DOCA/day by intramuscular injection. After 10 days, this animal's heart rate and blood pressure responses to exercise were 178% and 165% greater, respectively, than during exercise with potassium depletion alone. Unfortunately, the electromagnetic flowmeter in this dog failed after 12 weeks of implantation, so that cardiac output and oxygen consumption data could not be obtained. Blood plasma after exercise was pink and had markedly increased levels of creatine phosphokinase, indicating rhabdomyolysis.

Since these results suggested that the hemodynamic and chemical effects observed by others might be due to DOCA rather than potassium deficiency per se, studies of the effect of the two methods of inducing potassium deficiency were done in 8 additional dogs. These dogs were not exercised. One group of 4 dogs ate a potassium deficient diet for 78-215 days. One dog developed generalized weakness and died after 78 days. Muscle potassium content was decreased 35%, serum  $K^+$  was 2.1 mEq/L, and ECG was consistent with hypokalemia. Although the other dogs had hypokalemia, ECG evidence of hypokalemia and decreases in muscle potassium to a comparable degree (35%) they never exhibited paralysis. Muscle glycogen phosphorylase activity was unchanged for at least 4 months and varied inconsistently after then.

During high-sodium plus DOCA administration the "re-fed, re-depleted" dog described above and 4 additional dogs fed 60 mEq of sodium/day and given DOCA all developed paralysis and died after 18-35 days. Muscle potassium content in these dogs was decreased an average of 47 per cent at the time of death. All had chemical and ECG evidence of hypokalemia, and serum  $CO_2$  was increased dramatically in contrast to a slight fall in the group depleted by diet alone. Glycogen phosphorylase was decreased by an average of 45% ( $p < 0.01$ ), but this impairment did not always correlate with muscle weakness. Electromyograms were normal despite a preliminary pathological report of selective atrophy of white (i.e. fast contracting, glycolysis-dependent) muscle fibers. Analysis of muscle samples for 3 other key glycolytic enzymes, hexokinase, phosphofructokinase and pyruvate kinase, the latter two of which are regulated by potassium, has not been completed.

This study largely confirms the results obtained by other investigators who employ a regimen of high-sodium diet plus DOCA to produce potassium deficiency, but strongly suggests that the changes which

are observed are an artefact due to the steroid rather than potassium deficiency per se. Potassium deficiency of itself does not appear to increase the energy cost of performing physical work. This study in dogs, though not entirely applicable to man, does not suggest a need for change in Army basic combat training schedules.

## 2. Hemodynamic Responses to Coronary Insufficiency.

During this reporting period work on coronary collaterals was aimed at identifying the factor responsible for initiating collateral development. It is now clear that at least in the dog, the collateral circulation to an ischemic area of myocardium develops via the enlargement of pre-existing small (100-200  $\mu$ ) interarterial communications. The stimulus to this enlargement could be either hypoxic dilatation of these vessels or the pressure gradient between their "normal" and "ischemic" ends which is generated by coronary obstruction. Progressive stepwise coronary constriction leading to complete occlusion over a period of 24-48 h was performed in 7 conscious instrumented dogs 10-37 days postoperative while measurements were made of intercoronary pressure gradient, peripheral coronary pressure in the ischemic region, and the  $pO_2$  of venous blood draining from both normal myocardium and the ischemic bed. In each animal coronary constriction produced an interarterial pressure gradient before any changes in venous  $pO_2$  were observed. Upon complete occlusion the  $pO_2$  of the effluent of both the normal and the ischemic beds fell transiently. In 4 dogs the  $pO_2$  of venous blood from the ischemic region began to rise 24-48 h later in parallel with a rise in peripheral coronary pressure. These dogs had large myocardial infarcts at autopsy. One dog developed a fatal ventricular arrhythmia within 12 hours of coronary occlusion: peripheral coronary pressure and venous  $pO_2$  were unchanged, and infarction was found at autopsy. Two dogs showed no difference in venous  $pO_2$  between the normal and ischemic regions, had unusually rapid increases in peripheral coronary pressure, and did not have myocardial infarction. These tentative results do not support the hypothesis that the low  $pO_2$  of an ischemic region is the primary stimulus for collateral growth. Further studies will be needed to clarify the relationship of an interarterial pressure gradient to collateral development.

The beta radiation detector developed during FY 74 for the study of coronary flow distribution has been implanted on the epicardium of a dog and functioned well for 60 days. The sensitivity (or efficiency) was marginal, however, so a search was initiated for ultra-thin stainless steel shim material for use as a window in the encapsulating process. A foil only 0.15 mil thick was obtained from England, but this material was not totally free of pin holes; 0.5 mil thick steel was finally obtained and yielded an improved detector which had a

10-to-15-fold increase in sensitivity.

A new experimental preparation has been developed, with beta radiation detectors attached to both the epicardial and endocardial surfaces of the heart, without having to use cardiac bypass. A 3-pronged jig is used to insert 3 ligatures into the left ventricle, from a carefully delineated area of the epicardium, between the last left coronary ventricular branch and the interventricular branch, at about 1/4 of the distance from the apex to the base of the heart. A modified small hemostat is used to bring the 3 ligatures out of the ventricular cavity, via a stab wound at the apex. These ligatures are then attached to a beta radiation detector and pulled until the detector seats firmly against the endocardium. These same 3 ligatures are used to attach a second detector on the epicardium. This approach places the endocardial detector between the posterior papillary muscle and the septum, an area of relatively smooth surface, generally free of trabeculae carnae. Experiments on the transmural distribution of coronary flow using this preparation have begun.

### 3. Cardiac Metabolism and the Metabolic Regulation of Coronary Blood Flow.

The adenosine hypothesis proposes that adenosine is continuously released from myocardial cells in proportion to their need for oxygen. This nucleoside is thought to adjust oxygen delivery to demand by its powerful vasodilating effect on coronary arteries. A detailed understanding of the mechanisms underlying the coupling of coronary flow to cardiac oxygen requirements is important not only for interpreting the normal physiological responses of this vascular bed but also because the normal regulatory mechanisms appear to be lost in disease states such as certain bacterial toxemias and in hemorrhagic shock. Our research efforts during this reporting period have focused on how the catalytic activity of 5'-nucleotidase, the enzyme which produced adenosine, is regulated in the beating heart and also on how adenosine caused the coronary arteries to relax.

As indicated in the FY 74 Annual Report, cardiac 5'-nucleotidase is glycoprotein. Further studies with a wider variety of glycosidases and lectins have confirmed our observations indicating that the activity of this enzyme is absolutely dependent on the integrity of the oligosaccharide moiety, and show that this carbohydrate chain consists of sialic acid,  $\beta$ -galactose,  $\beta$ -N-acetylglucosamine,  $\alpha$ -N-acetylglucosamine, and probably  $\alpha$ -mannose residues. One such study is summarized in Table 1, and shows the remarkable dependence of the activity of this enzyme on amino sugars. Because these studies were carried out on cardiac microsomes, which probably contain a variety of glycoproteins other than 5'-nucleotidase, the

Table 1 Effects of Glycosidases and Lectins on Dog Heart Microsomal 5'-Nucleotidase

	Preliminary digestion with NA									
	<u>No enzyme</u>	<u>Lyz</u>	<u>NacGlc</u>	<u>NacGal</u>	<u>Gal</u>	<u>NA only</u>	<u>Lyz</u>	<u>NacGlc</u>	<u>NacGal</u>	<u>Gal</u>
No Lectin	100	37	25	0	0	42	24	7	8	7
Con A, 400 mg/ml	0	10	18	0	0	40	15	10	0	10
WGA, 15 mg/ml	49	25	0	0	0	50	27	23	0	9
SBA, 1.2 mg/m.	119	26	5	8	4	47	27	0	4	27
LCH, 3.6 mg/ml	28	5	6	8	12	4	6	5	8	0

Abbreviations: Con A, concanavalin A; WGA, wheat germ agglutinin; SBA, soybean agglutinin; LCH, lentil lectin; Lyz, Lysozyme; NacGlc,  $\beta$ -N-acetylhexosaminidase (mostly N-acetyl glucosaminidase); NacGal,  $\alpha$ -N-galactosaminidase; Gal,  $\beta$ -D-galactosidase, and NA, neuraminidase. Data are expressed as percentage of residual activity in (mg/min per mg protein) of untreated cardiac microsomes which in this experiment was 69.5  $\mu$ mol/min per mg protein at an AMP concentration of 69  $\mu$ M.

results cannot give more than a general picture of the structure of the oligosaccharide. Accordingly, we are developing methods for preparing the enzyme in highly purified form by means of affinity chromatography. The purification scheme exploits our earlier studies showing that lectins bind to 5'-nucleotidase and also that it is competitively inhibited in a pH-dependent fashion by the nucleoside phosphonate AOPCP, and calls for sequential chromatography on columns containing *Lens culinaris* lectin (LCH) and AOPCP as ligands. We chose LCH because it is inexpensive to prepare and has a relatively low affinity for the carbohydrate of 5'-nucleotidase, thus facilitating elution of the enzyme. Preparation of the lectin by the standard Howard and Sage method followed by additional purification on Sephadex G-150 yielded 620 mg of protein suitable for use as a ligand. Preliminary experiments (Figure 1) verify that it is possible to effect a single-step 4-fold purification of 5'-nucleotidase on Sepharose-LCH. The second purification step calls for separation

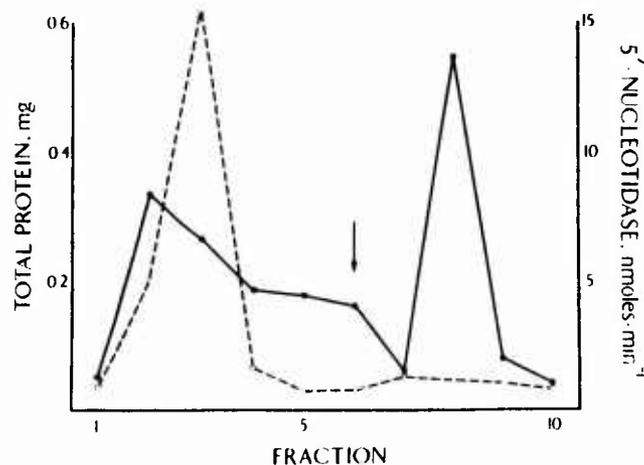


Figure 1: Affinity chromatography of deoxycholate-solubilized dog heart 5'-nucleotidase on Sepharose-LCH. A sample containing 1.2 mg protein was applied to the column, which was then eluted with 1% Na deoxycholate. At the arrow the eluant was changed to 2%  $\alpha$ -methylmannoside in 1% deoxycholate. Fraction

volume 5 ml. Protein content 0---0, 5'-nucleotidase activity 0---0.

of 5'-nucleotidase from other glycoproteins by chromatography on Sepharose-AOPCP. Our studies show that 5'-nucleotidase inhibition by AOPCP assumes a progressively more non-competitive character as pH is lowered below 7, but is a competitive inhibitor, and therefore suitable as a ligand for affinity chromatography, at  $\text{pH} \geq 7.5$ . A batch of Sepharose AOPCP was prepared and used in preliminary experiments with the 5'-nucleotidase eluted from the Sepharose-LCH column. These experiments failed because of the extreme pH- and temperature-dependent viscosity of solutions of deoxycholate, the detergent used to solubilize the enzyme. Other ancillary work has shown that Triton X-100 is a satisfactory detergent for this purpose, and have validated new methods for protein assay which circumvent the interference caused by sugars and detergents in standard assays.

The fact that adenosine production rates in beating hearts are inexplicably lower than would be predicted from the properties and total activity of the enzyme which be isolated from these hearts may be a clue to how its activity is regulated in vivo. There is unequivocal histochemical evidence that this enzyme is located in the plasma membrane (sarcolemma) of the cardiac cell, and, because it is a glycoprotein at least the oligosaccharide portion of the molecule is situated on the external surface of the membrane, raising the possibility that the catalytic site, too, is oriented to the exterior. Such enzymes are designated ectoenzymes. We tested this possibility by measuring the rate of hydrolysis of  $^{14}\text{C}$ -AMP infused intracoronary in anesthetized, open-thorax dogs. Additional studies of the penetration of AMP from coronary plasma into the cardiac interstitial space were carried out with  $^{14}\text{C}$ -adenosine-5'-phosphorothioate (AMPS), a poorly metabolized analog of AMP, which was synthesized by a published method. The corollary studies with AMPS showed that during intracoronary infusions of this nucleotide there was a plasma-interstitial fluid gradient of 60-100:1. Calculations based on this gradient and the kinetic properties by the uninhibited enzyme in vitro completely accounted for the rates of AMP hydrolysis observed in beating hearts (Figure 2), establishing that 5'-nucleotidase is an ectoenzyme and that the low rates of adenosine production observed previously are due to limited substrate availability. This is consistent with our earlier proposal that adenosine production may be regulated by a small, perhaps intra-membranal pool of 5'-AMP. Experiments to test this hypothesis further are planned for the coming year.

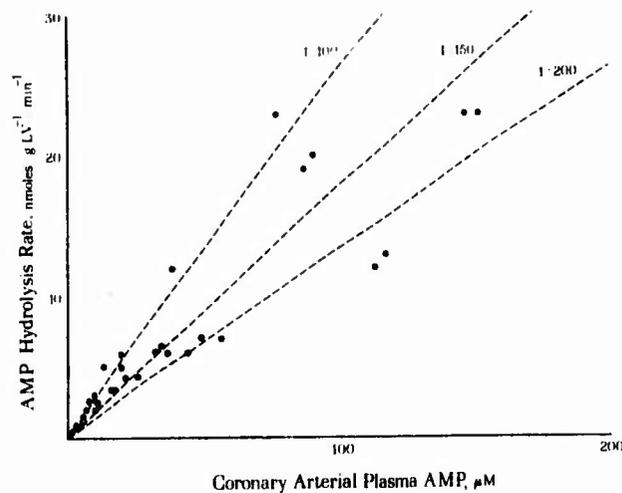


Figure 2: Hydrolysis of AMP infused intracoronary by beating dog hearts. The observed rate of hydrolysis is plotted as a function of the concentration of the nucleotide in coronary plasma water. The dashed lines are the rates of hydrolysis predicted from the total activity of 5'-nucleotidase in dog heart (447 nmole/g LV per minute) and  $K_m$  of the enzyme in cardiac microsomes (16  $\mu\text{M}$ ) assuming plasma: interstitial concentration gradients of 1:100, 1:150 and 1:200 and no inhibition of the enzyme.

The second line of work aimed at testing the adenosine hypothesis examined the mechanism by which adenosine causes coronary relaxation. The studies of the structure-activity relationships of purine nucleosides which were reported in the FY 74 Annual Report have been extended and, in collaboration with Dr. Friederick Hahn of the Department of Molecular Biology, will be used for a detailed theoretical analysis of the determinants of adenosine's vasoactivity by the "linear free energy" approach.

Vasoactive adenosine derivatives are congeneric by structural relatedness. They produce parallel log dosage-activity curves which are displaced relative to the log concentration axis. Hence, they also act in the manner of congeneric compounds which bind to one receptor with different affinities. Although the nature of the

adenosine receptor is incompletely understood, the adoption of the receptor hypothesis affords a direct comparison of activities of tested compounds and the derivation of structure-activity relationships.

Nearly one-third of all adenosine derivatives which have been considered carried different substituents at C<sub>2</sub>. They exhibit a more than 500-fold activity range and among them are halogen derivatives which are more active than adenosine itself. We are not aware of any C<sub>2</sub>-substituted adenosine derivatives which are entirely non-active.

The electronic Hammett constant,  $\sigma$ , is a relative measure of the electronegativity of substituents. A plot of the logarithms of per cent molar vasodilatory activity as a function of  $\sigma$  for a set of C<sub>2</sub>-substituted adenosines yielded a fairly satisfactory one-parameter regression and suggests that activity is a function of the relative electronegativity of substituents in position C<sub>2</sub>. Such substitutions decrease the electron density of the adjacent nitrogen atom N<sub>1</sub>. This correlation is important for the consideration of one speculation that adenosine derivatives inhibit the action of the enzyme adenosine deaminase, and, hence, act indirectly by protecting endogenous adenosine against enzymatic inactivation.

A plot of known affinity constants of C<sub>2</sub>-substituted adenosines for adenosine deaminase as a function of  $\sigma$ , showed that the affinity decreased with increasing electronegativity of the C<sub>2</sub>-substituents. Hence, the most active vasodilators had the least affinity for adenosine deaminase. Evidently, protection of endogenous adenosine from deaminase attack is not a mode of action of these derivatives and activity may be a direct consequence of receptor binding of intrinsically active adenosine congeners which mimic their natural prototype.

The amino group in position C<sub>6</sub> is important for activity. Replacing it by H, Cl or SH decreases activity to 2 per cent or less of that of adenosine. We are unable to correlate this activity difference with electronic or hydrophobic parameters and assume that the effect is steric in nature. It is conceivable that the amino group at C<sub>6</sub> is necessary for receptor binding by hydrogen bond formation. An alternate possibility would be binding to the receptor by electrostatic attraction, subject to steric hindrance by substituents as small as one methyl residue which has a van der Waals radius of 2.0 Å.

There exists an absolute requirement of the imidazole ring for vasodilatory activity of adenosine. Cytidin from which the imidazole moiety is missing is non-active. Replacement of CH in position 8 by N, yielding 8-azaadenosine, reduces activity to less

than one per cent. 8-azaadenosine is also deficient as an adenosine analog in other respects: it is not biosynthetically incorporated into RNA in lieu of adenosine. Two compounds in which either N<sub>7</sub> or N<sub>9</sub> are replaced by CH are the antibiotics tubercidin or formycin. Whereas they can be incorporated into RNA in cell-free systems and are, at least in this respect, competent adenosine analogs, their vasodilatory activities are less than one per cent of that of adenosine. While substitutions of CH for N in the imidazole moiety are isosteric, they dramatically lower the pK of the 5-membered ring; it is reasonable to assume that the low activity of these derivatives is related to their greater acidity.

The adenine and ribose moieties of adenosine rotate relative to each other around the N<sub>9</sub>-C<sub>1'</sub> bond. The resulting syn and anti conformations are in an equilibrium which favors the anti conformation. Substitution with Br at C<sub>8</sub>, adjacent to the N<sub>9</sub>-C<sub>1'</sub> bond, hinders sterically the rotation and constrains 8-bromoadenosine in the syn conformation. 8-Bromoadenosine as well as 8-thioadenosine is non-active. From this, we conclude that the anti-conformation is essential for vasodilatory action. Adenosine has a specific preference for the anti-conformation. Guanosine, in contrast, favors the syn-conformation and has less than one per cent of the vasodilatory activity of adenosine. The difference in the conformations preferred by the two nucleosides may be one factor contributory to the activity difference between adenosine and guanosine.

A most rigorous requirement for vasodilatory activity resides in the steric configuration of ribose. Arabinosyl, xylosyl and lyxosyl adenines are non-active. It is evident that there is no latitude for epimerizations at the pentose carbons 2' and 3'. This points to steric hindrance of receptor binding. In contrast, deletion of OH at position 3' in the antibiotic cordycepin which is 3'-deoxyadenosine, retains 11 per cent activity, while deletion of CH at position C<sub>2'</sub>, yielding 2'-deoxyadenosine, retains one per cent activity.

Steric hindrance may also be involved in the decline in activity which result from an extension of ribose at position C<sub>1'</sub> by CH<sub>2</sub>OH; the resulting ketohexosyl adenine is the antibiotic psicofuranine, which has less than one per cent activity.

Other parts of the ribofuranosyl moiety show greater latitude with respect to permissible molecular modifications. Substituting the furanose bridge oxygen by CH<sub>2</sub> yields the so-called "carbocyclic adenosine" which has 23 per cent activity. Substitutions at the ribose carbon 5' have been among the first molecular modifications to be investigated. Replacement of OH by Cl or methylsulfoxide

gives rise to compounds with activities similar to that of adenosine itself. Only when larger residues are introduced into the sulfoxide group such as isopropyl, benzyl or phenyl does the activity decrease. Evidently, there is limited bulk tolerance at C<sub>5'</sub> but the OH group at this position is not essential.

We are currently carrying out studies of the phase distribution of various nucleosides between aqueous and hydrophobic solvents in support of this theoretical analysis, have obtained a suitable computer program for the multiple regression analysis this study requires, and have either synthesized or obtained additional nucleosides as gifts from other investigators in order to critically evaluate certain predictions made from our existing intuitive analysis.

The well known coronary effects of adenosine, which are competitively antagonized by methylxanthines have been dealt with heuristically by pharmacologists in terms of an "adenosine receptor." While such a receptor is conceptually convenient, its existence lacks support. Indeed, the rapid uptake of purine nucleosides by a variety of cells admits an equally plausible alternative, that these nucleosides penetrate into the coronary smooth muscle cells where they (or the products of their metabolism) somehow modify metabolic processes critical to the maintenance of contractile tension. As the first step in gathering evidence for a coronary "adenosine receptor" we covalently linked adenosine to molecules whose size made it unlikely that the products, having MW > 1000 daltons, could penetrate cell membranes. Guided by the structure-activity rules discussed above, we reacted adenosine-5'-carboxaldehyde with polylysine nonapeptide and then reduced the resulting enamine with borohydride. After purification by repeated gel filtration on Sephadex G-10 the product, which contained an average of 2 adenosine residues per molecule, was infused into the coronary artery of a dog, whereupon it produced dose-dependent coronary vasodilation which was antagonized by the systemic administration of aminophylline. The expense of the starting materials and the number of synthetic steps involved led us to develop alternative compounds. Because 6-substituted ribosides are also active, we chose this position for linkage. Nucleophilic displacement of the chlorine of 6-chloropurine riboside by a bifunctional alkylamine seemed to be a promising route and, indeed, the reaction of this nucleoside with an excess of 3, 3'-iminobis (propylamine) proceeded smoothly in quantitative yield. Unreacted amine was removed by fractional crystallization of the hydrochloride salt from ethanol. This compound was reacted with the tetraaldehyde produced by controlled periodate oxidation of the galactosyl residues of the non-reducing tetra-saccharide stachyose ("digalactosyl sucrose"), and the resulting enamine was reduced with borohydride (Figure 3).

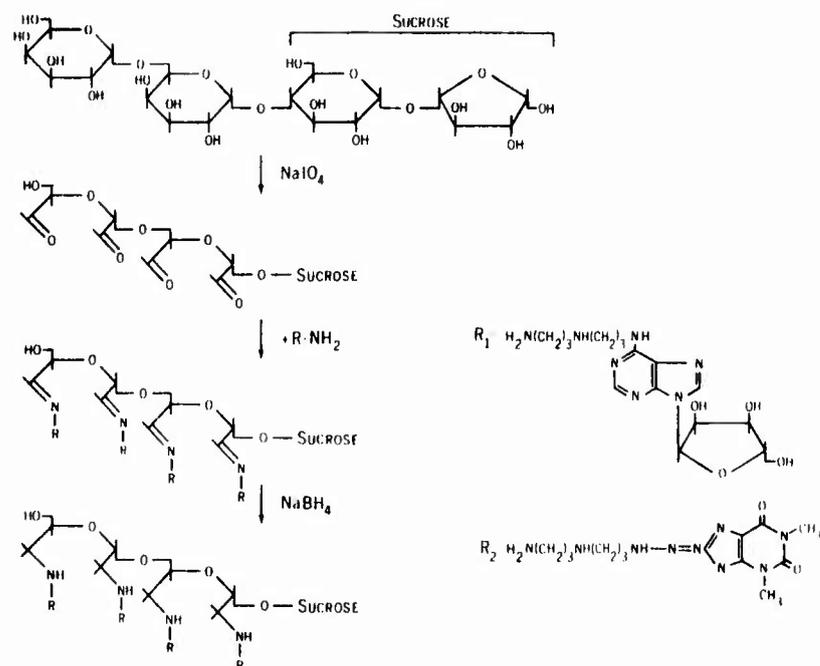


Figure 3: Synthesis of adenosine and theophylline derivatives of stachyose. It is evident from this diagram that stachyose may be considered to be "digalactosyl sucrose". The two galactose moieties, by virtue of their 3,4- cis-diol structure, can be selectively oxidized by periodate to yield a tetra-aldehyde. At pH 9.5 the aldehyde groups will react with primary amines, and the resulting enamine can be converted to a  $-\text{CH}_2\text{NH}-$  bond by borohydride reduction.  $\text{R}_1$  and  $\text{R}_2$  are the adenosine and theophylline derivatives, respectively, which have been coupled to oxidized stachyose.

This compound, like polylysine-adenosine, was vasoactive when infused intracoronary (Figure 4), and this activity was antagonized by aminophylline. In a parallel experiment 8-diazotheophylline was reacted with the product of the reaction of iminobis (propylamine) and periodate-oxidized stachyose, giving a product whose UV spectrum is consistent with the formation of a triazeno linkage between theophylline and the alkylamine chain. Intracoronary infusions of this compound antagonized the coronary vasodilatory effect of adenosine. These results suggest that adenosine initiates its coronary effects by interacting with a specific receptor on the

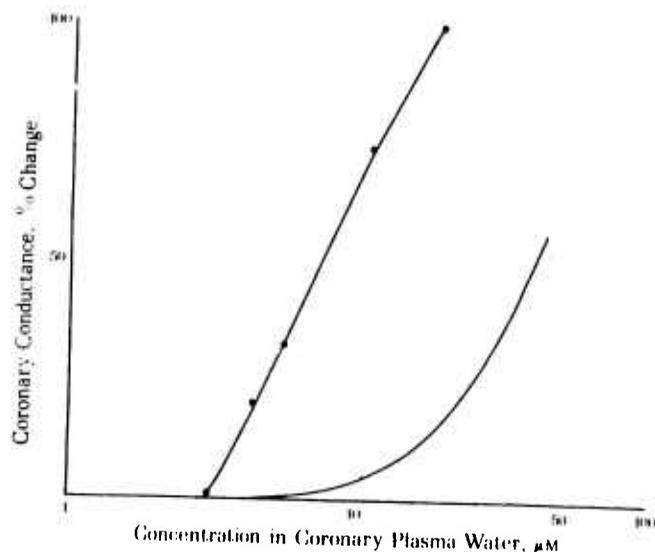


Figure 4: Comparison of the coronary vasodilatory effects of intracoronary infusions of adenosine (●---●) and adenosine covalently linked to periodate-oxidized stachyose (○---○) showing that the dose response curves are parallel. Data on methylxanthine inhibition are not shown.

surface of the coronary smooth muscle cell, and that this interaction is antagonized by methylxanthines, thus fulfilling some of the criteria for the existence of a specific receptor. At this time nothing is known of the chemical nature of the putative adenosine receptor or of the kind(s) of metabolic alterations which are initiated when adenosine binds to it. In order to have larger amounts of a more stable theophylline compound for continuing the animal studies, a supply of 8-(2-aminoethylamino) theophylline was recently synthesized in collaboration with Dr. Daniel Klayman, Division of Medicinal Chemistry, and coupled to oxidized stachyose. Purification and characterization of the product is in progress.

Cardiac sarcoplasmic reticulum contains endogenous cAMP-stimulated protein kinase activity which catalyzes the phosphorylation of several proteins in this membrane, including  $Ca^{++}$ -stimulated ATPase.

We have previously shown that the activity of this ATPase is stimulated by cAMP, and this year examined the hypothesis that this stimulation was due to phosphorylation of the ATPase itself. Discontinuous gel electrophoresis of phosphorylated membranes in polyacrylamide containing sodium dodecyl sulfate revealed several phosphoproteins. The amount of one which had a molecular weight of 20,000 daltons varied directly with the activity of  $\text{Ca}^{++}$ -ATPase as the latter was varied by manipulating  $\text{Ca}^{++}$  concentration over a range of 1-100  $\mu\text{M}$ , suggesting that this protein may be the ATPase or at least one of its subunits. These studies also revealed that there is a second protein kinase in this membrane. The previously described kinase is stimulated by cAMP, has an apparent  $K_m$  for ATP of 20-30  $\mu\text{M}$  and is partially inhibited by  $\text{Ca}^{++}$  concentrations as low as 10-100  $\mu\text{M}$ . The second protein kinase has a  $K_m$  for ATP of 120-140  $\mu\text{M}$  and is stimulated by  $\text{Ca}^{++}$  concentrations in the range of 10-100  $\mu\text{M}$ . The  $\text{Ca}^{++}$ -stimulated protein kinase has a  $V_{\text{max}}$  about twice that of the "low  $K_m$ " kinase, and appears to account for most of the membrane phosphorylation which occurs under conditions required to demonstrate cAMP stimulation of  $\text{Ca}^{++}$ -ATPase. It thus appears that the cAMP stimulation of  $\text{Ca}^{++}$ -ATPase is initiated by the phosphorylation of a  $\text{Ca}^{++}$ -stimulated protein kinase by a cAMP-stimulated protein kinase followed by phosphorylation of the ATPase by the  $\text{Ca}^{++}$ -stimulated kinase. This sequence is similar to the cAMP-mediated activation of phosphorylase b kinase.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 085 Circulatory responses to disease and injury

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL DD FORM (AR) 1498	
				DA OA 6450	75 07 01		
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8. ORG'S INSTR <sup>f</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
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b. CONTRIBUTING							
c. <del>XXXXXX</del>		CARDS 114F					
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Military Hematology							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
008800 Life Support 002600 Biology 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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e. KIND OF AWARD:				f. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered.				ASSOCIATE INVESTIGATORS			
				NAME: Ahr, MAJ D. J.			
				NAME:			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Coagulation; (U) Malaria; (U) Blood; (U) Blood Transfusion; (U) Anemia							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pursuit individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Studies of the pathophysiology of diseases with hematologic manifestations in soldiers and the investigation of those that occur as a direct military problem.							
24. (U) Studies of hematologic abnormalities produced by chemicals, drugs, and infectious agents encountered primarily in military populations and in natives of geographic areas of potential military operations. Studies of blood, blood products, and blood substitutes used for the treatment of casualties and the prevention and diagnosis of diseases in soldiers.							
25. (U) 74 07 - 75 06 Using plant lectins as structural probes, the surfaces of normal human T, B, and null lymphocytes, leukemic lymphocytes, lymphocytes transformed by plant mitogens, and isolated guinea pig intestinal cell brush borders were studied. Chemotactic factor-induced changes in human granulocyte surface charge were determined. Red cell metabolism of vitamin B6 was defined in subjects receiving prophylactic isoniazid, patients with sideroblastic anemia, and animals rendered B6-deficient by diet. Alterations in glycogen metabolism in the muscles of potassium-depleted dogs were studied. Alterations in intracellular metabolism and membrane biophysics were studied in erythrocytes from rhesus monkeys during the course of a synchronous infection with P. knowlesi. The hypercoagulable state was examined in a group of patients with long bone fractures and in another group with severe vascular disease. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.							

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DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 086 Military hematology

Investigators:

Principal: Michael J. Haut

Associate: David J. Ahr, Jeffrey L. Berenberg, Edgar C. Boedeker, David H. Boldt, John A. Kark, Robert G. Knodell, Stephen F. Speckart, Michael S. Steinberg, Harold L. Williams.

Description: Basic and clinical studies were performed to investigate the functions of blood and blood-forming organs. These focused on three specific areas: (1) examination of blood cell surface membrane composition and function in normal and disease states, (2) study of normal blood cell metabolism and its alteration by disease or by toxic agents, and (3) coagulation.

Progress:

1. Blood cell surface membranes in normal and disease states

A major effort of our department during the past year has been the use of plant lectins as structural probes of cell surfaces. Lectins are proteins from plants which are capable of binding specifically to unique cell surface glycoprotein receptor sites. Since each lectin has a different specificity, one can use a group of lectins with differing specificities to probe cell surfaces for the presence of these unique receptors. Furthermore, one can calculate number of lectin receptors per cell as well as affinity constants for lectin binding. Using these lectins, we have focused on four problems: (1) characterization of the surfaces of normal human T, B, and null lymphocytes; (2) examination of the surface changes of lymphocytes in chronic lymphocytic leukemia; (3) analysis of surface changes in lymphocytes transformed by plant mitogens; and (4) probing of the structural features of isolated guinea pig intestinal cell brush borders. The first two projects were done in collaboration with Dr. Richard MacDermott, Department of Gastroenterology; the fourth was done in collaboration with Dr. Edgar Boedeker, now with Department of Gastroenterology.

Using six purified plant lectins as probes of cell surface structure, human T, B, and null lymphocytes have been examined for cell surface differences. The results of binding experiments with

radioiodinated lectins indicate that these three lymphocyte populations--although functionally different--bind equivalent amounts of E and L phytohemagglutinin (PHA), lentil PHA, concanavalin A (Con-A), ricinus communis agglutinin (RCA-I), and wheat germ agglutinin (WGA). Since these lectins are capable of binding to at least four different cell surface oligosaccharides, our data indicate that human T, B, and null lymphocytes do not differ with respect to surface content of these carbohydrate structures. In addition, all six lectins were investigated for their ability to stimulate DNA synthesis in these human lymphocyte populations. Four of these lectins (E- and L-PHA, lentil PHA, and Con-A) were mitogenic for human lymphocytes, while RCA-I and WGA failed to stimulate significant DNA synthesis in any lymphocyte population. When incubated with the mitogenic lectins, both B and null cells displayed a delayed peak of DNA synthesis in comparison to T cells. Thus, despite virtually identical binding characteristics, the lectin-induced mitogenic response exhibited by B and null cells was markedly different kinetically from that of T cells.

Lymphocytes from patients with chronic lymphatic leukemia (CLL) carry B cell markers. To characterize the relationship between CLL cells and normal B lymphocytes, we examined binding of five purified plant lectins to normal human lymphocyte populations (mixed T and B cells, purified T, and purified B cells) and compared results with lectin binding to unseparated CLL lymphocytes. Normal T and B cells were separated on an immunoadsorbent column. Binding of  $^{125}\text{I}$ -lectins showed equal numbers of surface receptors for a given lectin on each normal lymphocyte population: purified B, T, and mixed T + B cells. Compared to normal human lymphocyte populations, CLL cells had significantly fewer receptors for E-PHA, WGA and Con-A and significantly more receptors for L-PHA. Binding of RCA-I was essentially unchanged. We conclude: (1) normal human T and B lymphocytes do not differ in receptor sites for five lectins; and (2) compared to normal B lymphocytes, CLL lymphocytes are characterized by extensive and consistent cell surface alterations detected by lectins.

Lectin binding was used to investigate surface changes in lymphocytes transformed by plant mitogens. Lymphocytes transformed with either PHA or Con-A bore increased receptor sites for six different lectins. The magnitude of this increase varied considerably among the various lectins, indicating selective replication of specific surface structures during the course of blast transformation. Studies using various specific inhibitors indicate that these surface changes do not depend on DNA, RNA, or new protein synthesis, nor do they appear to require an intact microtubular system. This information suggests that the process of blast transformation involves unfolding of the cell membrane with subsequent exposure of previously "cryptic" glycoprotein structures.

Since the ileal receptor for the intrinsic factor-vitamin B12 complex (IF-B12) is thought to be a glycoprotein, but documentation of the binding site structure is lacking, lectin binding was used to probe the structural features of brush border membranes (BBM) prepared from guinea pig small intestines. The interaction of a series of lectins with such BBM was studied first; then the effect of these lectins on IF-B12 interaction with BBM was studied. In the initial studies, the purified lectins WGA, RCA, and E-PHA were labeled with  $^{125}\text{I}$ . Saturable binding of WGA, RCA and E-PHA to proximal and distal BBM was observed. For distal BBM,  $K_a$ 's in the range  $1.5-1.9 \times 10^6 \text{ M}^{-1}$  with  $1.6-9.4 \times 10^{14}$  binding sites/mg BBM protein were observed for the three lectins. For IF-B12 studies, human gastric juice was mixed with  $^{57}\text{Co}$ -labeled vitamin B12, and saturable binding of the resulting IF-B12 complex to distal BBM, but not proximal BB, was demonstrated ( $K_a$   $0.5-1.0 \times 10^{10} \text{ M}^{-1}$ ;  $0.8-1.2 \times 10^{11}$  binding sites/mg BBM protein). When distal BBM were preincubated with E-PHA, competitive inhibition of subsequent IF-B12 binding occurred ( $K_i$   $1-5 \times 10^{-6} \text{ M}$ ). The same inhibition occurred even after excess lectin was washed from the mixture before IF-B12 incubation, indicating an effect of membrane-bound lectin, rather than an interaction between lectin and IF-B12. Further, when IF-B12 and distal BBM were incubated with 5 mg/ml of fetuin (a glycoprotein bearing oligosaccharides analogous to the known erythrocyte membrane receptor sites for E-PHA), IF-B12 binding was again competitively inhibited ( $K_i$   $3.5 \times 10^{-4} \text{ M}$ ). In contrast to the E-PHA results, WGA, RCA and Concanavalin A caused increased binding of IF-B12 to proximal and distal BBM. This new binding is mediated by interaction between membrane-bound lectin and IF-B12, since gel filtration experiments demonstrate lectin binding to IF-B12. Thus, our data indicate that the ileal IF-B12 receptor shares determinants with the known receptor sites for E-PHA.

Our studies on the mechanism of rosette formation between human T-lymphocytes and sheep erythrocytes continued. Previous work in this area (FY 74 report) demonstrated that rosette formation is mediated by glycoprotein receptors located at the surface of the sheep erythrocyte membrane. These receptors can be released in crude form by tryptic digestion or by extraction of the sheep erythrocyte membrane with lithium diiodosalicylate. The latter procedure releases three or four glycoprotein components from the sheep cell membrane and indicates that the receptor for rosette formation resides on a major membrane glycoprotein structure. A number of model oligosaccharide compounds were used as haptene inhibitors of rosette formation to derive structural information about the nature of this receptor site. Data from these studies indicate that compounds with the basic oligosaccharide structure: sialic acid galactose N-acetylglucosamine linked to a mannose core are effective inhibitors of rosette formation. Stepwise degradation of fetuin glycopeptide (the most effective of the inhibitory compounds tested) indicates that the galactose residues are the most important determinants of inhibition.

Further studies have focused on the ability of model compounds bearing the above structure to bind to human lymphocytes. Fetuin glycopeptide was treated with mild periodate oxidation followed by reduction using sodium borotritide to obtain a compound with a tritium label in the sialic acid residues. This compound was then used in binding studies with normal human lymphocytes. Results demonstrate  $7-8 \times 10^6$  saturable binding sites for this compound per lymphocyte. The compound does not bind to sheep erythrocyte membranes nor to guinea pig spleen lymphocytes (which do not form rosettes).

Interaction of leukocyte chemotactic factors with the granulocyte surface membrane was studied in collaboration with Drs. J. Gallin and A. P. Kaplan of the National Institutes of Health. The negative surface charge of human granulocytes was diminished after incubation with the chemotactic factors C5a, dialyzable transfer factor, and the enzymes kallikrein and plasminogen activator. No such change was observed after incubation with human IgG, albumin, horseradish peroxidase, or a mixture of prekallikrein and plasminogen proactivator. Hydrocortisone inhibited the effect of C5a upon granulocyte surface charge and inhibited its chemotactic activity, suggesting that steroids act at the cell surface. The chemotactic inhibitors colchicine and cytochalsin B had no effect upon granulocyte surface charge, consistent with their presumed effect upon microtubules and microfilaments, respectively. The data suggest that the decrease in cell surface charge may be a prerequisite for normal cell movement.

The role of membrane sialic acid in erythrocyte survival was studied by treating red cells with neuraminidase which specifically cleaves the glycosidic linkages between sialic acid and mucopolysaccharides. Reduction in sialic acid correlated with decreases in electrophoretic mobility and loss of PAS staining of membrane glycoproteins on polyacrylamide gels. No changes in ATP levels or deformability were found.  $^{51}\text{Cr}$  erythrocyte survivals in rats and rabbits showed rapid clearance of desialylated erythrocytes with sequestration by the liver. These results suggest that reduction in erythrocyte sialic acid is a mechanism of erythrocyte destruction and may be important in erythrocyte senescence.

## 2. Metabolism of normal blood cells and its alteration by disease or by toxic agents

The sideroblastic anemias are a group of disorders in which iron is not incorporated into heme, but instead accumulates within the mitochondria of bone marrow erythroid precursors. On bone marrow smears stained for iron, these iron-laden mitochondria surround the nuclei of erythroblasts, forming a pathologic entity known as a "ring sideroblast". This group of disorders is thought to be related to vitamin B6 metabolism because ring sideroblasts can be produced by

dietary B6 depletion in animals and because a significant fraction of patients with sideroblastic anemia are partially cured by pharmacologic doses of pyridoxine.

Vitamin B6 metabolism and its relationship to sideroblastic anemia was selected as an area of investigation because (1) very little work has been done on the metabolism of this vitamin in blood cells, in contrast to other vitamins such as folate or B12; (2) sideroblastic anemia often accompanies other hematologic disorders (myeloma, leukemia) and is a frequent manifestation of drug toxicity (ethanol, isoniazid); and (3) inhibition of metabolism of this vitamin may be a useful tool in the chemotherapy of diseases of military importance in a manner analogous to the use of antifolate drugs in malaria.

Patients taking isoniazid (INH) often have low plasma levels of pyridoxal-5-phosphate (PLP) and sideroblastic bone marrow morphology. Snell *et al* demonstrated that INH inhibits the activity of pyridoxal kinase (PL kinase) *in vitro* but were unable to demonstrate inhibition of the enzyme *in vivo* in INH-dosed rats. To determine the effect of INH on PLP metabolism by human red cells, blood was drawn from eight otherwise healthy people on prophylactic INH therapy (300 mg/d) and six controls). Activities of both the synthetic (PL kinase, pyridoxine phosphate oxidase) and degradative (B6 phosphatase) enzymes involved in B6 metabolism were measured using intact red cells, hemolysates, and washed membranes. Plasma INH and PLP levels were determined. The plasma PLP of the patient group was lower than that of the normals.

The three patients with the lowest PLP's had markedly diminished PL kinase activity. A fourth patient with a normal PLP level had a low PL kinase. All patients with normal PL kinase activity had normal plasma PLP. The activities of pyridoxine phosphate oxidase and B6-phosphatase were identical in red cells of patients and controls. A significant group of patients taking INH have decreased red cell PL kinase activity. Our data suggest that these are the same patients who subsequently cannot maintain normal plasma PLP. The inhibitory effect of INH on red cell PL kinase may be the mechanism by which isoniazid causes sideroblastic changes in the bone marrow.

To determine if non drug-related sideroblastic anemia also was caused by a decreased capacity for net synthesis of PLP, eight patients with idiopathic refractory sideroblastic anemia (IRSA), three patients with preleukemia-associated sideroblastic anemia, and eight controls were studied. Synthesis of PLP from pyridoxal (PL) or pyridoxine (PN) and degradation of PLP were measured in intact red cells at optimum substrate concentrations. In red cells of all SA patients, synthesis from PL was increased, as was synthesis from PN. These

changes were independent of B6 therapy or of plasma PLP, but were less pronounced in four transfused patients. B6-phosphatase was normal in all patients. Those patients not taking B6 (8/11) had low plasma PLP levels. Thus all patients had major abnormalities of B6 metabolism intrinsic to their own red cells. In contrast to patients with drug-induced SA, those with IRSA or preleukemia-associated SA showed increased capacity for PLP synthesis by their red cells.

In an attempt to elucidate the relationship between altered vitamin B6 metabolism and the development of sideroblastic anemia, nutritional vitamin B6 deprivation was studied in six young adult beagle dogs and six 2- or 3-year-old rhesus monkeys. The objectives of this study were to define the changes in (1) morphology of peripheral blood, bone marrow, and liver cells particularly in regard to iron deposition; (2) plasma, blood cell, and tissue levels of pyridoxal-5-phosphate (PLP), the active coenzyme form of B6; (3) activity of erythrocyte and liver enzymes involved in B6 metabolism; and (4) activity of some enzymes involved in heme synthesis in liver and bone marrow. Young adult animals were used to avoid the neurologic lesions seen in B6-deprived young animals; none of our 12 animals exhibited neurologic deficits. The onset of anemia was much later than described in the many previous studies of infant animals. In the dogs, moderate degrees of anemia appeared at 7 months, and the animals became severely anemic at 8 to 9 months. The monkeys developed moderate anemia only at 10 months; several monkeys developed granulocytopenia and died of infection. The anemia developing in both groups of animals showed classic features of B6-deficiency on both light and electron microscopy. Plasma PLP levels decreased to one-third control levels within the first 2 months. Thereafter they decreased more slowly to one-fifth control values at 10 months. The animals have just developed their severe anemia, and samples are being obtained for measurement of tissue B6 levels and for assay of enzymes involved in B6 metabolism and heme synthesis. Appropriate controls have already been obtained for (1) pyridoxal kinase, pyridoxine phosphate oxidase, and B6 phosphatase, and for (2) ALA synthetase, ALA dehydratase, and ferrochelatase.

Collaborative studies have continued with MAJ Patterson, Dr. Lowensohn, and COL Olsson (Dept Cardiorespiratory Diseases, Div Medicine) and MAJ Montgomery (Div Experimental Pathology) on the biochemical basis for muscle weakness in potassium deficiency. Eight dogs have been studied: four were depleted of potassium by diet alone and four were depleted with DOCA. The rationale for this study and the results are thoroughly summarized by Dr. Fatterson in his report (work unit 085).

Collaborative studies have been initiated with Dr. B. P. Doctor of Division of Biochemistry. The ultimate goals of these studies are (1) to develop the capability of the two units working together to investigate cell-free protein synthesis in blood cells, and (2) to analyze the effect of specific drugs and toxins of importance to military hematology on the protein synthetic mechanism. During the past year, this work primarily has been concerned with areas of interest to Dr. Doctor, so that Major Steinberg can develop some expertise in this area. Since this work is summarized extensively in Dr. Doctor's annual report (work unit 075), it will be treated only briefly here. Ribosomes were purified from rat brain, liver, and gut by using deoxycholate solubilization of the membrane-bound particles and high-speed centrifugation. A high-speed supernatant fraction was also prepared from rat brain. In the presence of the purified ribosomes, a 35%-70% ammonium sulfate fraction of the supernatant, ATP, GTP, and purified phenylalanine specific t RNA, polyadenylic acid was capable of directing the synthesis of labeled polyphenylalanine from a tritiated monomeric precursor. Studies of the effect of dilantin on this system indicated that dilantin did not alter the tritiated phenylalanine incorporation, or the activation of amino-acyl t RNA.

A collaborative effort has been initiated between Major Steinberg, Dr. Doctor, and LTC Diggs (from DCD&I) to study the effects of anti-malarial drugs on in vitro polyadenylic acid-directed protein synthesis by a cell-free system isolated from malaria-infected erythrocytes. This study will focus on three areas: (1) A number of drugs will be examined for activity against this system, and the mode of action of promising ones will be further investigated. (2) Drug-sensitive and -resistant strains of parasite will be examined for differences in drug sensitivity of the protein-synthesizing system, and for ribosomal structure and integrity. (3) Freshly drawn infected cells and frozen stabilates will be compared.

Alterations in erythrocyte metabolism and membrane biophysics in erythrocytes from monkeys infected with P. knowlesi were studied during the course of a synchronous infection. This study is being done in collaboration with CPT K. Webster (Div Biochemistry), MAJ L. Martin (Dept Medical Zoology, DCD&I), LTC P. Hildebrandt (Exper Path), and Dr. J. Durocher (Pa. Hospital). Young adult male rhesus monkeys are placed in a restraining chair and allowed to chair-adapt for 2 weeks. A subclavian indwelling catheter is inserted and kept patent by flushing daily with a small amount of heparinized saline. After blood has been drawn for baseline studies, the monkeys are inoculated with infected erythrocytes from a frozen stabilate. Daily samples are obtained for study until the first parasites appear, usually at about 4 days. At that time, blood samples are obtained from the monkey at 3- or 4-hour intervals for 2-3 cycles. To date, eight animals have been studied. Five of these were

infected and the infection was allowed to run its course. One was successfully treated with chloroquine after he had developed parasitemia. One was treated with chloroquine and served as a drug control. One was phlebotomized and served as an anemic control.

Studies performed on samples obtained from these animals included (1) assays of red cell enzymes, particularly the kinases; (2) measurement of the mono-, di-, and trinucleotides by high-pressure liquid chromatography; (3) measurement of red cell filterability and surface charge; and (4) assays of selected glycolytic intermediates. So far, our most complete data are for the enzymes. All animals showed an increase in activity of pyruvate kinase and hexokinase following infection. In some animals, glucose-6-phosphate dehydrogenase and phosphofructokinase were elevated as well. The most prominent elevation in all instances was the pyruvate kinase, which usually exceeded twice control values. Since these animals had no reticulocytosis, the enzyme is most likely that of the parasite. We are doing electrophoretic studies to prove that this is the case. The increase in enzyme activity occurred before parasitemia could be detected on the peripheral blood smear. This suggests that the parasite synthesizes these enzymes even before it can be detected microscopically.

### 3. Coagulation

Investigations in the coagulation laboratory were concentrated in three areas: (1) the hypercoagulable state, (2) studies on factor VIII and its role in von Willebrand's disease, and (3) clotting abnormalities in animals with parasitic infection.

Hypercoagulability is a serious complication of a number of disorders which might be encountered in a military situation. Patients with trauma, burns, or exposure to extreme environments (heatstroke, cold exposure, hyperbaric pressure) are particularly prone to develop this complication. The underlying cause of the hypercoagulable state is currently being investigated in patients with trauma, patients with severe vascular disease (in collaboration with the WRAMC vascular surgery department), and patients on birth control pills (in collaboration with the WRAMC Ob-Gyn department). Investigation of these patients includes thorough studies of platelet function and coagulation factors, as well as more specialized tests such as antithrombin-III.

From a military standpoint, the most highly relevant group is the trauma one. Coagulation studies and arterial blood gases were performed in five patients with long bone fractures who developed evidence of the fat emboli syndrome. Pertinent results from the acute phase included a markedly shortened PTT, elevated fibrinogen,

thrombocytopenia, and diminished pO<sub>2</sub>. All patients had pulmonary infiltrates and two had mental confusion. Heparin therapy was instituted in 4/5 patients and evidence of persistent hypercoagulability necessitated prolonged therapy in 3/4. Though the mechanism of activation of the coagulation system is not yet worked out for this condition, the data suggest that all patients with long bone fractures be evaluated for hypercoagulability and the institution of heparin therapy.

The following laboratory tests have been evaluated to determine their usefulness in conforming a clinical impression of hypercoagulability: hematocrit, prothrombin time, activated partial thromboplastin time, platelet count, platelet adhesivity, fibrinogen, antithrombin III, thrombin time, platelet factor III availability, platelet aggregation, factors II, V, VII, VIII, IX, XI, XII, fibrin degradation products, and fibrin monomer. The technical approach thus far has been quite simple in that a diagnosis of hypercoagulability is entered by an experienced observer and the laboratory tests being currently evaluated are then performed in an attempt to see if laboratory support for the clinical diagnosis can be obtained. This preliminary step has been necessary prior to initiating work on the protocol design in order to see which tests are actually useful.

We have evaluated 33 patients with severe vascular disease for hypercoagulability by the previously mentioned tests. Of these 33, 21 (64%) had two or more laboratory abnormalities. Another 7 (21%) had one abnormality only and 5 (15%) had no laboratory abnormalities. Of the five with no laboratory abnormalities, only two had a high clinical index of suspicion of hypercoagulability. We do not consider the laboratory tests confirmatory unless two or more are abnormal except when the single abnormality is platelet hyperaggregability. Based on these studies, the laboratory tests confirmed (two or more tests abnormal) the clinical impression of hypercoagulability in 73% of cases and supported it in another 12% of cases. Additionally, the clinical impression of no evidence of hypercoagulability was confirmed in another two patients (6%) so that 91% of patients were accurately screened. Thus, in this part of the study, we have established firm criteria for the laboratory diagnosis of hypercoagulability.

Transfusion studies in patients with VWD given normal plasma or cryoprecipitate have demonstrated dissociation of antihemophilic factor procoagulant activity (VIII<sub>AHF</sub>) from antihemophilic factor antigen (VIII<sub>AGN</sub>). We have examined this relationship in patients with VWD following the infusion of epinephrine, a stimulus which is free of the complicating presence of exogenous plasma proteins. VIII<sub>AHF</sub>, VIII<sub>AGN</sub> (determined by radioimmunoassay using a rabbit antibody to human factor VIII) and bleeding times were determined at

intervals following the intravenous injection of epinephrine (0.003 mg/kg) over a 30-minute period. The patients developed transient tachycardia and hypertension with a narrowed pulse pressure. In a severely affected patient there was minimal change in VIII<sub>AGN</sub> while VIII<sub>AHF</sub> increased tenfold. Although VIII<sub>AGN</sub> rose in a second patient with mild VWD, VIII<sub>AHF</sub> increased disproportionately. The bleeding time was not shortened in either patient. These studies indicate that patients with VWD are capable of responding to epinephrine with the release or activation of factor VIII<sub>AHF</sub>. The lower response of VIII<sub>AGN</sub> indicates that the concentration of the multifunctional complex does not parallel the VIII<sub>AHF</sub> increase, however, and is consistent with the failure of epinephrine to change the bleeding times. These studies suggest preferential release of the low molecular weight (LMW) protein in stimulated patients with VWD and may correspond to the in vitro observations that LMW VIII<sub>AHF</sub> activity can be dissociated from VIII<sub>AGN</sub>.

A collaborative study is currently underway with Medical Zoology on the alternate complement pathway and disseminated intravascular clotting (DIC) in Aotus monkeys infected with Plasmodium falciparum. Eight Aotus monkeys were bled on days 7 and 14 prior to infection of four animals with  $1 \times 10^8$  P. falciparum (camp) parasitized erythrocytes and on days 3, 6, 7, and 10 after infection. DIC was demonstrated in all four infected animals and contributed to the death of two animals. This study is still in progress, and the coagulation data have not been correlated with the studies on the alternate complement pathway. A new group of 12 animals will be studied in September.

#### 4. Other

a. In addition to his work on infectious hepatitis, MAJ Knodell pursued several studies in hepatic physiology and pharmacology.

The effects of chlorpromazine on aspects of bilirubin  $T_m$  and patterns of output, production, and conjugation were studied in male Walter Reed rats with external biliary fistulae. Acute CPZ treatment (25 mg/kg IV) produced hemolysis and resulted in increased bilirubin output and stimulation of hepatic heme synthesis. Both unconjugated bilirubin and a gamma azopigment found primarily in obstructed rat bile were seen. Acute infusion of hemoglobin solutions to simulate the hemolysis produced by acute CPZ treatment resulted in increased bilirubin output and increased hepatic heme synthesis, but no alterations in the normal conjugation pattern of bilirubin in bile were seen. Chronic CPZ treatment for 4 and 8 days (25 mg/kg IP) increased bilirubin output without elevating plasma hemoglobin levels and increased the fraction of monoconjugated bilirubin in bile; the 8-day chronic treatment regimen also caused unconjugated bilirubin to appear in bile. No change in liver weight, bile salt, phospholipid,

or cholesterol output was seen with chronic CPZ therapy. Changes in bilirubin production and conjugation caused by CPZ may be a contributing factor in this agent's hepatotoxicity.

In collaboration with MAJ Kinsey and MAJ Boedeker (Dept Gastroenterology, Div Medicine), deoxycholic acid metabolism was examined in five patients with alcoholic cirrhosis and two normal subjects. This project is discussed in detail in the Gastroenterology Dept's report (work unit 130).

The effect of increasing bile flow on biliary elimination of pentobarbital was studied in rats with biliary fistulae. A significant increase in pentobarbital excretion was seen with bile salt perfusion, despite the fact that rats have high basal bile-salt independent flow and lack gallbladders that decrease enterohepatic bile salt circulation and reduce bile salt-dependent flow. Greater change with potential therapeutic usefulness might be expected in species with lower basal rates, such as man.

b. Our department's studies on endogenous carbon monoxide production were completed this past year. Heme turnover in man can be accurately measured by quantifying endogenous CO production because the degradation of mole of heme results in the production of 1 mole of CO as well as 1 mole each of iron and bilirubin.

Endogenous carbon monoxide (CO) production rates and total body hemoglobin content were determined simultaneously in blood and gas phase in 15 individuals. At 24% oxygen concentration in the closed rebreathing system, a correlation was obtained which revealed that a 1 umole rise in CO in the gas phase of the system was the result of  $48.94 \pm 5.3$  (SE) umoles of CO rise in the body. Three individuals had heat-damaged red cells reinjected intravenously to determine if the recovery rate of the subsequently produced CO was proportionate in both the gas phase and blood phase. Changes in blood CO concentration were not consistently detected by gas phase analysis. Prediction of CO production rates utilizing gas phase data alone did not reflect accurately CO production rates determined from blood carboxy-hemoglobin rises.

Changes induced in measurements of endogenous carbon monoxide production by blood in the lumen of the gut were studied in five normal volunteers. The study was undertaken because exogenous heme is absorbed by intestinal mucosal cells where the porphyrin ring is split with the release of CO that could contribute to blood CO levels and lead to a fallacious diagnosis of hemolytic disease. Volunteers who consumed 200 ml of their own blood doubled their endogenous production of CO (0.69 versus 0.34 umoles/kg/hr). This suggested that at least 3% of the ingested heme was degraded and recovered as CO within 2½ hours. Measurements of serum bilirubin also showed a

significant increase after ingestion of blood. These data indicate that blood in the gastrointestinal tract can interfere with quantification of heme and bilirubin turnover from measurements of either endogenous CO production or bilirubin and suggest that this might occur with the ingestion of meat.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 086 Military hematology

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Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL DD-DR&E(A) 10 26	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. REGRADING <sup>e</sup>	8. DRG <sup>f</sup> INST <sup>g</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM A WORK UNIT
74 07 01	D. Change	U		NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES <sup>h</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	6110.3A	3A-1102B		088			
b. CONTRIBUTING							
c. OPERATIONAL	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>i</sup>							
(U) Military Nursing Research							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>j</sup>							
003500 Internal Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
58 03		JUN 75		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: NA				FISCAL YEAR		75 3.5 15	
c. TYPE: NA				CURRENT		76 0 0	
d. KIND OF AWARD: NA				e. CUM. AMT			
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buscher, COL. H. L.				NAME: DELL, Margaret L., LTC, ANC			
TELEPHONE: 202-576-3222				TELEPHONE: (202) 576-2191			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: FELTON, Geraldene, LTC, ANC			
				NAME: PETRELLO, Judith M., MAJ, ANC			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Nursing; (U) Clinical Assessment; (U) Patient Teaching; (U) Survey; (U) COMPSY							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Develop rational underlying military nursing and provide a basis for military nursing practitioners to improve the quality of health care services; determine the effectiveness of patient teaching; and use of computers in military psychiatry.							
24. (U) Assessment of methods enhancing sleep, nurse-patient communications, biorhythmicity of heart rate in MI patient, structured versus unstructured preop teaching, nurses' perceptions of satisfying and dissatisfying factors affecting their ability to render quality care, and testing the use of computerized nursing applications.							
25. (U) 74 07-75 02 Data analysis is in progress for assessment of methods enhancing sleep; nurse-patient communications has been completed, data collected and now awaiting completion of time series analysis of pilot study, biorhythmicity of heart rate in MI patient; structured versus unstructured preop teaching and nurses' perceptions of satisfying and dissatisfying factors affecting their ability to render quality care are in progress; computerized nursing applications in psychiatry continue to be used and assessed. Portions of this work have been submitted to Walter Reed Army Medical Center under Work Units 9026, 9027, 9028, 9032, 9033, and 9076. For technical reports see Walter Reed Army Medical Center and Walter Reed Army Institute of Research Annual Progress Reports, 1 July 1974 - 30 June 1975.							
As of 1 February 1975, RDT&E funds were discontinued for the Division of Nursing. The Division now operates totally under OMA funding.							

PII Redacted

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DO FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing Research

Investigators:

Principal: LTC Margaret L. O'Dell, ANC

Associates: LTC Geraldene Felton, ANC

MAJ Waltraut M. Hurd, ANC

MAJ Judith M. Petrello, ANC

Description.

Research in military nursing is concerned with both direct and indirect patient care, and with identifying and testing principles underlying nursing care. The research endeavors being reported consist of the following: continuous heart rate monitoring of myocardial infarction patients; structured versus unstructured pre-operative nursing preparation of patients for surgery; hospitalized adults' understanding of selected terms and abbreviations used by nursing personnel; nurses' perceptions of satisfying and dissatisfying factors affecting their ability to render quality care; and computer support in military psychiatry.

Progress and Results.

1. Continuous Heart Rate Monitoring of Myocardial Infarction Patients.

Within the last two decades, numerous studies have demonstrated the existence of biological rhythmicities in living organisms at all levels of structural organization. Recognition of the importance of the temporal aspects of biological functioning has led to the emergence of a discipline known as biorhythmology or chronobiology. The purpose of this study is to investigate the circadian and ultradian rhythms of heart rate in myocardial infarction patients and to determine if there are detectable factors which alter these rhythms. The pilot study consisting of four subjects has been completed. Data awaits time series analysis. This research is being reported under Work Unit Number 9031.

2. Structured Versus Unstructured Pre-Operative Nursing Preparation of Patients for Surgery.

This study was conducted to determine the outcome of three nursing approaches to the preoperative preparation of the

surgical patient on frequency of postoperative complications, ventilatory function, manifest anxiety level, and patient's perceptions of psychological well being. Sixty-two surgical patients (49 males and 13 females) operated on under general anesthesia were studied from January to December 1974. Subjects were distributed into three groups (experimental, communication, and control) by stratified random assignment holding constant factors of sex, age, and site of surgery. All subjects had measurements of vital capacity (by use of a Vitalor) 24 hours preoperatively and 24, 48, and 72 hours postoperatively. The Personal Orientation Inventory (to measure psychological well being) and the Multiple Adjective Affect Check (to measure anxiety) were administered 24 hours preoperatively and on the 4th and 5th day postoperative. Nursing intervention with each group occurred under the following study conditions. Two different nurses worked with experimental and control subjects. A third nurse assigned patients to groups and collected the data. The experimental group (N=25) was presented with a previously established and administratively approved protocol for teaching the patient before surgery at a time and place established by the nurse. Teaching consisted of demonstration and practice of stir-up activities presented in two cassette films, Prevention of Postoperative Pneumonia and Preventing Postoperative Circulatory Complications, included diaphragmatic breathing, support of incision, deep coughing, foot and leg exercises, turning in bed, getting out of bed. Control group (N=25) had preoperative preparation routinely provided by unit nursing staff. Communications group (N=12) had nurse attention for one hour to provide atmosphere in which the patient could verbalize his feelings and utilize the problem-solving process. A Physician Philosophy Scale to determine physicians' perceptions of how much information surgical patients want and need indicated the 18 physician respondents revealed a philosophy of high informativeness ("A surgical patient should be told everything he will experience"). There were no significant differences among groups on factors of age range, normalcy of preoperative vital capacity, site of operation, length of operation, duration of anesthesia, presence of postoperative complications, or days of postoperative hospitalization. All groups had substantial mean decrease in vital capacity for the first three postoperative days (Experimental: -34, -28, -16; Communications: -35, -30, -24; Control: -35, -21, -12). By the third postoperative day the vital capacity for all groups was still 8-25% below normal. All groups had high percent postoperative complications (fever, productive cough, atelectasis, consolidation: 80%, 92%, and 50%). The experimental group showed increased postoperative psychological well being ( $p \leq .01$ ) (Mann-Whitney U Test) and greater decrease in anxiety. Two out of four hypotheses were supported. Indication

of the quality and effectiveness of nursing service is not easy to identify or measure. It obviously requires ingenuity and familiarity with the service, understanding of expected outcomes and manifestations of health and illness in the population to be studied in order to be able to define measurable health outcomes clearly and appropriately related to effectiveness of nursing care. It is possible not enough emphasis was placed on achieving maximum voluntary sustained inspiration for a long enough period of time and recording the number of times respiratory maneuvers were carried out postoperatively. Building a relationship with the patient, presenting him with information he needs and wants, and assisting him to accept some of the responsibility for his postoperative course would appear to favorably influence client satisfaction, and hence, psychological well being and more marked decrease in anxiety. The experimental approach could be equally valuable with groups of patients and should be combined with close postoperative supervision of the patient's performance of the activities. This research is being reported under Work Unit Number 9032.

3. Hospitalized Adults' Understanding of Selected Terms and Abbreviations Used by Nursing Personnel.

This study was conducted to determine hospitalized adults' understanding of selected health care terms and abbreviations used by nursing personnel in verbal communication with patients. Data were obtained using a 50 item (40 words and 10 abbreviations) open-ended interview schedule requiring 200 randomly selected adult male and female patients from non-intensive medical and surgical wards to define the terms. Data were analyzed by scoring the responses and obtaining percentages for "correct," "partially correct," "incorrect," and "I do not know" categories. The percentages were then related to demographic variables. None of the 200 respondents defined all words or abbreviations correctly. Twenty words and three abbreviations were defined correctly by less than 50% of the respondents. The 40 words and 10 abbreviations were identified by the respondents as having been used by nursing personnel in verbal communication with them. Correct responses increased with (1) increase level of education of respondents, (2) the respondent's number of prior hospitalization, and (3) the respondents placement in the unit (patients in private rooms had more correct responses). The findings from this study demonstrated that hospitalized adults do not understand many of the health care terms and abbreviations being used by the nursing staff in verbal communications. This would indicate that blocks are occurring in the communication process which would have the potential to impede a mutual understanding between nursing personnel and patients. From this realization, nursing personnel must

move towards assessing and improving their communication pattern. This research is being reported under Work Unit Number 9027.

4. Nurses' Perceptions of Satisfying and Dissatisfying Factors Affecting Their Ability to Render Quality Patient Care.

The quality of patient care in many of our health care facilities over the years has decreased as our operating systems do not allow for it. Nurses are carrying out non-nursing functions which prevent them from accomplishing the purpose for their existence - rendering patient care. Consumers are now demanding that changes be initiated. Managers and administrators, in some health care facilities, are cognizant of the problems that exist. They have been and are taking action to make the necessary changes to free the nurses so they can again direct their efforts to providing quality patient care. Others have either not recognized the existing problems or they have refused to admit that problems exist. It must be realized that no single approach is going to bring about a universal solution. Each health care facility has a unique environment and must be analyzed and evaluated on this basis. The purpose of this study is to identify the satisfying and dissatisfying factors as perceived by nurses working in a large medical center which affect their ability to render quality care for their patients and to categorize, analyze, and synthesize the content. A random stratified sampling of 24 registered nurses' perceptions of satisfying and dissatisfying factors affecting their ability to render quality care was obtained employing a nondirective interviewing technique. Data was analyzed by content analysis. Final report is in progress. This research is being reported under Work Unit Number 9033.

5. Computer Support in Military Psychiatry.

The Computer Support in Military Psychiatry (COMPSY) study was a project of the Walter Reed Army Medical Center's Department of Psychiatry and Neurology with full-time nurse support from the Division of Nursing, Walter Reed Army Institute of Research until January, 1975. It is being reported under Project 9076, WRAMC.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing Research

Publications

1. O'Dell, Margaret L. "Vital Signs of Surgical Patients on Routine Admission to the Hospital and Three and Six Hours Post-Admission." Military Medicine, 139: 719-721, September 1974.
2. O'Dell, Margaret L. "Physicians' Perceptions of an Extended Role for the Nurse." Nursing Research, 23: 348-351, July-August 1974.
3. O'Dell, Margaret L. "Are Routine Admission Signs Really Reliable?" RN Magazine, Vol 38, No. 4, pp. 23-24, April 1975.
4. Felton, Geraldene. "Increasing the Quality of Nursing Care by Introducing the Concept of Primary Nursing: A Model Project." Nursing Research, 24:27-32, 1975.
5. Felton, Geraldene. "Body Rhythm Effects of Rotating Work Shifts." Journal of Nursing Administration, 5: 16-19, 1975.
6. Nichols, G.A., Anna Koneck, Eunice Kennedy, Judith Petrello, and Waltraut Hurd. "Patients' Perceptions of Important, Satisfying and Dissatisfying Aspects of Army Hospitalization." Military Medicine, 139:871-878, December 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup>	2. DATE OF SUMMARY <sup>2</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMRY 74 07 01	4. KIND OF SUMMARY D. Change	5. SUMMARY ACTY <sup>3</sup> U	6. WORK SECURITY <sup>4</sup> U	7. REBRADING <sup>5</sup> NA	8A. DRG'S INSTR <sup>6</sup> NL	8B. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
9. NO./CODES <sup>7</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	61102A	3A161102B71R		02	039		
b. CONTRIBUTING							
c. CONTRIBUTING	CARDX 114F						
11. TITLE (Precede with Security Classification Code) <sup>8</sup>							
(U) Pathogenesis of Renal Diseases of Military Importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>9</sup>							
012900 Physiology 003500 Clinical Medicine 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PREVIOUS		b. FUNDS (In thousands)	
b. NUMBER:				FISCAL YEAR		75	
c. TYPE:				CURRENT		11	
d. KIND OF AWARD:				76		10	
e. AMOUNT:				650			
f. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Div of Medicine			
				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2265			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Flamenbaum, MD, Walter			
				NAME: DA			
22. REVISIONS (Precede EACH with Security Classification Code)							
(U) Renal Failure; (U) Renal Hemodynamics; (U) Heat Stress (U) Shock; (U) Fluid and Solute Homeostasis; (U) Dialysis; (U) Kidney Function							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To investigate mechanisms for maintaining fluid, electrolyte and hemodynamic homeostasis in response to disease, injury and environmental stresses of military significance, such as acute renal failure, shock, infectious disease, heat stress, and gastrointestinal disorders in order to provide rational basis for prevention and treatment.							
24. (U) Clearance methods, dialysis, externally monitored isotope methods, isotope dilutions, experimental models, in vivo micropuncture, in vitro renal microperfusion, membrane transport, radioimmunoassay, light and electron microscopy, and chromatographic techniques.							
25. (U) 74 07-75 06 Using uranyl nitrate induced acute renal failure evidence has been obtained interrelating direct effects of uranyl nitrate on tubular epithelium and the characteristic renal hemodynamic and renal functional abnormalities. Heavy metals, both uranyl nitrate and mercuric chloride inhibit active sodium transport in vitro without altering the structural integrity of the urinary epithelium or passive ion movement. Increased distal tubule [Na <sup>+</sup> ] demonstrated by micropuncture and increased juxtaglomerular apparatus renin activity result in decreased glomerular plasma flow and nephron filtration (tubuloglomerular feedback). The direct heavy metal effects on sodium transport in vitro are completely reversed by dithiothreitol, and, in vivo, this agent ameliorates heavy metal induced acute renal failure. Investigations of other renal vasoactive hormones indicate that pyrogen induced renal hyperemia is mediated by a balance of increased renin-angiotensin system and renal prostaglandin activities. Heterogeneity of solvent and solute transport in the proximal tubule has been demonstrated. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 June 75.							

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DD FORM 1498  
1 MAR 68

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PROJECT 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 089 Pathogenesis of renal diseases of military importance

Investigators.

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Description: Studies are directed at investigations of mechanisms for maintaining body fluid, electrolyte and hemodynamic homeostasis or their correction in response to disease, injury and environmental stress of military significance such as acute or chronic renal failure, shock, heat stress, infectious disease and gastrointestinal disorders. The role of adaptive homeostatic mechanisms, including renal and extrarenal mechanisms, whereby body fluid and solute balance is achieved and maintained in the face of stress has been emphasized in order to provide a rational basis for the development of improved methods for prevention and treatment of altered fluid, electrolyte and hemodynamic states and acute and chronic renal failure induced by these stresses.

Progress:

1. Acute Renal Failure:

Previous investigations from this laboratory have resulted in a schema for the pathophysiologic basis for the initiation of acute renal failure (1-6). Based upon these previous studies efforts have been maintained at delineating the role of tubuloglomerular feedback in the initiation phase of uranyl nitrate induced experimental acute renal failure, a nephrotoxic model developed in this laboratory. Figure 1 depicts the schema outlining the role for tubuloglomerular feedback in the pathogenesis of the renal functional abnormalities characteristic of the early, initiation phase of uranyl nitrate induced acute renal failure. As proposed in this schema, uranyl nitrate directly inhibits sodium reabsorption in proximal nephron segments, resulting in an increased distal tubule sodium concentration (or an associated alteration in tubular fluid milieu). This stimulus, via the macula densa segment of the distal nephron, results in increased renin-angiotensin system activity characterized by increased juxta-glomerular apparatus renin activity and increased local generation of angiotensin. As a consequence of these events, there is an increase in arteriolar tone (presumably at the preglomerular level, (3)) and diminution in glomerular plasma flow resulting in decreased glomerular

capillary hydrostatic pressure and single nephron glomerular filtration rate. Although in the intact nephron the diminished single nephron glomerular filtration rate and decreased tubular fluid flow rate would result in increased sodium reabsorption and "turn-off" the feedback, the direct effects of uranyl nitrate on sodium reabsorption by tubular epithelium precludes any significant feedback. Therefore, there is continued depression of renal blood flow and glomerular filtration. For the mechanism of tubuloglomerular feedback to be operative in acute renal failure, it must be demonstrated that the macula densa segment of the distal nephron will respond to different sodium stimuli. Using a four micropipette technique which functionally separates the glomerulus and proximal nephron segments from the macula densa and distal nephron segments, studies were performed to ascertain the effect of high and low sodium concentration on glomerular function early (6 hours) after the administration of uranyl nitrate (7). In control rats, figure 2, perfusion of the macula densa segment with Ringer's solution containing 150 mM sodium resulted in an early proximal tubule fluid flow rate of  $6.73 \pm 0.46$  nl/min/100 g body weight, significantly lower than the value obtained when perfusion was performed with sodium free isotonic mannitol solution. In experimental rats, studied 6 hours after the administration of uranyl nitrate (10 mg/kg body weight) a similar but blunted differential response was observed in high and no sodium perfusions. These results demonstrate that tubuloglomerular feedback is operative in this model of experimental acute renal failure. To further characterize the operation of the tubuloglomerular feedback mechanism in the initiation phase of acute renal failure, determinations of single nephron glomerular filtration rate, distal tubule sodium concentration and both plasma and juxtaglomerular apparatus renin activities were performed in control and experimental rats (9-11). In association with a 30-40% decrease in both total kidney and single nephron glomerular filtration rates there was a twofold increase in tubular sodium concentration, measured using helium glow photometry (fig. 3). In support for the role of the renin-angiotensin system as the mediator of the alteration in glomerular filtration, a twofold increase in plasma renin activity and increases in both superficial and deep juxtaglomerular apparatus renin activity was observed (11,12). The results of these studies firmly suggest that tubuloglomerular feedback, initiated by alterations in sodium reabsorption and mediated by the renin-angiotensin system, is the primary pathophysiologic mechanism responsible for the pathogenesis of the initiation phase of acute renal failure.

b. To further exclude the possibility that other mechanisms participate in the pathogenesis of acute renal failure, additional studies designed to evaluate tubular fluid leak and glomerular structure were performed. If transtubular leak of a normally formed glomerular filtrate is responsible for the observed diminution in glomerular filtration rate, then radioactively tagged inulin microinjected into early superficial proximal convoluted tubules should be

incompletely recovered from the ipsilateral injected kidney and should appear in the urine of the contralateral non-injected kidney. In control rats,  $97.9 \pm 0.9\%$  of injected radioactivity was recovered from the injected side, and  $0.5 \pm 0.1\%$  from the contralateral kidney (7,8). After uranyl nitrate the recoveries,  $97.4 \pm 1.0\%$  and  $0.3 \pm 0.1\%$  from ipsilateral and contralateral kidneys respectively, were not significantly different from control. These results exclude a significant role for transtubular leakage of glomerular filtrate in the pathogenesis of this model of acute renal failure. Furthermore, these results confirm our previous micropuncture studies of uranyl nitrate induced acute renal failure (3) in which single nephron glomerular filtration rate did not decline at site more distant from the glomerulus, including the distal nephron. The structure of the glomerulus was examined using scanning electron microscopy at 6 and 24 hours after the administration of uranyl nitrate (8). At 6 hours, the visceral epithelium and pedicels revealed no abnormalities as compared to control rats (fig. 4A), suggesting that altered glomerular permeability does not play a significant role in the pathogenesis of the initiation phase of this model. In contrast, 24 hours after uranyl nitrate the visceral epithelium of the glomerulus revealed swelling and broadening of the trabeculae and the interdigitating pedicels (fig. 4B). The physiologic consequence of this observation during the later, maintenance phase of this model of acute renal failure has not yet been evaluated.

c. As proposed above, and depicted in figure 1, the first event in activation of the tubuloglomerular feedback mechanism must be a direct effect of uranyl nitrate on sodium transport by tubular epithelium. To study the direct effects of heavy metals on transport function, and avoid any secondary alterations resulting from the almost immediate perturbations in renal hemodynamics or glomerular filtration rate (1), investigations were performed utilizing an in vitro renal epithelial preparation. The urinary bladder of the freshwater turtle, Pseudemys scripta elegans, a mesonephric derivative, was utilized in these studies (13-18). Exposure of the urinary (mucosal) surface of the bladder to 0.1 mM uranyl nitrate resulted in a prompt decrease in active sodium transport demonstrated by a 70% decline in short-circuit current and isotopically labeled sodium flux (fig. 5). Since heavy metals are known to react with sulfhydryl groups the alteration in short-circuit current as a consequence of membrane sulfhydryl-uranyl nitrate complex formation may have occurred. To evaluate this possibility, dithiothreitol, a dithiol sugar capable of complexing heavy metals and regenerating sulfhydryl groups, was added to the mucosal side of the bladder after the addition of uranyl nitrate. As demonstrated in figure 5, dithiothreitol promptly and completely reversed the inhibition of short-circuit current induced by uranyl nitrate. Because of the prompt onset of inhibition of short-circuit current by uranyl nitrate without change in the passive fluxes of sodium or chloride and lack of change in electrical ionic conductance of the membrane as well as the prompt reversibility with

dithiothreitol, it is probable that the inhibition resulted from a decreased sodium entry into the cell from the mucosal surface. To evaluate this proposal amphotericin B, an antibiotic which increases the ionic conductance of the mucosal surface of urinary epithelium, was added after uranyl nitrate inhibition of short-circuit current. As depicted in figure 5, amphotericin B also reversed, transiently, uranyl nitrate inhibition of short-circuit current, demonstrating that this heavy metal alters sodium transport by decreasing sodium entry into the cell without affecting the active site of sodium transport. To demonstrate that this is not a singular effect of uranyl nitrate additional studies were performed with  $\text{HgCl}_2$ , another heavy metal salt which has also been used to produce nephrotoxic acute renal failure. As depicted in figure 6, 10  $\mu\text{M}$   $\text{HgCl}_2$  had similar effects on short-circuit current as did uranyl nitrate. Similarly, this inhibition was reversed with both dithiothreitol and amphotericin B. Thus, heavy metals can directly inhibit sodium transport without disrupting the integrity of a urinary epithelium as a barrier to the passive movements of solute and solvent.

d. In view of the prompt and complete reversal of heavy metal induced alterations in transport functions by dithiothreitol, additional studies were performed in vivo (19-21). In preliminary studies dithiothreitol, 15.4 mg/kg intraperitoneally, was administered at various intervals from -60 to +180 minutes with reference to the time of uranyl nitrate administration. Equal protection, as determined from the level of azotemia at 48 hours after the administration of uranyl nitrate, was observed when dithiothreitol was given between -30 and +30 minutes. In view of this observation, all further studies were performed by administering dithiothreitol 30 minutes after the injection of uranyl nitrate. At this time interval, equal protection as judged by the blood urea nitrogen concentration at 48 hours after uranyl nitrate was obtained with 7.7-30.8 mg/kg intraperitoneally. Dithiothreitol did not alter renal function in sham injected control rats when administered in this dose range, but 308 mg/kg of dithiothreitol was immediately lethal. Determinations of plasma creatinine, creatinine clearance and the fractional sodium excretion in treated and untreated rats are shown in figure 7. In rats not receiving dithiothreitol uranyl nitrate resulted in increased plasma creatinine concentration, decreased creatinine clearance and increased fractional excretion of sodium during both 24 and 48 hours after the induction of acute renal failure. In contrast, rats receiving 15.4 mg/kg of dithiothreitol 30 minutes after the injection of uranyl nitrate had significantly smaller alterations in these parameters. In particular, the marked increase in the fractional excretion of sodium observed in untreated rats after uranyl nitrate did not occur in the group treated with dithiothreitol. Similar results were obtained with dithiothreitol in rats with  $\text{HgCl}_2$  induced acute renal failure, indicating that this protection was not specific for uranyl nitrate but could be obtained with an alternate heavy metal model of acute renal failure. Thus, dithiothreitol both in vivo and in vitro could

reverse the inhibition of transport and acute renal failure induced by heavy metals, lending support to the proposed schema outlined in figure 1 and proposed above.

e. Alterations in proximal phosphate transport have been observed after alterations in sodium transport. Inasmuch as tubular transport defects occur in acute renal failure, and calcium and phosphate homeostasis are disturbed in renal failure, studies were performed to characterize phosphate reabsorption in nephron segments (22-25). The results of these studies, performed in anatomically defined segments of the superficial proximal convoluted tubule of the rabbit kidney, are depicted in figure 8. As compared to control rates of net fluid absorption, both dibutyryl cyclic AMP and either intact bovine parathyroid hormone or the active 1-34 fragment of parathyroid hormone resulted in marked and significant decreases in net fluid absorption in segments of the early proximal superficial convoluted tubule. In contrast, no changes were noted with these agents in either late proximal convoluted tubule segments or in pars recta segments of the superficial convoluted tubule. These results suggest there is a heterogeneous response to both dibutyryl cyclic AMP and parathyroid hormone along the length of the proximal convoluted tubule. They further suggest that the elevations in parathyroid hormone activity observed in renal failure may also contribute to the decreased proximal tubular fluid and sodium reabsorption observed in acute renal failure. These alterations in solute and solvent handling may participate in the stimulus received at the macula densa and help perpetuate the activation of tubuloglomerular feedback.

f. Although increased vasoconstrictor activity, mediated by the renin-angiotensin system, has been proposed (fig. 1) to account for the renal hemodynamic abnormalities characteristic of acute renal failure, additional studies designed to evaluate the potential role of diminished vasodilator activity in the pathogenesis of acute renal failure are in progress. These studies are being performed to assess the role of decreased vasodilator activity in addition to the already demonstrated increased vasoconstrictor activity in the renal hemodynamic alterations in acute renal failure. Initial studies were designed to evaluate prostaglandin-renin interrelationships in pyrogen induced renal hyperemia, a model of renal dysfunction characterized by increased total renal blood flow (26,27). The administration of typhoid toxin to dogs results in an initial (70 minutes after toxin) increase in total renal blood flow, in association with a maintained glomerular filtration rate and a modest decrease in systemic blood pressure. As noted in figure 9, during the early ( $T_1$ ) and late ( $T_2$ ) intervals after the administration of typhoid toxin there is a progressive redistribution of intracortical blood flow from the outer (I+II) to the inner (III+IV) cortical areas. Determinations of renin, prostaglandin E and F secretory rates revealed (fig. 9) marked increased after the administration of typhoid toxin. These results suggest that the diminution in outer cortical blood

flow observed after typhoid toxin is the result of increased renin-angiotensin system activity, and the increased inner cortical blood flow is the consequence of increased prostaglandin synthesis and secretion. The increase in total renal blood flow during the initial phase of typhoid administration would be the direct result of the predominance of vasodilator over vasoconstrictor effects. It is of note that similar alterations in the intracortical distribution of blood flow are observed in acute renal failure. The results of these studies suggest that increases in both renin and prostaglandin mediate the alterations in renal blood flow observed in pyrogen induced renal hyperemia, and lay the foundation for studies of a model of decreased renal blood flow, namely, acute renal failure.

## 2. Chronic Renal Disease and Transplantation:

### a. Drug metabolism in chronic renal disease:

Patients with both acute and chronic renal failure (28,34-36, 43,44), especially those undergoing hemodialysis treatment, frequently require modification of various therapeutic agents (29-31,33,41,42). Studies continue to be done in stable chronic renal failure rather than in high-risk unstable acute renal failure patients. The extraction and assay techniques developed for procainamide (PA) last year (38,39) have been shown to require particular attention to detail with regard to how long the specimen remains acidified prior to measurement. As can be seen in figure 10, acid hydrolysis of the acetylated metabolite increases with time yielding spuriously high levels of PA. The improved methodology in PA measurements has also been applied to a pharmacokinetic study of the major metabolite, N-acetylprocainamide (NAPA), in renal failure patients, yielding interesting and important information. First, a direct correlation between patient INH acetylation phenotype and the conversion of PA to NAPA has been demonstrated (fig. 11) (37). Because significant elimination of NAPA occurs by renal excretion, it would be anticipated that renal failure patients of rapid acetylation phenotype would accumulate larger quantities of NAPA with chronic dosing. Since NAPA has been shown to be pharmacologically active, toxicity is a potential problem. More importantly, we have recently demonstrated that whereas, after single dosing, PA is no longer detectable in patients sera by 24 hours, peak NAPA serum concentrations are sustained up to 72 hours, despite hemodialysis. This observation compounds the risk of marked accumulation and toxicity from the metabolite in renal failure patients given chronic dosing of PA, even if the dose is modified in view of the presence of renal failure.

Since most clinical laboratories are not equipped to undertake serum drug level measurements by gas liquid chromatography or thin layer chromatography with densitometry, a simple, rapid fluorometric assay for separate quantitative analysis of PA and NAPA was developed. Figure 12 demonstrates the effect of various ratios of PA to NAPA on

the measurement of NAPA at pH 1, excitation 288 nm, emission 341 nm= curve a-1:1 ratio; curve b-10:1, P<sup>a</sup> excess; curve c-10:1, NAPA excess. The effective linear range in serum is 0.1 to 10.0 ng/ml, regardless of the ratio of NAPA to PA.

Our earlier studies of propranolol pharmacokinetics in renal failure patients suggested that although there might be some impairment of the "first-pass" extraction, elimination rate constants were not different from those observed in normal subjects (32). Thus studies were undertaken to evaluate the effectiveness of propranolol with standard chronic dosage regimens as an antihypertensive agent in patients with chronic renal failure. A high incidence of noncompliance was noted in these patients and was associated with poor control of blood pressure (40). In compliant patients, propranolol was an effective, safe antihypertensive agent in most patients manifested primarily by a satisfactory blood pressure response to hemodialysis. Plasma renin activity (PRA) determinations were done to examine the potential relationship between PRA suppression and blood pressure response. Although compliant patients as a group did demonstrate PRA suppression (fig. 13), blood pressure response was dissociated from the latter in that some patients had good blood pressure control despite elevated PRA and others remained hypertensive despite PRA suppression.

b. Potential adverse effect of hemodialysis

Since the polyvinyl tubing used to connect patients to hemodialysis units contains plasticizer material, predominantly di-2-ethylhexylphthalate (DEHP), a study was undertaken to determine where DEHP was delivered into patients during the procedure. Indeed, it was found that as much as 150 mg of DEHP may be delivered into patients over a standard 5 hour hemodialysis (see table below) (46). In contrast to patients undergoing a limited number of exposures to DEHP from cardiopulmonary bypass or transfusion, patients requiring long term hemodialysis might be exposed to as much as 11 to 14 grams of DEHP per year of dialysis. Although DEHP toxicity has been demonstrated in tissue culture and other experimental studies, the chronic toxicity of DEHP in man is unknown. Thus, additional information regarding volume of distribution, metabolism, tissue distribution and concentrations and additional toxicity studies are necessary.

TABLE

Estimated amount of DEHP (mgs)  
delivered to each patient per dialysis  
in relation to length of dialysis

Patient	Hours			
	0.25	3	4	5
1	1.3			
2				102
3		68		
4				129
5			74	
6				150
7				11
8			74	
9				9

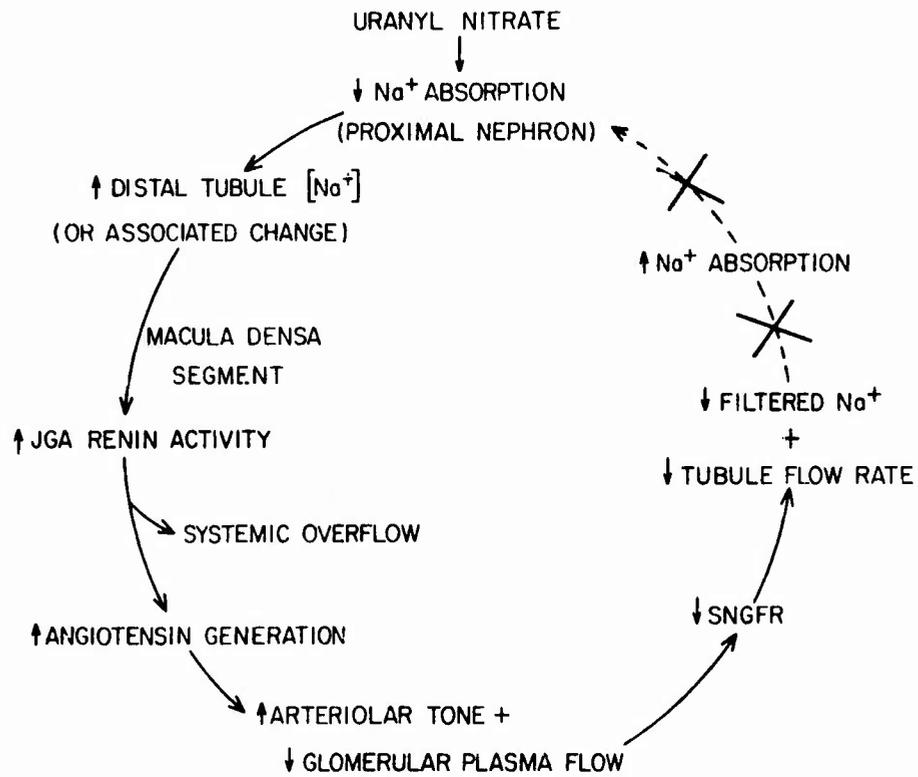


Figure 1. Proposed schema outlining the role for tubuloglomerular feedback in the pathogenesis of uranyl nitrate induced acute renal failure.

TUBULOGLOMERULAR FEEDBACK CONTROL AND EXPERIMENTAL

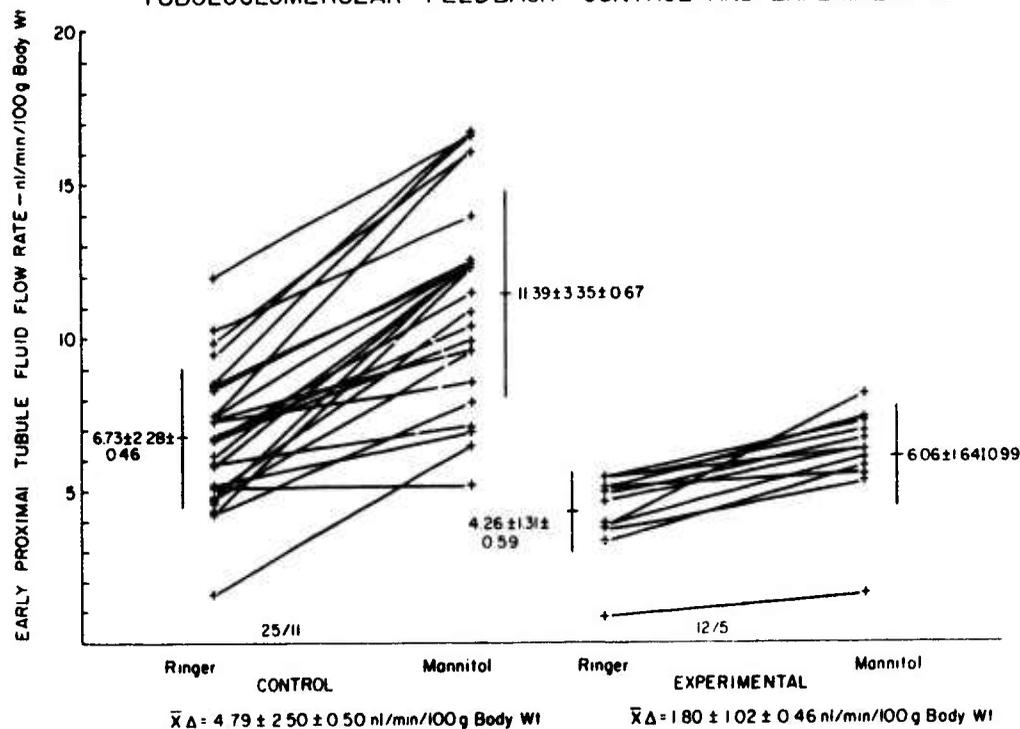


Figure 2. Results of retrograde perfusion of the macula densa segment of superficial nephrons with Ringer's solution (150 mM sodium) or sodium free isotonic mannitol solution in control animals or rats studied up to 6 hours after uranyl nitrate.

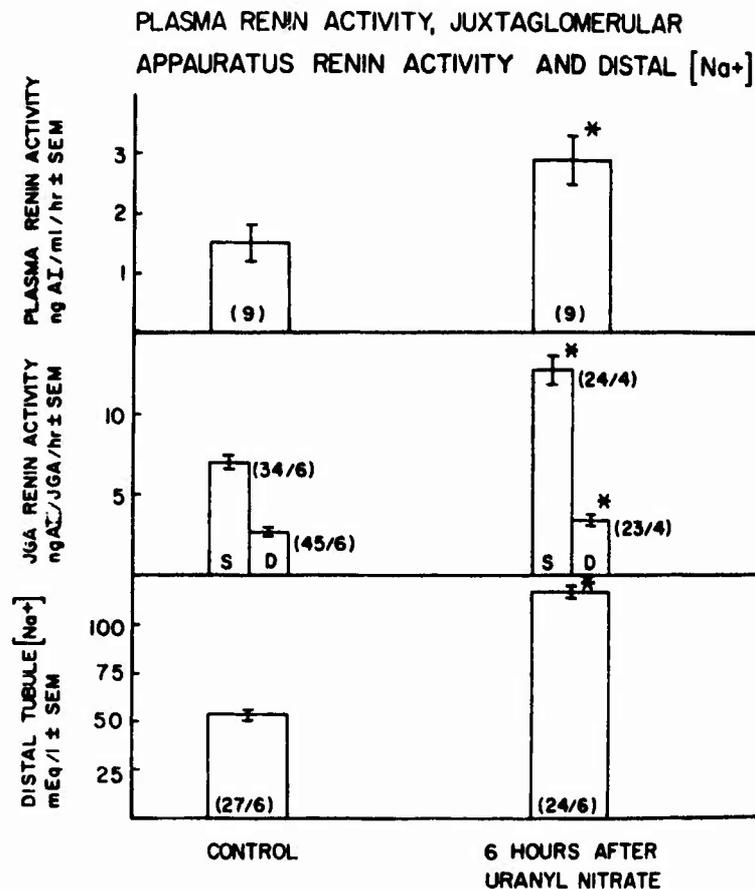


Figure 3. Results of determinations of plasma renin activity, juxtaglomerular apparatus (JGA) and distal tubule sodium concentration in control rats and animals studied up to 6 hours after uranyl nitrate. The number in parenthesis refers to the number of animals studied or the number of determinations made over the number of animals studied. S and D refer to superficial and deep JGA's respectively. An asterisk indicates significantly different from control.



Figure 4A. Photomicrograph of a glomerulus obtained from a rat 6 hours after administration of uranyl nitrate (20,500x).



Figure 4B. Photomicrograph of a glomerulus obtained from an animal 24 hours after the administration of uranyl nitrate (7,000x).

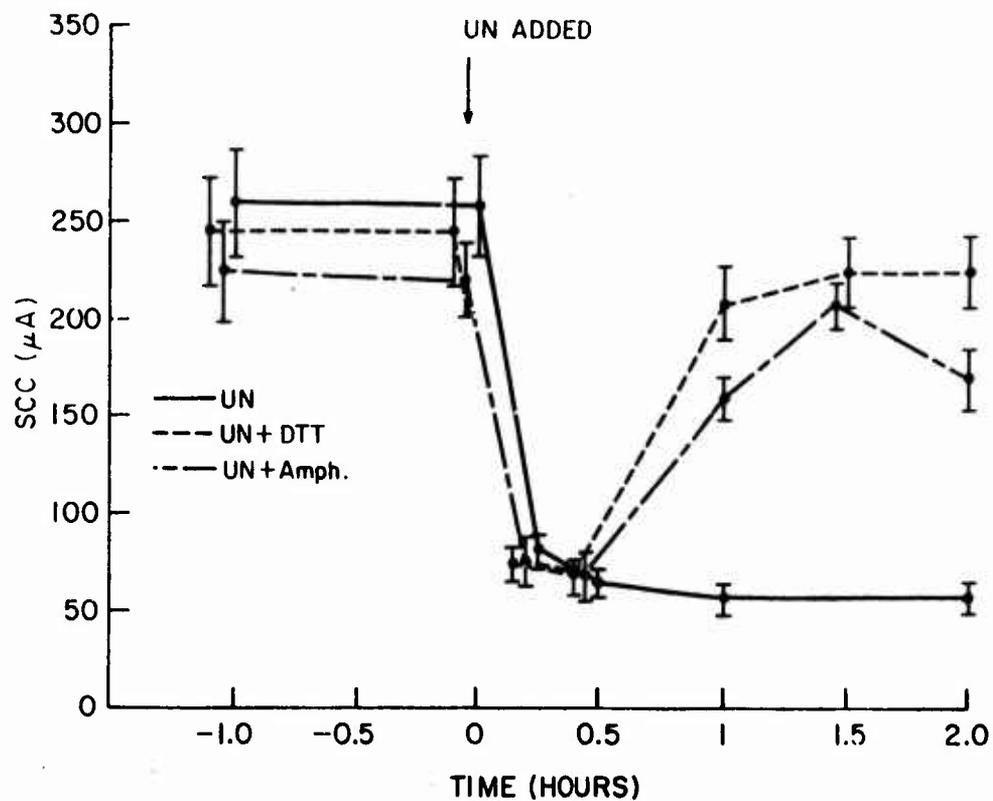


Figure 5. Alterations in short-circuit current (SCC) induced by uranyl nitrate alone, uranyl nitrate plus dithiothreitol (DTT) and uranyl nitrate plus amphotericin B (AMPH) as a function of time.

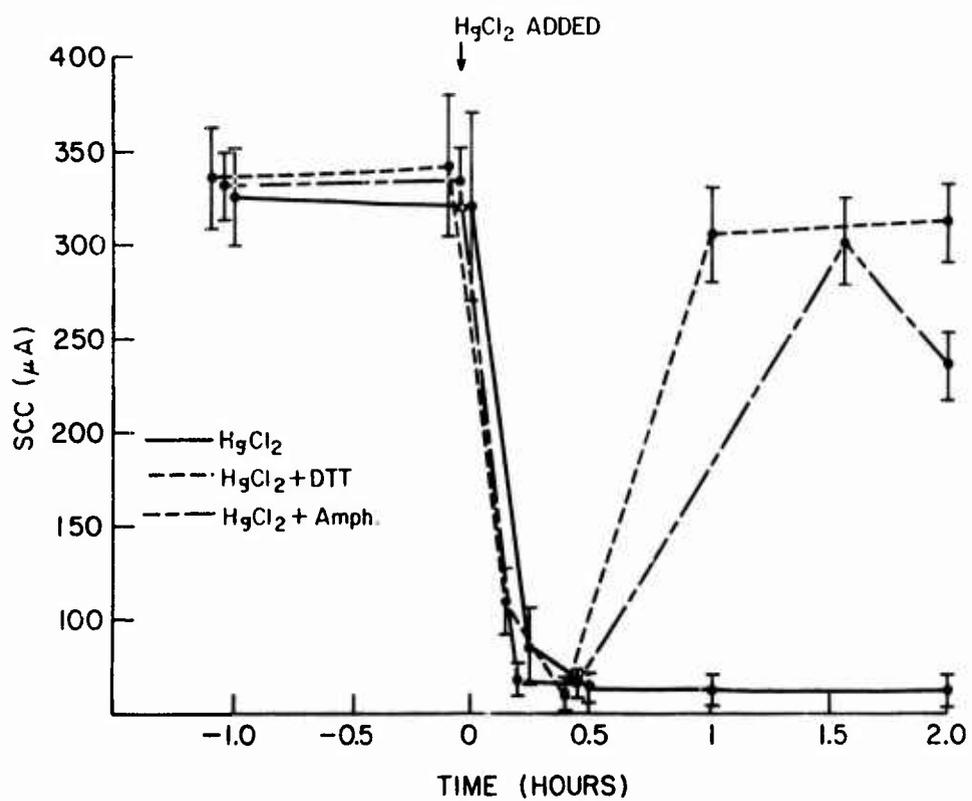


Figure 6. Alterations in short-circuit current (SCC) induced by HgCl<sub>2</sub> alone, HgCl<sub>2</sub> plus dithiothreitol (DTT) and HgCl<sub>2</sub> plus amphōtericin B (AMPH) as a function of time.

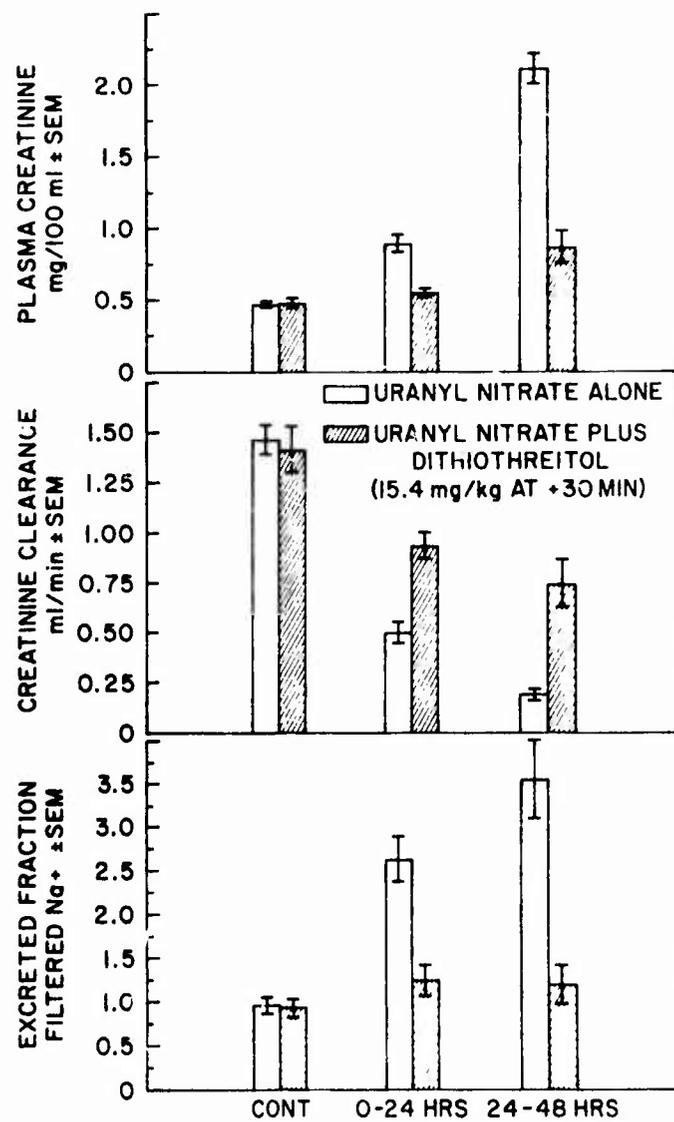


Figure 7. Alterations in plasma creatinine, creatinine clearance and the excreted fraction of filtered sodium during control periods and for up to 48 hours after the administration of either uranyl nitrate alone or uranyl nitrate plus dithiothreitol.



ALTERATIONS IN BLOOD FLOW DISTRIBUTION, PROSTAGLANDIN  
AND RENIN SECRETORY RATES AFTER TYPHOID TOXIN

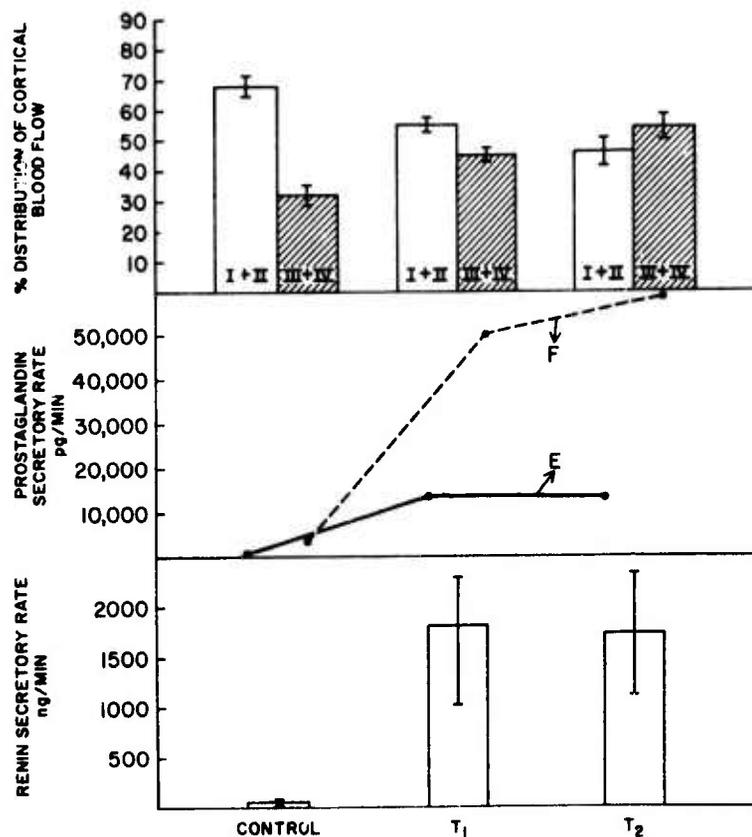


Figure 9. Alterations in blood flow distribution, prostaglandin and renin secretory rates after typhoid toxin. Studies were obtained both early (T<sub>1</sub>) and late (T<sub>2</sub>) after the administration of toxin. Blood flow was determined for both the outer (I+II) and inner (III+IV) renal cortex.

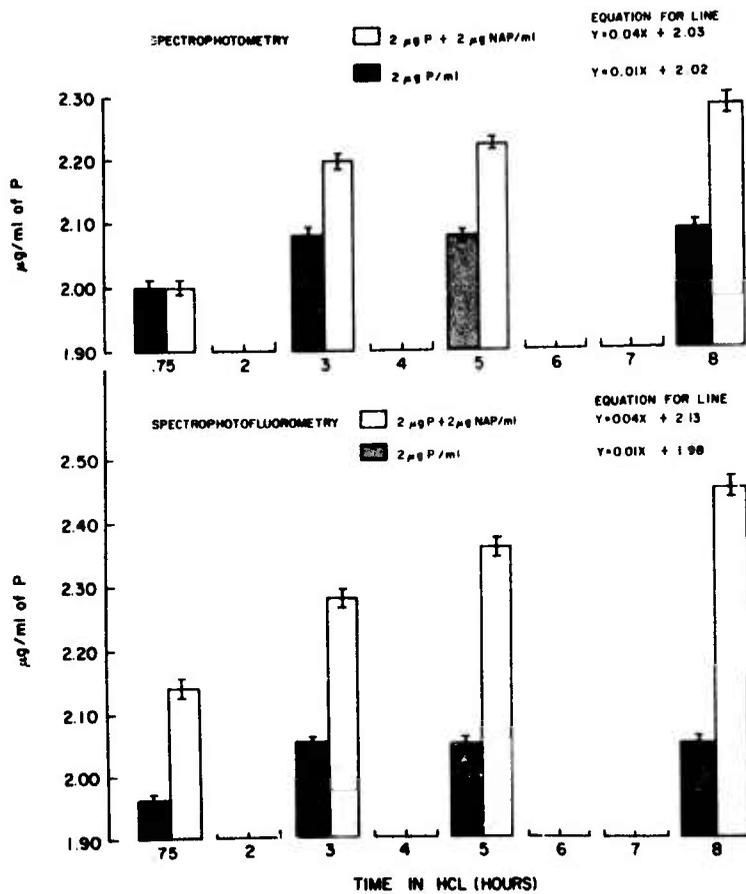


Figure 10. Effect of acid hydrolysis with time on the determination of procainamide (PA) or its N-acetyl derivative (NAPA).

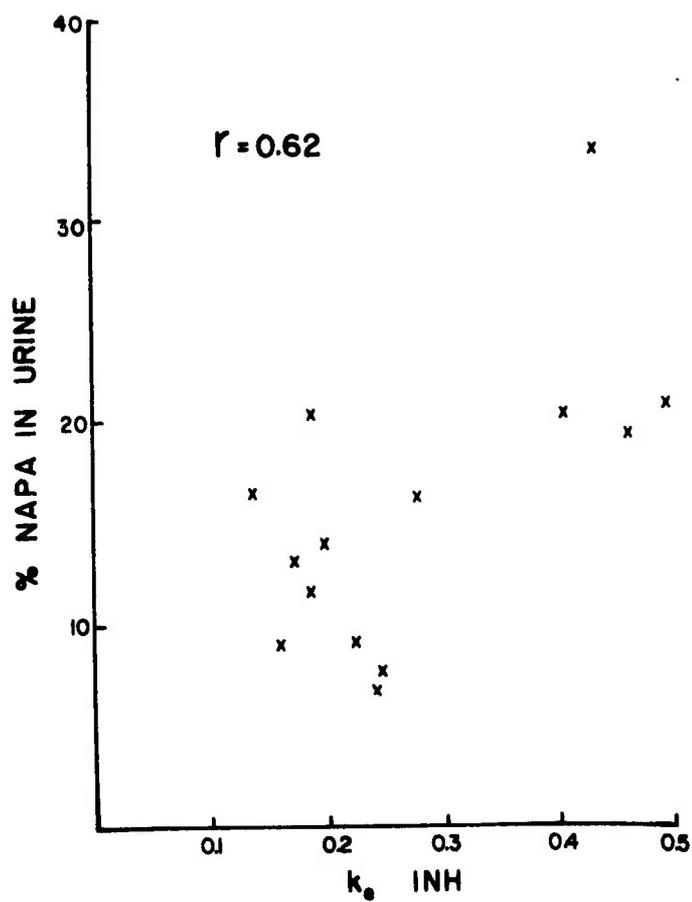


Figure 11. Correlation of INH phenotype and procainamide conversion to N-acetylprocainamide (NAPA).

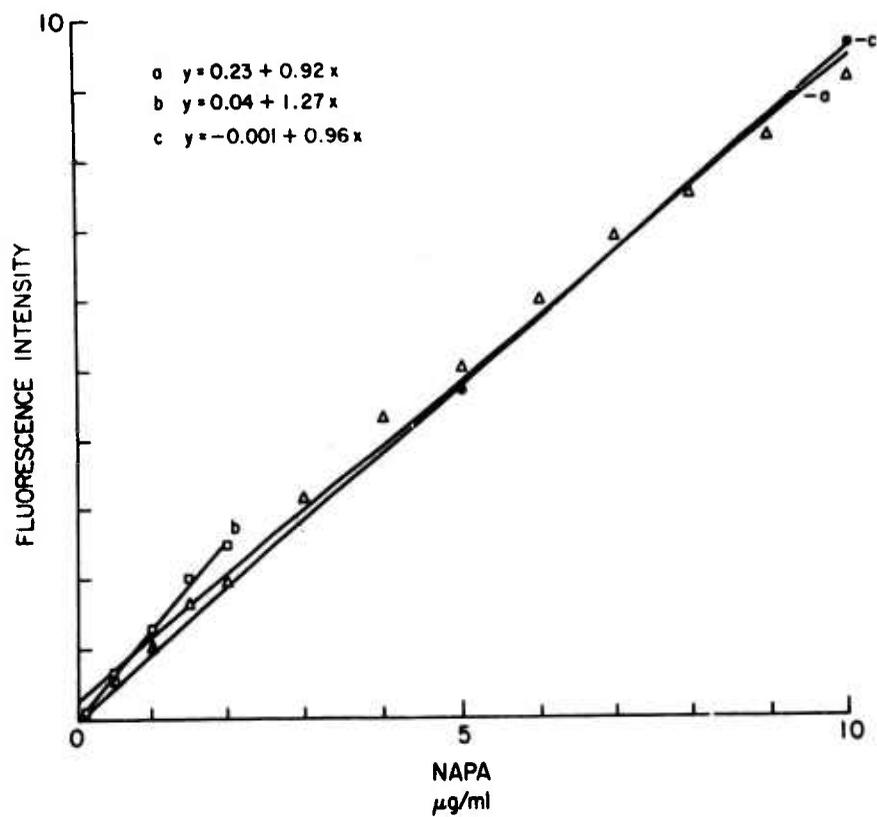


Figure 12. The effect of various ratios of procainamide to NAPA on the measurement of NAPA. The ratios are: curve a, 1:1, curve b, 10:1, PA excess; and curve c, 10:1 with NAPA excess.

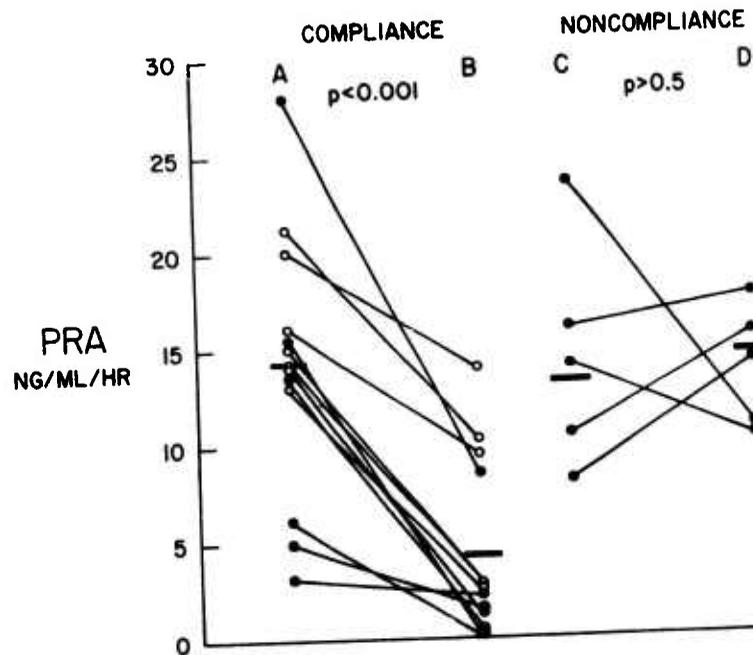


Figure 13. The effect of propranolol on plasma renin activity (PRA) in compliant and noncompliant subjects before (A and C) and after (B and D) hemodialysis.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 089 Pathogenesis of renal diseases of military importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION NO.	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
					DA OB 6462	75 07 01	DD-DR&E(AR)436
3. DATE PREVIOUS SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ICITY	6. WORK SECURITY	7. REGRADING	8. ORIGIN INSTN	9. SPECIFIC DATA - CONTRACTOR ACCESS	
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10. NO./CODES		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		61102A	3A161102B71R	02	090		
b. CONTRIBUTING							
c. COPY/REF		CARDS 114F					
11. TITLE (Precede with Security Classification Code)							
(U) Cellular Mechanisms of Diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
016200 Stress Physiology 017100 Weapons Effects							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
70 01		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PREVIOUS		b. FUND (\$ thousands)	
b. NUMBER:				75		2.0	
c. TYPE:				CURRENCY		350	
d. KIND OF AWARD:				76		2.0	
e. CUM. AMT.						263	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Res.			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Wray, LTC, H.L.			
				NAME: Warren, K.R., PhD			
				DA			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Adaptive growth, (U) Cell Culture, (U) Cell Cycle, (U) Cell Synchrony, (U) Cyclic Nucleotides, (U) Growth Activation, (U) Energy Metabolism							
24. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Precede individual paragraphs identified by number. Precede last of each with Security Classification Code.)							
23. (U) Health impairment associated with the mission of the military is due to stress inflicted by the energy of weapons or exposure to adverse environments and pathogenic microorganisms. Survival is insured through increased function of the appropriate physiological systems, the elementary working units of which are specialized cells. The demand for increased function is met by activation of reserve cells and, depending on the intensity and duration of the stress, by increase of the cell population, i.e. adaptive growth which has far greater survival value and is the object of this study.							
24. (U) The mechanisms controlling adaptive growth are studied by means of model cell culture systems with well defined phases of growth and differentiated function.							
25. (U) 74 07 - 75 06 The essential sequence of adaptive growth, i.e. activation of DNA replication and mitosis with a concurrent decline of specialized function and the reversal of these changes upon restoration of the original cell population density, was successfully reproduced in suspension cultures of fibroblastic cells. Investigation of the possible regulatory role of cyclic nucleotides in this system revealed that levels of cGMP tend to peak during transition between growth (a) and resting (b) states whether the direction of the transition is a + b or its reverse b + a and whether the inhibition of growth is due to high cell density or to manifest nutrient depletion. Cyclic GMP thus appears as an indicator of the synchronous passage of a sizeable fraction of the cell population through a particular subdivision of the G1 phase of the cell cycle, rather than as a specific growth regulator. The behavior of cAMP is similar albeit somewhat complicated because of sensitivity to nutrient depletion. In high density cultures, ATP precursor levels were not sufficiently depressed to account for this effect; determination of the entire nucleotide pool and estimation of the adenylate and nonadenylate energy charge is in progress. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 74 - 30 June 75.							

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DD FORM 1498  
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Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 090 Cellular mechanisms of diseases

Investigators.

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Description

Health impairment associated with the mission of the military is due to stress and injury inflicted by the energy of weapons or exposure to adverse environments and pathogenic microorganisms. Survival is insured through the heightened functional activity of the appropriate physiological systems, the elementary working units of which are specialized cells. The demand for increased function is met by activation of reserve resting cells and, depending on the intensity and duration of the stress, by increase of the cell population, i.e. adaptive growth. As the restoration of the soldier's health and combat capability depends on these processes, it is the objective of this study to uncover the underlying mechanisms and to develop means for increasing their effectiveness.

Background

The problem of the mechanisms controlling adaptive growth can be stated in the form of the following two questions: 1) What is the nature of the changes in the cellular environment which following injury and cell loss first induce cells to proliferate and later limit cell division to the maintenance of a functionally active cell population? 2) What is the nature of intracellular molecular interactions which occur in response to these extracellular changes and result in the early phase of DNA replication and general protein synthesis followed later by a marked reduction of these activities while specialized cellular functions increase?

In the past, a great number of clinical and experimental studies have failed to provide satisfactory answers to these two questions because of the great complexities of the clinical situation in man and of the experimental conditions in the whole animal. Even the discovery that dense cultures of fibroblasts attached on glass or plastic surfaces respond to injury and cell loss by activation of DNA synthesis and cell division which terminates when the initial cell density is restored, i.e. adaptive growth, has failed to provide the desired answers. This is due to the fact that in attached cultures, it is impossible to distinguish between physical growth regulatory mechanisms necessitating cell-to-cell contact and humoral regulation

operating through decreased uptake of substances essential for growth, by dense cultures.

We resolved this difficulty by using WRL-10A fibroblasts in suspension cultures where cell-to-cell contact is limited to transient collisions. We have previously shown that maintenance of these cultures by daily media renewal without cell dilution results in population densities up to  $10^7$  cells/ml which are viable for extended periods and manifest the following characteristics: 1) mitosis and DNA synthesis are inhibited approximately 95% with the majority of cells either arrested or greatly retarded in the  $G_0$  or early  $G_1$  phase of the cell cycle; 2) total protein synthesis is markedly depressed, while the synthesis of specialized proteins such as membrane ectoenzymes or collagen is increased; and 3) cell loss is followed by a 24-hour latent period during which protein synthesis, DNA replication and cell division are activated and continue until the initial population density is restored.

The suspension culture model therefore shares with attached tissue cells in the body or in solid surface cultures, the essential characteristics of the transition from exponential growth to resting, stable functional cell populations capable of reverting to active growth in response to injury and cell loss. Recent work with attached cells has led to the suggestion that changes in the cellular levels of cyclic nucleotides may constitute the first link in the chain of molecular interactions responsible for these transitions between growth and resting states. Accordingly we determined the concentrations of cyclic AMP and cyclic GMP in suspension cultures of WRL-10A fibroblasts in low density exponentially growing cultures and in high density growth inhibited populations paying particular attention to the reversible transition between these two states.

Cyclic nucleotides were assayed by a slight modification of the radio-immunoassays described by Steiner et al. using highly specific antisera. Samples were obtained by centrifugation of aliquots of culture medium plus cells at 750 g for 2.5 min at room temperature followed by decantation of the supernatant medium. The cell pellets and media were immediately frozen in dry ice-ethanol and stored at  $-40^\circ\text{C}$  or lower. Frozen media or frozen cell pellets plus ice cold 50 mM Na acetate pH 6.2 were heated for 10 min at  $90^\circ\text{C}$  followed by 2 min sonification. The suspensions were filtered using ultrafiltration membrane cones (Amicon Centriflo, CF50A) and filtrates added directly to the assay. The recovery of cyclic nucleotides added to cell pellets was determined by radioimmunoassay and found to be 108 - 110% for cyclic AMP and 77 - 85% for cyclic GMP. Recovery of immunoassayable cyclic nucleotides in cell extracts after treatment with cyclic nucleotide phosphodiesterase was determined by comparison of the extracts treated with active enzyme to those treated with heat-inactivated phosphodiesterase. Cyclic nucleotide standards for these assays were similarly treated. The cyclic AMP and cyclic GMP recoveries were <5% and <20% respectively. The cyclic nucleotide values reported in this study

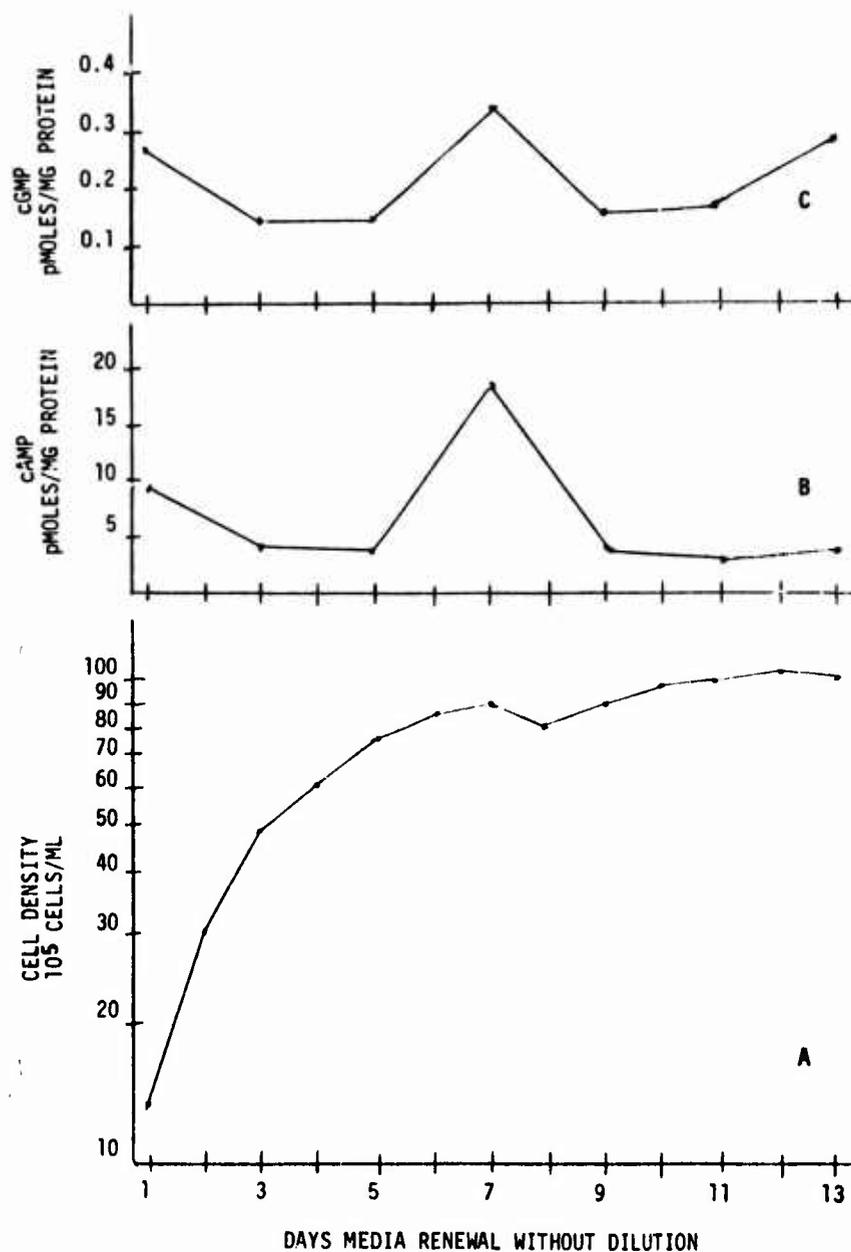


FIGURE 1. Cyclic nucleotide levels during the progression of a low density exponentially growing culture to the high density resting phase. Media renewal without dilution of the culture was carried out daily and the ensuing cell population kinetics are shown in A. Aliquots for cyclic nucleotide and protein assays were taken at the times indicated and the results obtained in regard to cAMP and cGMP are shown in B and C, respectively.

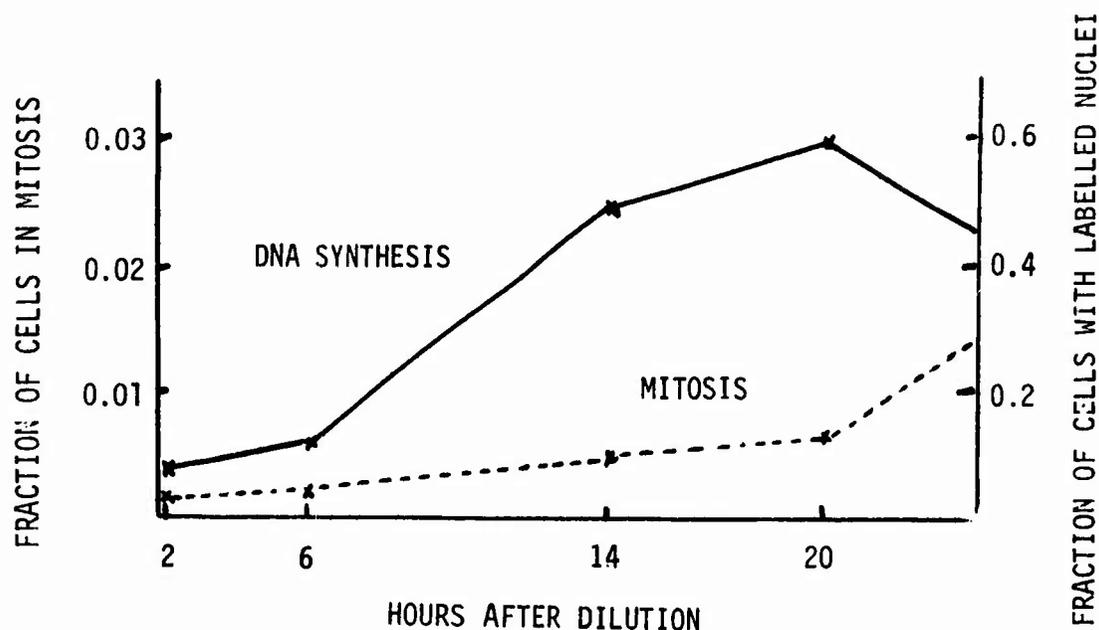
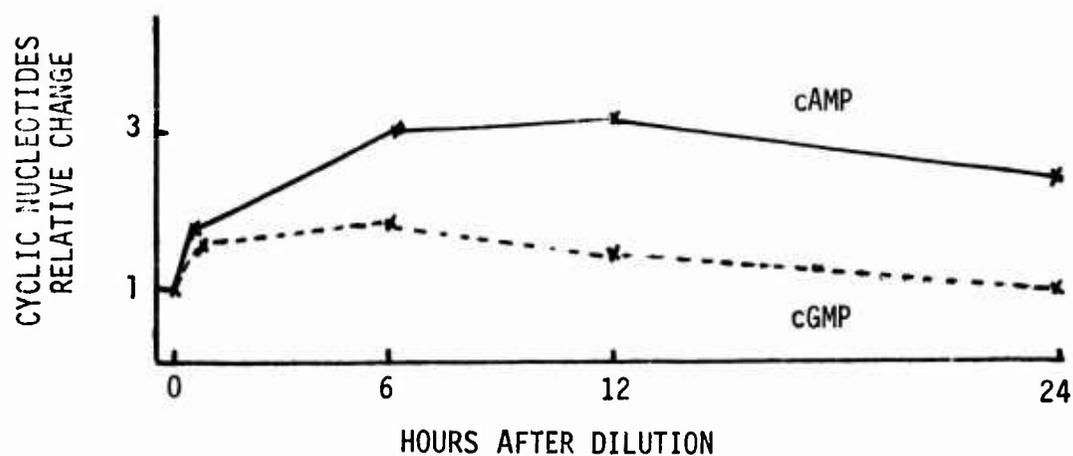


FIGURE 2. Cyclic nucleotide levels following activation of high density resting cell populations by dilution. Two experiments were performed and the mean values obtained are shown in the upper graph. Relative change was calculated on the basis of the levels of the nucleotides in the high density resting populations prior to dilution, which were 2.69 pmoles/mg protein for cAMP and 0.162 pmoles/mg protein for cGMP. Cyclic nucleotide changes are shown superimposed on the known kinetics of DNA synthesis and mitosis following activation<sup>1</sup>, shown on the lower graph.

were calculated directly from the radioimmunoassay standard curves without corrections for the above recoveries.

### Progress and Results

The cultures grown in Eagle's spinner minimum essential medium supplemented with 10% horse serum were maintained and manipulated as previously described<sup>1</sup>. Low density cultures were maintained in exponential growth by daily medium renewal accompanied by dilution to  $0.4 - 0.6 \times 10^6$  cells/ml of medium. High density ( $7 - 12 \times 10^6$  cells/ml) growth inhibited populations were obtained by daily medium renewal without dilution and Fig. 1A illustrates typical cell kinetics of a culture treated in this fashion. From an initial population of  $1.3 \times 10^6$  cells/ml the culture grew to a final cell density of  $10^7$  cells/ml within 2 weeks. Between these two end points there is an exponential growth period from day 1 to day 3, a period of declining growth rate from day 3 to day 7 and prior to reaching the stable resting phase the culture is shown to traverse a transition period between day 7 and day 11, when the cell population fluctuated between  $8$  and  $10 \times 10^6$  cells/ml. Changes of the cellular levels of cAMP and cGMP during this progression are shown in Figures 1B and 1C. It may be seen that the levels of both cyclic nucleotides declined by approximately 50% during the first 3 days of rapid exponential increase of the cell population, that they remained relatively stable during the period of declining growth rate and that during the ensuing transition from the active growth to the resting stable phase showed an abrupt rise which in the case of cyclic AMP exceeded the initial level on day one by a factor of approximately two. Following the establishment of the stable resting phase the levels of the two cyclic nucleotides declined again, but while the final level of cGMP was not significantly depressed, cyclic AMP values remained markedly below initial levels.

In contrast to the slow progression of low density exponentially growing cells to the high density resting phase, the reverse, i.e. activation of cells in the resting phase in response to a sudden decrease of the cell density by dilution of the culture, is completed within 24 hrs. As this activation obviously involves cellular events preceding the actual increase of the cell population, it can best be followed by using as indicators DNA synthesis and mitosis. Figure 2 shows that during the first 5 minutes after dilution of the high density cultures and long before the activation of DNA synthesis, there was a very steep rise of both cyclic nucleotides. Beyond this time the rise continued, although at a much lower rate, and cGMP reached its peak with an 80% increase over its initial level at approximately 6 hrs, still prior to the activation of DNA synthesis; after this time cGMP decreased slowly returning to its initial level at about 24 hrs. Cyclic AMP reached its peak with an approximate 3-fold increase over its initial level at 12 hrs and then gradually declined so that at 74 hrs there was only a 2-fold elevation over its initial level.

The results obtained so far indicate, first, that cyclic AMP and cyclic GMP exhibit peak levels during transitional states between low density actively growing cultures (*a*) and high density resting populations (*b*) regardless of the direction of the transition  $a \rightarrow b$  or its reverse  $b \rightarrow a$ , second, that these peaks are more pronounced in the case of cAMP than cGMP and third, that in established high density resting populations there is no significant change in the level of cGMP while the level of cAMP is significantly lower than in actively growing cultures.

TABLE I

CYCLIC NUCLEOTIDE LEVELS IN ESTABLISHED HIGH  
AND LOW DENSITY SUSPENSION CULTURES

Culture No.	High Density 7-12 x 10 <sup>6</sup> cells/ml		Low Density 0.7-1.2 x 10 <sup>6</sup> cells/ml	
	cAMP	cGMP	cAMP	cGMP
1	2.75	0.187	8.92	0.240
2	6.52	0.414	9.80	0.392
3	2.91	0.166	7.25	0.144

Each culture was maintained at high density by daily medium renewal without dilution for at least 5 days before aliquots were taken for cyclic nucleotide and protein assays. Subsequently the cultures were diluted and maintained at low density by daily medium renewal and dilution. All samples were obtained at 24 hrs after medium renewal. Cyclic nucleotides are expressed in pmoles/mg protein.

This latter finding is further supported by the data summarized in Table I where three different cultures are compared in regard to their cyclic nucleotide content in the high density resting phase and after growth activation by means of dilution. It may be seen that although the level of cyclic nucleotides was observed to vary among different cultures by a factor of almost three, in each case the level of cAMP was significantly lower in the high density cultures while cGMP remained unchanged. At variance to the high cAMP levels reported to accompany inhibition of growth in many cases of attached cultures, the elevation of cAMP in suspension cultures appears, therefore, to be limited to the transition from active growth to the resting state (Fig. 1). Upon establishment of the latter state in suspension cultures, cAMP is shown to be significantly lower (Fig. 1, Table I) and after reactivation of the resting cells cAMP shows an early rise (Fig. 2) instead of the decrease reported for attached cultures.

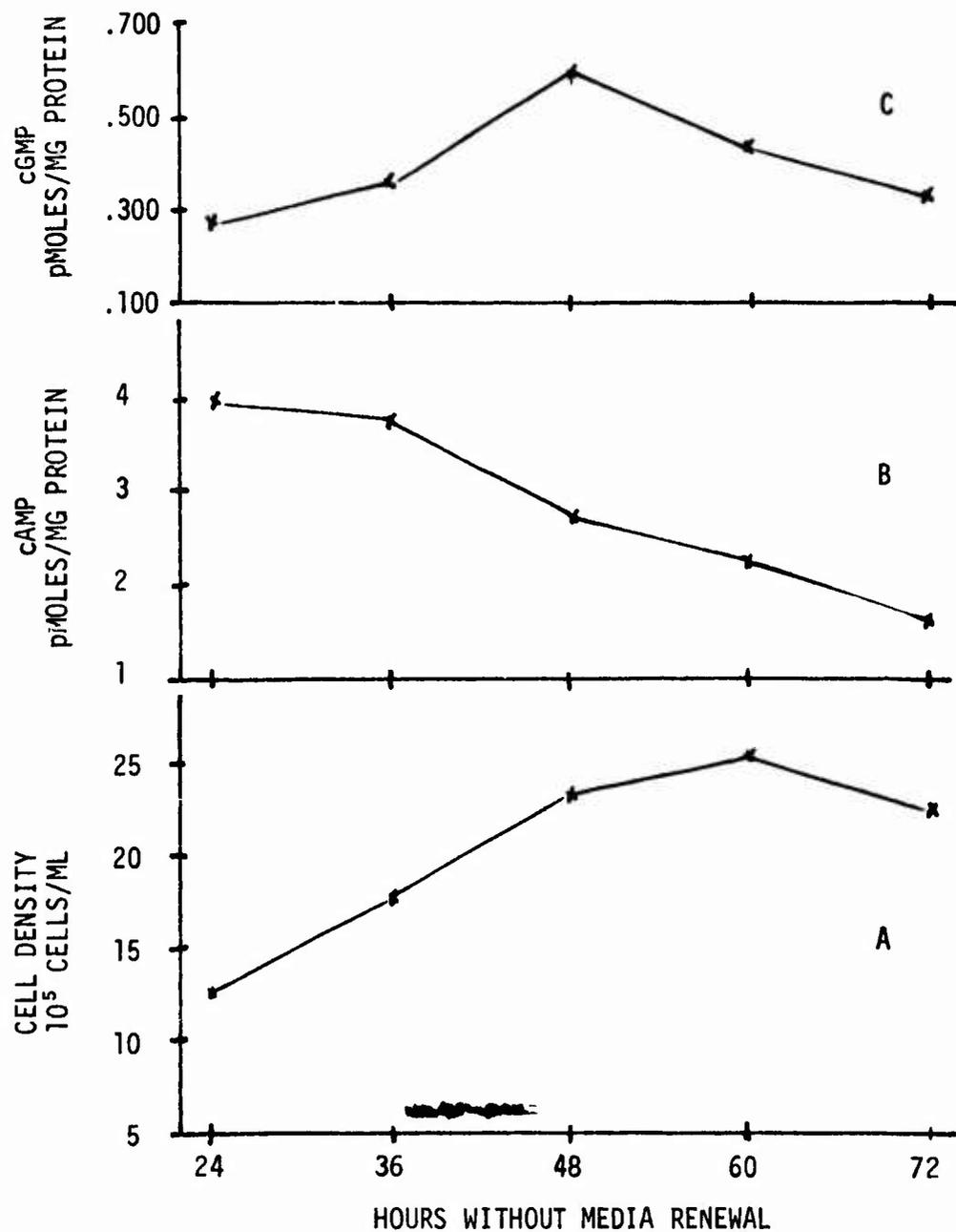


FIGURE 3. Cyclic nucleotide levels during the progression of low density exponentially growing populations to the starvation plateau. The daily media renewal was omitted and the ensuing cell population kinetics are shown in A. The cell counts shown as well as the values for cAMP (B) and cGMP (C) represent means of three experiments.

With the possible exception of microenvironmental gradients (1) the concentration of small molecular weight nutrients in the media does not become limiting in dense attached cultures; in contrast, it is one of the main factors regulating growth in suspension cultures<sup>1,4,5</sup>. To investigate whether limiting media components could be responsible for preventing maintenance of high cAMP levels in growth inhibited populations, cyclic nucleotides were determined during the starvation plateau induced in low density cultures by omission of the daily media renewal<sup>4</sup>. Fig. 3A shows that if at the end of 24 hours after the setting up of low density exponentially growing cultures, the media are not renewed, growth continues for another day until the onset of the starvation plateau at cell densities of the order of  $2 - 3 \times 10^6$  cells/ml. Figures 3B and 3C show that during this time cAMP declines progressively to very low levels while cGMP peaks at 48 hrs. with a 2-fold increase and then gradually declines reaching its initial level at 72 hrs. The decrease of cell density seen at that time is due to loss of cellular viability and signals the beginning of the end of the starvation plateau.

The behavior of cyclic nucleotides in suspension cultures, therefore, appears to be nearly the same regardless of whether the inhibition of growth is due to high cell density or to manifest nutrient deprivation with the significant difference that in the latter even the transient increase of cAMP during the transition from active growth to the resting phase was eliminated. Cyclic AMP levels thus appear to be greatly affected by the availability of extracellular nutrients while exactly the opposite appears to be true for cGMP the level of which was not affected even in the severe nutritional deprivation associated with the end of the starvation plateau.

The peaking of the cGMP during transitional states between growth and no growth both in high density (Fig 1C, Fig. 2) as well as starvation-plateau cultures (Fig. 3C) raised the question as to whether the cells were arrested in the same phase of the cell cycle in both cases. Phases of the cell cycle associated with inhibition of growth may be identified through the kinetics of the mitotic activity of the cells after reactivation of the growth inhibited population. It may readily be seen that upon reactivation, a cell population uniformly delayed in all three stages of the interphase, i.e.  $G_1$ , S and  $G_2$  will yield an immediate and sustained rise of the mitotic index. A population delayed primarily in  $G_1$  and  $G_2$ , as is commonly the case with nonspecific growth inhibition because of the relative insensitivity of DNA synthesis once begun, will yield two distinct mitotic peaks, the first, occurring early because of the division of cells delayed in  $G_2$ , followed later by a second due to cells originally delayed in  $G_1$  and which must traverse both the S and the  $G_2$  periods before dividing. Finally, a population selectively arrested in an early  $G_1$  or  $G_0$  period will result in a single mitotic wave which will be considerably delayed since all the cells will have to traverse the S and  $G_2$  period before mitosis. On this basis, Fig. 4 conclusively demonstrates that in high density

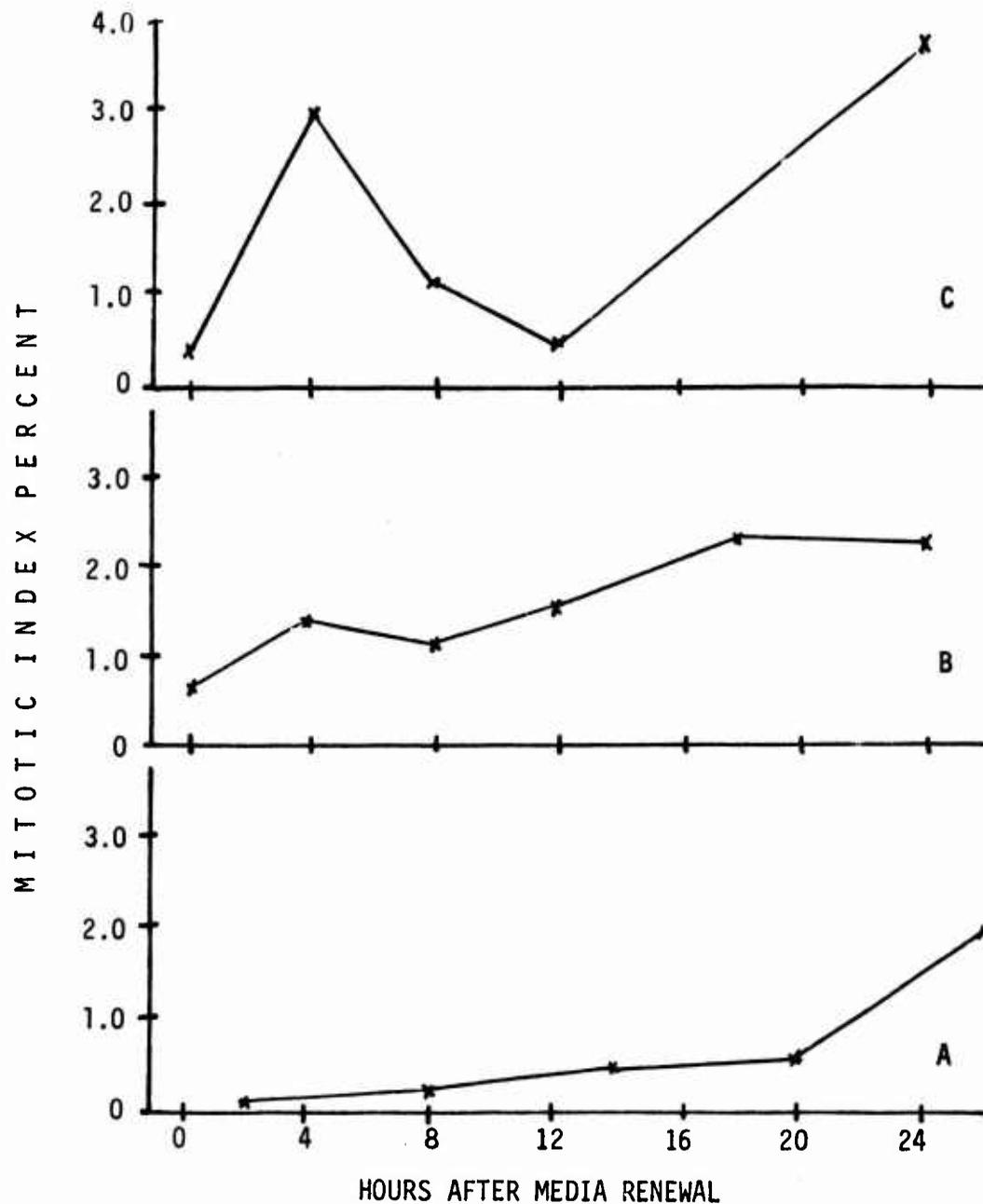


FIGURE 4. Kinetics of cell division following activation of cell populations in the high density resting phase and in the starvation plateau. Activation was carried out by diluting a suitable aliquot from the cultures with fresh media to yield a cell concentration approximately  $4 \times 10^5$  cells/ml. This procedure was carried out 24 hrs. after media renewal in the case of the high density populations shown in A and at 48 and 72 hrs. in the case of the starvation plateau cultures shown in B and C respectively. For each point the percent mitotic index was calculated on the basis of counts performed on 3000 cells.

populations (Fig. 4A) the cells were arrested selectively in an early  $G_1$  or  $G_0$  phase while in the starvation plateau cultures (Figs. 4B and C) the cells were delayed in a nonspecific way both in  $G_1$  and  $G_2$ . As expected, the biphasic mitotic activity indicative of this delay was more pronounced when reactivation of the growth inhibited cultures was carried out at 72 hours (Fig. 4C), rather than at 48 hours (Fig. 4B), because of the progressively increasing accumulation of cells in the  $G_1$  and  $G_2$  phases with time.

It may be concluded that with the exception of the depression of cAMP levels in established high density populations and during nutritional deprivation which will be discussed later, the outstanding feature of the behavior of cyclic nucleotides we have observed consists of the temporary elevation of their levels during transitions between growth and resting states. This is most strikingly demonstrated in the case of cGMP which peaked in all the transitional states investigated in this study whether from active growth to the resting state or the reverse and regardless of whether growth inhibition was due to high cell density or to manifest nutrient deprivation (Figs. 1-3). The only common denominator in all these transitional states is accumulation and partial synchronization of a sizeable fraction of the cells in the  $G_1$  phase of the cell cycle (Fig. 4). Accordingly it is proposed that one of the numerous subdivisions of  $G_1$ <sup>6</sup> is characterized by relatively high concentrations of cGMP and that the peaking of this cyclic nucleotide in the situations described in this report is simply due to the simultaneous passage of a relatively large number of cells through this particular  $G_1$  segment.

While the present work was in progress, increase of cGMP during passage of cells through the  $G_1$  phase was also reported for resting attached cells following their activation by addition of serum<sup>7</sup>. In these attached cultures the increase of cGMP during the  $G_1$  phase occurred not only immediately after activation but also during the subsequent division of already growing cells<sup>7</sup>. Also, in the suspension cultures described in this report, cGMP increase was observed not only during the transition of cells from the resting to the growing state but also during the reverse transition from active growth to the growth inhibited state. This is in excellent agreement with the recently described movement of cells through the early  $G_1$  or  $G_0$  phases not only away but also toward the resting state<sup>8</sup>. Taken together, these observations on attached and suspension cultures are consistent with the view of cGMP as an indicator of a particular  $G_1$  segment but offer no support for the claim that this cyclic nucleotide represents a specific signal for the activation of resting cells<sup>7</sup> and others.

Changes of the level of cAMP during the cell cycle have also been described<sup>9,10</sup>. While there is agreement that during the  $G_1$  phase, cAMP levels tend to rise, information as to variations among different cell types in regard to the specific  $G_1$  segments involved and the magnitude of the rise, is lacking. This probably accounts for the fact that

while in many cases low and high cAMP levels have been associated with growing and resting states, respectively, other observations indicate that tumors have higher cAMP levels than normal tissues<sup>11,12</sup>, that cyclic AMP rises after cells are stimulated to divide<sup>13,14</sup>, and that it fails to rise or even declines during density dependent inhibition of growth<sup>15,16</sup>.

Synchronous passage of our WRL-10A cells through the G<sub>1</sub> period during the transition of a high density culture to the resting state was accompanied by a rise in cAMP (Fig. 1) but this rise did not occur when growth was inhibited through nutrient depletion (Fig. 3). Also, in both, established high density populations and starvation plateau cultures cAMP levels were depressed (Table I and Fig. 3). To investigate the possibility that this was mediated through alterations of the ATP precursor pool because of nutrient limitation, the luciferine-luciferase method was used to determine cellular ATP levels in cultures of varying cell densities at 2 and 24 hours after media renewal. Preliminary results are summarized in Table II where it can be seen that although ATP

TABLE II  
CELLULAR ATP LEVELS IN SUSPENSION CULTURES

CULTURE DENSITY	LOW		INTERMEDIATE		HIGH	
TIME POST MEDIA RENEWAL (hrs)	2	24	2	24	2	24
CELL DENSITY (X10 <sup>5</sup> cells/ml)	3.7	7.7	5.5	11.3	117.5	115.5
FACTORIAL INCREASE <u>Cell Density 24 hrs</u> <u>Cell Density 2 hrs</u>	2.1		2.1		1.0	
ATP (fmoles/cell)	8.9	7.9	7.6	6.8	5.9	5.0
MEAN ATP (fmoles/cell)	8.4		7.2		5.5	

Three cultures of varying cell density as shown were used. For a period of 3 consecutive days media renewal and cell dilution were carried out in the low and intermediate density cultures and media renewal without cell dilution in the high density population. Cell counts and ATP determinations were performed at 2 and 24 hrs. after media renewal and the values obtained averaged over the three-day observation period and tabulated as shown.

levels appeared to decline during the 24-hour media renewal cycle, in no case was this decline significant. On the other hand, the mean ATP level of high density resting populations was lower than in low density growing cultures by approximately 35 percent. This, however, is considerably less than the 83 percent reduction of the ATP pool shown to be necessary in order to interfere with the formation of cAMP<sup>13</sup>. Accordingly, the possible role of energy metabolism in the inhibition of growth and the reduction of cAMP reported here will be investigated further by expanding our determinations to include the entire nucleotide pool and the estimation of the adenylate as well as the nonadenylate energy charge of the cells in low density growing, high density growth inhibited and starvation plateau populations.

#### Summary and Conclusions

The essential sequence of adaptive growth, i.e. activation of DNA replication and mitosis with a concurrent decline of specialized function and the reversal of these changes upon restoration of the original cell population density was successfully reproduced in suspension cultures of fibroblastic cells. Investigation of the possible regulatory role of cyclic nucleotides in this system revealed that levels of cGMP tend to peak during transition between growth (*a*) and resting (*b*) states whether the direction of the transition is  $a \rightarrow b$  or its reverse  $b \rightarrow a$  and whether the inhibition of growth is due to high cell density or to manifest nutrient depletion. Cyclic GMP thus appears as an indicator of the synchronous passage of a sizeable fraction of the cell population through a particular subdivision of the G<sub>1</sub> phase of the cell cycle, rather than as a specific growth regulator. The behavior of cAMP is similar albeit somewhat complicated because of sensitivity to nutrient depletion. In high density cultures, ATP precursor levels were not sufficiently depressed to account for this effect; determination of the entire nucleotide pool and estimation of the adenylate and nonadenylate energy charge is in progress.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 090 Cellular mechanisms of diseases

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2. Wray, H.L. and Vail, J.M.: Density dependent regulation of growth in L cell suspension cultures: Levels of cyclic nucleotides. *Fed. Proc.* 34: 264, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>c</sup>	6. WORK SECURITY <sup>d</sup>	7. GRADING <sup>e</sup>	8A. DOD'S INSTR <sup>n</sup>	8B. SPECIFIC DATA- CONTRACTOR ACCESSION <sup>f</sup>	8. LEVEL OF RUM A. WORK UNIT
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10. NO./CODES <sup>g</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	61102A	3A161102B71R	02	092			
b. CONTRIBUTING							
c. SUPPORTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>h</sup>							
(U) Microbial Genetics and Taxonomy							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>i</sup>							
010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
63 08		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE:		b. EXPIRATION:		PRECEDING		a. FUNDS (in thousands)	
NA				75		3	
c. NUMBER:		d. AMOUNT:		CURRENT		275	
				76		3	
e. TYPE:		f. CUM. AMT.				283	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Wohlhieter, J.A.			
				NAME: [REDACTED]			
				DA			
22. KEYWORDS (Precede Each with Security Classification Code) (U) Vaccine; (U) Enteric Bacteria; (U) Antigens							
(U) Virulence; (U) Salmonella; (U) Drug Resistance							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Definition in genetic and molecular terms of the properties of gene transfer antigenicity, and virulence of pathogenic enteric bacteria which because of their disease producing capabilities, are of importance to military medicine concerned with the prevention and treatment of such infections in Army personnel. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and pathogenicity to serve as vaccine strains or as tools to study the infectious process.</p> <p>24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.</p> <p>25. (U) 74 07-75 06 Salmonella typhimurium hybrids expressing somatic antigen 9 after mating with either S. typhosa or S. enteritidis Hfr donors were shown not to differ from their virulent S. typhimurium parent with respect to the number of organisms required to produce death in mice inoculated intraperitoneally. Two transmissible genetic elements conferring the ability to ferment sucrose upon Salmonella strains isolated from clinical sources were characterized by genetic and molecular procedures. The DNA of the fertility factor, F, of Vibrio cholerae was shown to have a 35 to 40 per cent homology with V. cholerae chromosomal DNA. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 74 - 30 Jun 75.</p>							

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DD FORM 1 MAR 68 1498

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Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 092 Microbial genetics and taxonomy

Investigators.

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Description.

1. Salmonella typhimurium hybrids expressing somatic antigen 9 after mating with either S. typhosa or S. enteritidis Hfr donors were shown not to differ from their S. typhimurium parent with respect to the number of organisms required to produce death in mice inoculated intraperitoneally.

2. The deoxyribonucleic acid of the fertility factor, P, of Vibrio cholerae was shown to have 35 to 40% homology with V. cholerae chromosomal DNA and little or no homology with the DNA of the Escherichia coli sex factor F.

3. Two transmissible genetic elements conferring the ability to ferment sucrose upon Salmonella strains isolated from clinical sources were characterized by genetic and molecular procedures.

Progress.

1. Intraperitoneal mouse virulence of Salmonella typhimurium hybrids expressing somatic antigen 9.

a. In our previous studies of Salmonella typhimurium hybrids expressing S. typhosa somatic antigen 9 as the consequence of genetic exchange (Annual Report, WRAIR, 1973, 1974), we had not observed among them any loss of mouse virulence (determined by intraperitoneal inoculation) as compared with their S. typhimurium parent. We were concerned therefore, by reports elsewhere (2,3) that other S. typhimurium hybrids expressing the somatic 9 antigen as the result of inheritance of S. enteritidis genetic determinants, showed a 10-fold decrease in their

virulence for mice upon intraperitoneal injection. This decrease was considered to have resulted from the qualitative change in the lipopolysaccharide side chain, i.e., the substitution of tyvelose (conferring 9 antigen specificity) for abequose (conferring the native S. typhimurium 4 antigen specificity) in the repeating oligosaccharide units. We could see no reason why the genetic source of the 9 antigen determinants, whether S. typhosa or S. enteritidis, should be a factor (so long as only a qualitative, as opposed to a quantitative, change in the lipopolysaccharide was involved) and thought it worth comparing, within the same maternal S. typhimurium strain, 9-antigen-expressing hybrids of each paternal (with respect to the antigen determinants) genotype.

b. The S. typhimurium recipient strain employed was WR5005, a streptomycin-resistant mutant of the histidine-requiring, mouse virulent strain used in our previous studies. In matings with the S. typhosa Hfr strain WR4000, and also with the S. enteritidis Hfr strain WR4050, S. typhimurium WR5005 hybrids were selected for receipt of the histidine synthesis (his) genetic determinant of the donor to which the genes determining the somatic 9 antigen are closely linked in S. typhosa as well as in S. enteritidis. As the genetic locus occupied by the determinants of the 4 antigen of S. typhimurium is allelic with the locus of the 9 antigens of S. typhosa and S. enteritidis, S. typhimurium hybrids expressing the 9 antigen derived from either of these donors have lost their native 4 antigen. The somatic 5 antigen, which can not be expressed in the absence of the 4 antigen, also disappears from these 9-antigen-expressing hybrids.

c. Three S. typhimurium WR5005 hybrids expressing somatic antigen 9 after mating with the S. enteritidis Hfr, two hybrids expressing somatic antigen 9 as the result of hybridization with the S. typhosa Hfr, and the parent S. typhimurium WR5005 were examined for mouse virulence by intraperitoneal inoculation. Groups of 10 mice (BALB/c females weighing 14 to 18 g) were inoculated with 0.5 ml saline suspensions of various dose levels of each organism. These doses ranged from a high of 50,000 organisms to a low of 10 organisms. In three or more virulence titration experiments conducted with each of these hybrids and with their S. typhimurium WR5005 parent, no differences were observed among them with respect to the number of organisms capable of causing death of at least half of the animals tested, within a period of 15 days. At a 30-organism dose level, 50% end points were achieved, in all instances, within 13 days, and usually were achieved by day 15 at the 10-organism dose level.

d. In terms of the time required to attain their 50% end points, the three S. enteritidis-derived S. typhimurium hybrids appeared fairly similar to their WR5005 parent. Thus, at a dose level of 50 organisms, these hybrids achieved their end points in 7 to 10 days, the S. typhimurium WR5005 parent in 8 to 11 days. The two S. typhosa derived hybrids generally took longer, reaching their end points at the 50-organism dose level in 10 to 13 days. We would be hesitant about assigning any particular significance to these differences. We would not hesitate, however, to conclude that the qualitative change from 4 antigen to 9 antigen specificity is not, per se, responsible for any alteration of mouse virulence in hybrids of S. typhimurium WR5005 that can be measured by intraperitoneal inoculation.

2. Homology between the DNA of the Vibrio cholerae fertility factor P and V. cholerae chromosomal DNA.

a. In a previous study (Annual Report, WRAIR, 1972) we isolated and characterized the DNA of the Vibrio cholerae fertility factor P. This DNA exists as supercoiled circular molecules which are 41  $\mu$  in length and have a molecular weight of  $80 \times 10^6$ . The average base composition of this DNA was determined to be 42% guanine plus cytosine (G+C) as compared with 48% G+C for V. cholerae chromosomal DNA. Average G+C values, however, are not very informative in terms of the genetic relatedness of plasmid DNA to the DNA of its host. Thus, the E. coli fertility factor F, which happens to have the same average G+C composition as its host (50%), shows, in hybridization studies, only a 40% homology with E. coli chromosomal DNA. In similar studies described here, we have found almost the same amount of homology between the P factor DNA and the DNA of the V. cholerae chromosome.

b. Supercoiled circular DNA associated with the fertility factor P was labeled with  $^3\text{H}$  thymidine and isolated from the chromosomal DNA of V. cholerae strain V58  $\text{P}^+$  by dye-buoyant density centrifugation. The P factor DNA was then incubated at various temperatures with DNA from different bacterial strains. The extent of reassociation was determined by adsorption of reannealed DNA to hydroxyapatite columns. When the labeled P factor DNA was incubated with an excess of DNA extracted from strain V58  $\text{P}^+$ , reannealing occurred between the labeled P factor DNA and the P factor DNA present in the extracted DNA from the V58  $\text{P}^+$  strain. When labeled P factor DNA was incubated with an excess of DNA extracted from the V58  $\text{P}^-$  strain, some reassociation still occurred due to reassociation of labeled P factor DNA to the V. cholerae chromosomal DNA.

c. The results of these reassociation experiments are shown in Table I. The amount of labeled P factor DNA bound to the hydroxyapatite column after incubation is expressed as the percentage of DNA reannealed. The values in parentheses are the relative percentages of DNA reannealed based on 100 percent for reassociation of P factor DNA with DNA from strain V58 P<sup>+</sup>. Thus the amount of reassociation between the isolated P factor DNA and the V. cholerae chromosomal DNA was between 42% and 50% depending on the temperature at which the hybridization reactions were performed. These values, however, overestimate the amount of homology between the P factor DNA and the V. cholerae chromosomal DNA because they have not been corrected for the binding observed in controls. Binding of P factor DNA due to self-annealing was 6% or less under conditions and various incubation temperatures used in these hybridization experiments. Reannealing of the isolated P factor DNA with a non-related DNA such as S. marcescens DNA indicates that the amount of such reannealing is very small and cannot be distinguished from self annealing. Circular DNA molecules with a molecular weight of 40 million are present in a number of V. cholerae strains including both the V58 P<sup>+</sup> and the V58 P<sup>-</sup> strains used in this study. The function of these smaller circular DNA molecules is at present unknown, but they are isolated with the P factor DNA and comprise about 10-20% of the DNA in the P factor preparations. This small amount of circular material which is not P factor DNA will probably anneal with similar DNA in the chromosomal preparations, causing an overestimate in the amount of homology between P factor DNA and the V. cholerae chromosomal DNA. When the appropriate corrections are made, the value estimated for the homology between the P factor DNA and the V. cholerae chromosome is between 35 and 40%.

d. Competition experiments using nitrocellulose filters were performed according to the procedure of Denhardt (1) and <sup>3</sup>H labeled P factor DNA was annealed with V. cholerae DNA from strain V58 P<sup>+</sup> bound to nitrocellulose filters. Various DNA's were added to this reaction mixture to see whether they would compete and interfere with the binding of the labeled P factor DNA. If these DNA's are homologous to the DNA on the filter, then they will reanneal and prevent binding of the labeled P factor DNA. DNA from V. cholerae V58 P<sup>+</sup> was a good inhibitor and prevented binding even when added in low concentrations. Serratia marcescens DNA which has a G+C composition of 58%, was chosen

as a strain which should have little or no homology with P factor DNA. It did not interfere with the binding of P factor DNA to DNA from V. cholerae V58 P<sup>+</sup>, even at 2000 µg/ml, the highest concentration of DNA used, a ratio of 40,000 to 1. When E. coli F<sup>-</sup> or E. coli F<sup>+</sup> DNA was added, little or no interference with binding was observed. This finding indicates that little or no homology exists between the DNA of the F-factor of E. coli and that of the P factor of V. cholerae.

3. Characterization of transmissible genetic elements derived from clinically isolated sucrose-fermenting Salmonella strains.

a. Several sucrose-fermenting Salmonella strains isolated from clinical sources were examined for their ability to transfer, by conjugation, the Scr<sup>+</sup> property to E. coli WR3026. From two of these Salmonella strains, an S. typhimurium, and an S. senftenberg, low frequency transfer (ca 10<sup>-7</sup>) of the Scr<sup>+</sup> property was observed. E. coli WR3026 recipients of the Scr<sup>+</sup> character of the S. typhimurium strain (designated scr-53) were then capable of high frequency conjugal transfer (5 x 10<sup>-4</sup> to 5 x 10<sup>-3</sup>) of scr-53 to E. coli WR3028 which, in turn, transferred it at a similar frequency to E. coli WR3070. E. coli WR3026 recipients of the Scr<sup>+</sup> character of the S. senftenberg strain (designated scr-94) were capable of transferring the scr-94 element to E. coli WR3028, but at a lower frequency (10<sup>-6</sup> to 10<sup>-5</sup>) than that of scr-53. E. coli WR3028 recipients of scr-94 transferred this element in turn to E. coli WR3070 at frequencies similar to those with which they had received it.

b. As was the case with six previously studied transmissible lac elements (Annual Report, WRAIR, 1974) neither of the scr elements affected the streptomycin sensitivity of their Salmonella hosts. After their transfer to E. coli WR3026, the scr elements were examined additionally for possible effects on their host's sensitivity to ampicillin, chloramphenicol, tetracycline, kanamycin, gentamycin, furadantoin and polymixin B. No indication of resistance to any of these antibiotics was detected in WR3026 containing either scr element. Each of the six lac elements, which had not been tested previously for drug resistance markers (other than streptomycin), also was examined in a WR3026 host for possible ability to confer resistance to any of the above listed antibiotics and, again, none was detected.

c. Each of the scr elements was transferred from E. coli WR3026 to an E. coli WR3070 strain containing either the E. coli episome, F-lac, or one of each of the six previously characterized lac elements. From each cross, 20 Scr<sup>+</sup> exconjugants were examined to determine whether they still contained the resident lac element.

In most crosses no displacement of the resident lac element or F-lac by the incoming plasmid was observed. In those instances where it was observed, the occurrence was no more frequent than one or two of the 20 hybrids examined, and examination of additional exconjugants in these instances revealed no increases in this frequency. Similar results were obtained in the reciprocal experiments in which E. coli WR3070, containing either scr-53 or scr-94, served as recipients of the six lac elements and F-lac which were transferred by E. coli WR3026 donors. Thus, there was no indication of incompatibility (which would have indicated relatedness) between either of the scr elements and the six lac elements or F-lac. In all of these crosses, a plasmid free E. coli WR3070 recipient also was employed for comparison with the plasmid-containing recipient in order to observe any possible effect of a resident plasmid on transfer frequency of the incoming plasmid. In no instance was any consistent or significant reduction in transfer frequency (entry exclusion) observed. When either scr-53 or scr-94 were contained in E. coli WR3028, together with an F-lac or with any of the six lac elements, their transfer frequencies in matings with E. coli WR3070 did not differ from those obtained with an E. coli WR3028 donor containing either scr element by itself. Likewise, no reduction in the frequency of lac<sup>+</sup> transfer from these double plasmid donors was noted when compared with those obtained with an E. coli WR3028 donor harboring only F-lac or the particular lac element.

d. E. coli WR3026 exconjugants containing either scr-53 or scr-94 responded as females when tested with the female specific phage,  $\phi$ 11. In this regard their behavior was the same as that of E. coli WR3026 exconjugants containing any of the six lac elements which, likewise, do not show a male response to this phage. Neither scr-53 - nor scr-94 - containing WR3026 exconjugants showed lysis or plaque formation when tested with the male specific phage, R-17. However, when experiments were carried out to detect possible increase in phage titer on these exconjugants, using E. coli Hfr WR2017 as the indicator strain, a titer increase of 10,000-fold was observed on the WR3026 exconjugant containing scr-53. No increases in titer were observed in these experiments for WR3026 exconjugants containing scr-94 or any one of the six lac elements.

e. E. coli WR3026 containing the scr-53 element was susceptible to lysis by PI vir, as were WR3026 exconjugants containing any of the six lac elements. Transduction of scr-53 from a WR3026 exconjugant to WR3026 was accomplished with PI vir lysates having titers in excess of  $10^9$  pfu per ml. The E. coli WR3026 scr-53 transductants

were capable of transferring scr<sup>-53</sup>, by conjugation, to E. coli WR3028 at frequencies similar to those exhibited by E. coli WR3026 scr<sup>-53</sup> exconjugants. Thus, there was no apparent loss of transfer function of the scr<sup>-53</sup> element as a consequence of the transduction process. Moreover, molecular studies (see below) showed no difference in the size of the scr<sup>-53</sup> element isolated from a transductant as compared with one isolated from an exconjugant. E. coli WR3026 exconjugants that contained the scr<sup>-94</sup> element were not susceptible to lysis by PI vir. Similarly, the E. coli recipients WR3028 and WR3070 also lost their susceptibility to lysis by this phage upon receipt of the scr<sup>-94</sup> element. In experiments performed to determine whether phage adsorption was affected, it was found that WR3026 exconjugants containing scr<sup>-94</sup> adsorbed PI vir as well as did WR3026. However, whereas PI vir increased in numbers 100,000-fold on WR3026 in experiments carried out to measure phage propagation, no increase in the titer of PI vir was detected with the WR3026 scr<sup>-94</sup> exconjugant. We are currently investigating the nature of this scr<sup>-94</sup> effect on the lytic activity of PI vir, and have established, thus far, that it does not involve DNA restriction. Whatever its nature, however, it is clear that this effect is dependent upon the continuing presence of the scr<sup>-94</sup> element; segregants of any of these exconjugants from which scr<sup>-94</sup> was lost were found also to have regained their susceptibility to lysis by PI vir.

f. Supercoiled circular DNA was detected in scr<sup>-53</sup> containing exconjugants and transductants of E. coli WR3026 by dye bouyant density centrifugation. Examination of Scr<sup>-</sup> segregants of a scr<sup>-53</sup> containing WR3026 transductant by the same method showed that the loss of its Scr<sup>+</sup> character was reflected in the loss of its contained circular DNA. Sedimentation in a neutral sucrose gradient, of circular DNA isolated from an E. coli WR3026 transductant and from an E. coli WR3026 exconjugant, allowed the calculation of a molecular weight of  $61 \times 10^6$  for the scr<sup>-53</sup> element in the exconjugant and  $58 \times 10^6$  for the scr<sup>-53</sup> element in the transductant. However, experiments in which <sup>14</sup>C-labeled circular DNA isolated from a WR3026 scr<sup>-53</sup> exconjugant was sedimented in the same sucrose gradient with <sup>3</sup>H-labeled circular DNA isolated from a WR3026 scr<sup>-53</sup> transductant indicated an identical molecular weight for exconjugant and transductant scr<sup>-53</sup> DNA. When examined by electron microscopy, the circular DNA molecules isolated from the WR3026 exconjugant exhibited a mean contour length of  $26.64 \pm 1.35 \mu\text{m}$  corresponding to a molecular weight of  $51.1 \times 10^6$ . Those isolated from the WR3026 scr<sup>-53</sup> transductant had a mean contour length of  $27.01 \pm 1.83 \mu\text{m}$  corresponding to a molecular weight of  $51.9 \times 10^6$ . Thus, there was no difference in the size of these scr<sup>-53</sup> elements, whether transferred by conjugation or by transduction.

g. In spite of repeated attempts to detect it, supercoiled circular DNA was not observed in scr<sup>-94</sup> containing exconjugants of E. coli WR3026. Nor was it observed in scr<sup>-94</sup> containing exconjugants of either E. coli WR3028 or E. coli WR3070. Attempts to detect this DNA form in the S. senftenberg 5494-57 strain in which the scr<sup>-94</sup> element was initially discovered likewise failed to result in its detection. The consistent conjugal transmissibility of this element from one recipient to another indicates an extrachromosomal mode of conservation, as we see no reason, a priori, for considering extrachromosomal conservation of a transmissible sex factor in a form other than covalently closed circular. At present, however, we are not able to explain our apparent failure to demonstrate this DNA form in organisms containing scr<sup>-94</sup>.

#### Summary.

1. Salmonella typhimurium WR5005 hybrids expressing somatic antigen 9 as the consequence of mating with an S. typhosa Hfr strain and S. typhimurium WR5005 hybrids expressing this antigen as a result of mating with S. enteritidis Hfr strain were compared in intraperitoneal mouse virulence tests with their S. typhimurium WR5005 parent. Regardless of the genetic source of the somatic 9 antigen, the hybrids did not differ from their virulent S. typhimurium parent with respect to the number of organisms required to cause death of at least half the animals inoculated within a period of 15 days.

2. The DNA of the Vibrio cholerae fertility factor P was isolated by the dye buoyant density centrifugation procedure and hybridized to V. cholerae chromosomal DNA. The DNA of this fertility plasmid had between 35 to 40% homology with the V. cholerae chromosomal DNA. Little or no homology was detected between the P factor DNA and the DNA of the Escherichia coli sex factor F.

3. Two clinically isolated sucrose fermenting Salmonella strains were found capable of conjugal transfer of the sucrose fermentation (Scr<sup>+</sup>) property to Escherichia coli WR3026. The genetic elements conferring this Scr<sup>+</sup> property, designated scr<sup>-53</sup> and scr<sup>-94</sup>, were then conjugally transmissible from E. coli WR3026 Scr<sup>+</sup> exconjugants to other strains of E. coli at frequencies of  $5 \times 10^{-4}$  to  $5 \times 10^{-3}$  for the scr<sup>-53</sup> element, and  $10^{-6}$  to  $10^{-5}$  for the scr<sup>-94</sup> element. In E. coli hosts, both of these elements were compatible with F-lac and with each of six previously characterized transmissible lac elements, and no antibiotic resistance markers were discovered to be associated with either of them. Neither scr element generated a male host response to the female specific phage,  $\phi 11$ , but the scr<sup>-53</sup> element rendered its E. coli host sensitive to the male specific phage, R-17. E. coli hosts

containing scr-53 were susceptible to lysis by PI vir, and transduction of the scr-53 element was accomplished with this phage. The scr-53 element was isolated from E. coli WR3026, Scr<sup>+</sup> transductants, and from E. coli WR3026 Scr<sup>+</sup> exconjugants as a covalently closed circular DNA molecule with a molecular weight (determined by electron microscopy) of approximately  $52 \times 10^6$ . Receipt of the scr-94 element rendered E. coli hosts of this element unsusceptible to lysis by PI vir, although adsorption of the phage by an E. coli WR3026 scr-94 containing exconjugant occurred as efficiently as it did on WR3026 itself.

Table 1. Extent of Homology between Isolated P factor DNA and *V. cholerae* Chromosomal DNA

*Hybridization reaction	Incubation temperature					
	50 C		60 C		70 C	
	DNA reannealed (%)	T <sub>m</sub> (°C)	DNA reannealed (%)	T <sub>m</sub> (°C)	DNA reannealed	T <sub>m</sub> (°C)
P	6		5		4	
P / <i>S. marcescens</i>	5		6		4	
P / <i>V. cholerae</i> V58 P <sup>+</sup>	76 (100) <sup>1</sup>	81	78 (100)	81	69 (100)	81
P / <i>V. cholerae</i> V58 P <sup>-</sup>	32 (42)	78	38 (48)	76	35 (50)	77

\* Isolated P factor DNA was labeled with tritium and used at an input of 2,000 cpm/ml as described in Materials and Methods section.

<sup>1</sup>Figures in parenthesis are normalized to the binding observed in the P / *V. cholerae* V58 P<sup>+</sup> reaction, which was arbitrarily designated as 100%. These figures were not corrected for the background.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 092 Microbial genetics and taxonomy

Literature Cited.

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PROJECT 3A161102B71R  
RESEARCH IN BIOMEDICAL SCIENCES

Task 03  
Psychiatry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OA 6454	75 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGRADING <sup>c</sup>	8. USEFN INSTR <sup>d</sup>	9. SPECIFIC DATA-CONTRACTOR ACCESS	10. LEVEL OF DUM
74 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES <sup>e</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	61102A	3A161102B71R		03	025		
b. CONTRIBUTING							
c. <del>CONTRIBUTING</del>	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>f</sup>							
(U) Analysis and Management of Behavior and Stress in Military Environments							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>g</sup>							
013400 Psychology 012600 Pharmacology 012900 Physiology 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
63 08		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE: NA				PREVIOUS			
b. NUMBER: NA				FISCAL YEAR		205	
c. TYPE:				75		4	
d. KIND OF AWARD:				76		4	
e. CUM. AMT.						200	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with U.S. Academic Institution)			
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				NAME: Hursh, CPT S. R.			
23. KEYWORDS (Precede with Security Classification Code) <sup>h</sup>							
(U) Operant Behavior; (U) Respondent Conditioning; (U) Reinforcement; (U) Conditioning; (U) Military Psychiatry							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRAM (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Experimental analysis and development of complex behavioral models isolating variables likely to contribute to psychiatric decompensation or disease and subsequent ineffective performance in military environments. Functional relationships among physiological and behavioral variables governing the individual-environment interaction are defined and manipulated under controlled conditions to permit more precise specification of their role in the pathogenesis of behavioral and organic disorders.							
24 (U) Techniques of experimental psychology, principally operant and respondent conditioning, combined with those of endocrinology, pharmacology, physiology and anatomical sciences are used to define variables that maintain and control both adaptive and dysfunctional behavior.							
25 (U) 74 07 - 75 06 Several studies of circadian behavioral rhythmicities in accuracy, work output and generalization gradients have begun to document the role played by behavioral variables in the production and maintenance of performance variability across the circadian time frame. Variables, such as probability reinforcement, have been shown to have an impact on performance that can be functionally related to day-night cycle. A study of temporal factors in the disruptive effects of aversive control of behavior suggests a complete disorganization of behavior may occur even under conditions of only minimal performance demand. Preliminary data from studies of the role of differential probability of reinforcement in establishing control over behavior indicate that control cannot be established if the existing repertoire of the organism is reinforced with a probability of reinforcement of .20 and the probability for a change in behavior does not exceed .50. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 75 - 30 June 75.							

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Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03 Psychiatry

Work Unit 025 Analysis and management of behavior and stress  
in military environments

Investigators.

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Description.

Research managed within this work unit can be classified into two broad categories: (1) Experimental analysis of the individual-environment interaction as it relates to the maintenance and control of behavior; and (2) Experimental analysis of drug behavior relationships. It should be emphasized that this categorization is principally for purposes of organizing this report. The study of drug-behavior relationships is a specialized approach to the more general problem of the experimental analysis of the maintenance and control of behavior.

EXPERIMENTAL ANALYSIS OF THE INDIVIDUAL-ENVIRONMENT INTERACTION AS IT  
RELATES TO THE MAINTENANCE AND CONTROL OF BEHAVIOR.

The studies in this section are directed at description of functional relationships between environmental variables and the behavior and physiology of the organism. Their purpose is: (1) To develop, analyze and apply complex behavioral models which permit examination of the interaction of the organism with its environment under conditions likely to lead to psychiatric decompensation; (2) To specify general principles governing the maintenance and control of behavior; (3) To develop and maintain the technology required to objectively address questions of a behavioral nature arising from problems in military medicine and bearing on the health of the individual soldier.

The role of reinforcers in choice behavior. Studies are being conducted, addressed to the problem of how an organism distributes its behavior between various alternatives which produce different reinforcers. These studies can be described as investigations of the principles governing choice behavior. Previous researchers have described all behavior as choice behavior in the sense that

engaging in one response precludes many other responses and represents a choice to do one thing as opposed to another. Examination of the factors controlling such choices is fundamental to understanding the control of behavior; for example, the choice to self-administer drugs as opposed to emitting some other behavior. To date, however, nearly all studies of choice have avoided investigation of competing responses maintained by different reinforcers. Most of these early studies were concerned with factors which were best studied with behaviors maintained by similar reinforcers. As a consequence, little is known about the principles which might govern a choice between qualitatively different reinforcers, heroin and money for example. Much that is known about choice remains tentative until it is extended to comparisons of different reinforcers. It is possible that such comparisons introduce variables which have, until now, been overlooked. The first study is a fundamental comparison of choice between similar and dissimilar reinforcers. The purpose is to discover those characteristics of choice which are unique to comparisons of dissimilar reinforcers. The result has been that reductions in the availability of one food source increases responding for the other food source and decreases responding for water. These changes in responding occur even when there is no change in overall intake of food or water. These observations are consistent with the economic principles of substitutes and substitutable and complementary commodities. A change in the supply of one food increases demand for the substitute (the other food) while it decreases the demand for the complement (water). This dichotomy of reinforcers in this situation is not predicted by current learning theory although it appears consistent with the current physiological theory of food and water regulation.

In another study, rats are being exposed to nine months of continuous access to concurrent schedules of food and intracranial self-stimulation (ICS). The results of this study thus far indicate that as the availability of both reinforcers was decreased the responding for food increased, maintaining constant intake, while responding for postero-lateral hypothalamic brain stimulation decreased and daily ICS trains decreased. These results are interpreted as a parallel to the economic principle of demand elasticity. Food, a primary "need", has an inelastic demand curve. Electrical brain stimulation appears to have an elastic demand curve - the subject will not pay a higher price in order to prevent a reduction in consumption. Three additional subjects with other electrode placements will replicate this experiment. The results of this experiment are analogous to results obtained in other studies that indicate that demand for food is inelastic when concurrent with heroin while demand for heroin appears to be more elastic in that consumption varies with the price extracted by the schedule.

Parameters of safety. It is a well-known fact that avoidance of noxious stimulation will maintain performance. Usually the reinforcer in these experiments is the postponement of an aversive stimulus. If responding does not occur at some minimal frequency, the stimulus occurs; if the responding is rapid enough, it does not occur. In this setting, the reinforcer is present or not present. Yet, outside the laboratory setting, various degrees of safety are often available and an organism must often select what appears to be the safest alternative. Very little is known of the parameters of safety and what factors make one situation more safe (or less stressful) than another. These principles are of fundamental importance to an understanding of behavior under stress. A effort is now underway to fashion a setting in which an animal can be stressed and, by manipulating the duration, the amount of aversive stimulus reduction, and the frequency of availability of safety, it is hoped that a systematic theory of avoidance behavior can be developed which is as complete as current theories of appetitive behavior. A study was undertaken in which a Sidman avoidance paradigm was used and the duration of the response-shock interval was gradually lengthened to one hour. In the next to last condition with the session length of 3 hr and R-S = 1 hr. the subject responded continuously and avoided all shocks. When the session length was extended to 12 hrs to coincide with the day-night cycle, responding quickly deteriorated over the first 5 days of the condition until by the sixth day the animal received all programmed shocks (twelve) and stopped eating and drinking. This general disruption of behavior persisted for the next five days until the experiment was terminated to avoid harm to the animal. The observation of complete disruption of behavior by the relatively mild administration of only twelve shocks (an event easily tolerated in other situations such as punishment or conditioned suppression) represents an important psychological stress paradigm that will be replicated in other subjects. It is not known as yet whether the long session (12 hr) alone is sufficient or if the combination of prolonged R-S intervals and long sessions is necessary.

Behavioral variables in the production and control of circadian rhythmicity of performance.

A study of variation in auditory discriminations of rats requires rats to respond on one lever in the presence of tone bursts of one duration and on another lever in the presence of a second tone duration in order to obtain their entire daily ration of food pellets. Eight sessions are run, equally spaced throughout the day, each consisting of 100 discrimination trials. Several different measures of performance are being recorded, but only two have been analyzed in detail at the present. Total response output varies with time of day, peaking in the dark portion of the 12/12 light/

dark cycle. Accuracy of the performance also shows a circadian rhythm, with greatest accuracy corresponding with periods of highest response output. In other words, the greater the tendency to respond, the greater the accuracy. Amplitude of the circadian variations is being manipulated by varying the probability with which correct responses produce food. The greater the reinforcement/correct ratio, the greater amplitude of the time-of-day effect. Further experiments will investigate other variables affecting the degree to which circadian variability in behavior may be modified by behavioral variables.

Principles of Behavioral change - Behavioral change in the context of alternative reinforcement.

The monkey most advanced in this study has been participating since the middle of March in a study of behavioral change in the context of alternative reinforcement. The procedure is as follows: each day a selected sequence of responses (chain) is programmed to pay-off with some probability which started at 100% reinforcement. Any other chain executed will pay-off only 20% of the time. Thus, the subject has a choice between learning a new chain (changing his behavior) for some favorable percentage reinforcement versus continuing to make self-selected chains (not changing) for some less favorable percentage reinforcement. The strategy of the experiment was to gradually reduce the percentage favoring behavioral change to determine the point at which the advantage in reinforcement does not offset the cost of changing behavior or, put another way, to see if a stable pattern of learning will persist even when it provides no advantage in terms of primary reinforcement. Note that throughout the experiment any part of the new chain emitted by the subject produces a distinctive signal. This insures that the subject makes frequent contact with stimuli signalling correct members of the new chain and can change behavior even when the entire new chain is not emitted or is not followed by food.

With this subject, the percentage reinforcement for behavioral change was lowered from 100% to 50% to 35%. At 35% learning was inhibited but continued to occur at an inefficient level. When the percentage for learning was lowered to 20%, equal to not changing, learning occurred during the first four sessions. By the fifth session, learning ceased to occur and occurred only partially during two sessions of the next 18 sessions. Even though frequent signals informed the subject of correct members of the new sequence, he did not choose to emit them when they provided no advantage in getting food. Apparently, the cost of continuing to

learn is between 35% and 20% when other behavior also provides 20% reinforcement. In less formal terms, the pattern of emitting new chains each day did not generalize to a setting that provided no differential primary reinforcement for learning.

The next phase of the experiment involved gradually increasing the percentage reinforcement for behavioral change to determine the conditions necessary to induce learning once it has ceased to occur. The percentage reinforcement for new behavior has now been increased to 30% then 40% to 50%. Each probability was in effect for sixteen days. Yet, no signs of learning have occurred. The cost of inducing learning once it has ceased is greater than the cost of maintaining learning when it is an on-going pattern. The next step will be to select some chain which the subject currently emits with some regularity as the "new" chain. We will leave it in effect with a 50% pay-off for an extended period to see if prolonged exposure to the same learning problem will eventually lead to learning. In effect, this procedure will simulate the traditional "shaping" process used to produce behavioral change. Once learning occurs, a new but similar chain will be required. Gradually the learning process may be reinstated and a new series of decreasing probabilities will be studied to determine more precisely the cost of behavioral change.

Monkeys are involved in a study that requires discrimination of interoceptive stimuli. These animals work on a procedure that requires counting of responses to earn their daily ration of food. The animals each have two press plates. Responding on one (press plate B) is reinforced with food and the feeder light only following 8 consecutive presses on the other (press plate A). B responses following fewer than 8 A responses produce a 5 sec timeout, as does a sequence of 9 or more consecutive A responses. Average accuracy is around 50-60% for both animals. Each animal has a 30 minute session every four hours (six session per day). As with the rats, work output varies with time of day, with a peak during the day rather than the night. Accuracy also varies with time of day, but with a very small amplitude. Measurement error is also great, due to the low work output during the night. Current efforts are to increase the consistency of work output (i.e., reduce the amplitude of time of day effects on work output) by reducing the probability of food presentation for correct responses. This has a minimal effect on average accuracy and seems to be having the desired effect of increasing work output during the nighttime sessions. Future work will involve manipulation of other variables that are potentially important in producing circadian variability.

### Nonspatial memory after selective prefrontal lesions in monkeys.

Rhesus monkeys were trained on one of three tasks requiring differential responding to a visual cue not present at the time of responding: object alternation, delayed color matching, or delayed object matching. Subsequent bilateral lesions of the inferior convexity resulted in severe and lasting impairments on all three tasks, but lesions confined to the principal sulcus produced only small transient disruptions. Although the contributions of the inferior convexity cannot yet be specified, these results support the view that principalis cortex serves chiefly spatial functions.

### Limbic system involvement in visual match-to-sample behavior.

In studies conducted in collaboration with Dr. Mortimer Mishkin of NIMH we have previously shown that damage to the ventral portions of prefrontal cortex produces a severe and lasting deficit in performance of a color delayed match-to-sample problem. More recently we have produced equally severe deficits on this task with lesions of the cortex on the inferior surface of the temporal pole. Current plans revolve around the question of whether the two lesions produced equivalent impairments because they involve a common system. A crossed-lesion experiment should provide evidence in this regard: Only bilateral lesions are effective, so if the prefrontal and inferior temporal pole lesions produce independent deficits, production of the former in one hemisphere and the latter in the other should not produce an impairment. If these two areas of limbic cortex ordinarily participate in a common circuit, an orbital frontal lesion in one hemisphere and an inferior temporal pole lesion in the other should produce a severe deficit, particularly if the forebrain commissures are severed as well. This work is currently underway.

### Behavioral effects of lesions in the dorsomedial thalamus.

This nucleus (DM) is a major source of afferent fibers to, and efferent fibers from, the primate frontal cortex. The medial portions are interconnected with orbital frontal cortex and the lateral portions with the dorsolateral frontal area. Other workers have had some success mimicking behavioral effects of dorsolateral frontal cortex ablation with stereotactic lesions of DM. We have been attempting to produce a prominent "orbital" symptom by selective lesions of medial DM. Considerable effort has been expended to date on developing and improving techniques for reliable localization of small subcortical lesions in monkeys. Considerable effort remains ahead. "Control" animals have produced very interesting findings on

our battery of behavioral tests however. After learning a successive visual discrimination involving button-pressing for intermittent delivery of food pellets during one of two visual stimuli, and extinction (no pellets) during the other, the stimulus-reward contingencies are reversed. When the subject learns this new relationship, they are reversed again, and so on for a total of 5 reversals. As expected, normal monkeys and those previously given lesions of dorsolateral frontal cortex are equally adept at this task. Surprisingly, removal of the lateral portions of orbital cortex (the inferior convexity) also produces no reliable deficit. Lesions which include this cortex, but also extend medially across the surface of the frontal lobe produce a profound ventral impairment however. Future plans include removal of this medial area while sparing the inferior-convexity. We hope this will verify a functional localization not at all evident with present histological techniques.

#### Effects of lesions of the locus coeruleus on food and water intake.

Bilateral lesions of the locus coeruleus occasionally produce urinary disorders, aphagia, and general debilitation in rats, although the frequency of incidence of this syndrome in this laboratory has been low and appears to correlate well with damage ventral to the nucleus of the coeruleus itself. A recent study demonstrated hyperdipsia in coeruleus-lesioned rats as well as aphagia and excessive urinary retention. The lesions in this study were rather large, however, and appeared to include substantial tissue damage ventral to the coeruleus cells. An attempt was made to determine a more exact anatomical localization for these lesion effects. Two different types of lesions were made in rats, and food and water intake were monitored during the recovery period. One group of rats received lesions designed to destroy only the cells of the nucleus of the locus coeruleus itself. Another group received lesions designed to spare the cells of the locus coeruleus, but to damage the subcoeruleus area with comparably sized lesions. A third group of animals received electrode penetrations of the brain but were not lesioned (sham-operated controls). A fourth group was not operated. Preliminary results of this study indicate that neither lesion reliably produces hyperdipsia and urinary retention. However, it appears that if both the coeruleus and subcoeruleus areas are damaged, the syndrome is more likely to appear.

#### Tests of the radioprotective properties of antihistamine and histamine.

Rats exposed to ionizing radiation after drinking a palatable substance such as saccharin solution will subsequently avoid drinking that particular substance. Such conditioned taste aversion learning

is assumed to be supported by the aversive consequences of the radiation-illness experienced by the rat following exposure. The taste aversion paradigm, then, offers a fortuitous opportunity for the study of the effects of proposed radiation protection drugs, under the assumption that rats protected from the toxic effects of radiation by such a drug would not show a conditioned taste aversion following irradiation.

Using this technique, others have recently reported that rats pretreated with an antihistamine do not develop radiation-induced taste aversions. On this basis they suggested that radiation-induced histamine production is the primary event responsible for the aversive after effects of X-irradiation. This conclusion, however, appeared to be subject to some question due to the particular experimental design of the study. In particular, it was noted that the antihistamine treatments occurred before the rats experienced either the saccharin or the radiation. Since the antihistamine treatment itself could possibly be an aversive event, this raised possibility that the administration of the drug interfered with the conditioning process by making the animals ill before exposure to saccharin. This would amount to a backward conditioning paradigm under which it might be expected that conditioning would not occur.

Two studies were conducted in an effort to determine if the antihistamine effect could be shown to be related to experimental design. In the first study, rats were given the antihistamine treatment after drinking the saccharin solution, instead of before drinking. It was assumed that if histamine production was responsible for the aversive effects of subsequent X-irradiation, then the antihistamine-treated rats in this study would not develop aversions. The taste aversion tests revealed that the antihistamine treatments potentiated, rather than attenuated, the formation of aversions in the irradiated rats. In addition, for rats treated with antihistamine the treatment itself was aversive. These results supported the suspicion that the failure to observe conditioning in the study in question was related to the aversive nature of the antihistamine treatments and not to the inhibition of histamine production.

This hypothesis was further tested in a second study. One group of rats was pretreated with antihistamine before conditioning as in the original study. Another group of rats was pretreated with a dose of histamine that was known to be aversive for the animals. The effects of the two treatments upon the taste aversion conditioning were then compared. The results showed that, when given prior to exposure to saccharin, histamine was just as effective in inhibiting the formation of aversions as was the antihistamine, and provided further evidence that the original antihistamine

effect resulted from the flawed experimental design employed. Taken together, the results of these two studies cast serious doubt on the assertion that histamine production has been shown to be the primary aversive consequence of radiation exposure.

#### Limbic system structures and taste aversion learning.

Several brain areas which are traditionally included in the classification of limbic system structures have been implicated as being functionally important in conditioned taste aversion learning, including the olfactory bulbs, the amygdala, the hippocampus and the septum. Studies investigating the effects of lesions of the hippocampus or the septal area in rats on taste aversion learning have produced somewhat conflicting results, with some studies finding deficits with lesions of these areas and others finding no effects. The hippocampal and septal areas are closely related anatomically, each receiving reciprocal connections from the other via the fornix, a thick fiber bundle connecting the two. Studies are presently underway investigating the hypothesis that the integrity of the hippocampal-septal connections is importantly involved in taste aversion learning.

A study has been conducted investigating the effects of knife cuts of the fornix on the acquisition of conditioned taste aversion. Twelve rats underwent an operation in which a stereotaxic neurosurgical knife was lowered into the brain and knife cuts made through the body of the fornix. After recovering from the operation, these rats and a group of twelve non-operated control rats were water deprived and adapted to drinking water during a one-hour daily session. Half of each group was then trained in a conditioned taste aversion paradigm in which they were injected with 0.6 mg/kg of .15 M lithium chloride after consuming 0.1% saccharin solution during their daily drinking session. The other half of each group served as saline-injected controls. Two days later all animals were again offered saccharin solution during their drinking session instead of water. Both operated and non-operated rats injected with saline drank normal amounts of the saccharin solution as expected (means = 18.5 and 17.9 ml). The lithium-injected, non-operated rats also drank large amounts of saccharin (18.8 ml), showing that they did not develop taste aversions as a result of the injections. The lithium-injected, operated animals, however, drank significantly less saccharin than any of the other three groups (mean = 12.5 ml). In other words, the fornix-lesioned animals learned tasted aversions at drug doses that were not strong enough to produce aversions in non-operated rats. This result was unexpected, in view of the fact that

hippocampal lesions have been shown to impair taste aversion learning, and both septal and hippocampal lesioned rats invariably have difficulty acquiring shock-motivated passive avoidance responses. The possibility exists that the fornix-lesioned rats were simply more sensitive to the toxic effects of lithium chloride than normal. The uniqueness of the results of this experiment calls for replication, perhaps using the more conventional electrolytic lesioning technique to produce fornix damage.

#### EXPERIMENTAL ANALYSIS OF DRUG-BEHAVIOR RELATIONSHIPS.

For purposes of organization, studies of drug-behavior relationships are being presented as a separate subsection of this report. Studies in this category are conducted for two principal purposes: (1) The use of drugs in behavioral research is a particularly useful tool for exploring and defining principles which govern the interaction of the organism with its environment. As such, the study of drug-behavior relationships is an extension of the technology applied in the basic work unit; (2) Work unit 102 requires not only direct study of Biomedical Aspects of Drug Abuse, but also the development and critical analysis of an appropriate behavioral technology. Therefore, studies in this work unit (025) yield data directly applicable to the drug abuse work units while, at the same time, they evaluate approaches to the drug problem, and assess the adequacy of the technology and feedback data on the generality of findings with respect to broader issues related to the maintenance and control of behavior. In this context, research in this section utilizing drugs in the study of behavior may be directly related, tangentially related, or unrelated to drug abuse. However, as a matter of policy, all studies in this section have employed drugs of abuse whenever these compounds would suffice in the examination of the issues being addressed.

Drugs as a discriminatory stimulus. Frequently, drug levels required to produce performance changes are relatively high compared to those reported to be associated with subjective experiences. This discrepancy in dose-level raises questions as to the generality of results obtained in studies directed at drug-related performance deficits. The argument has frequently been made that performance decrements are observed at doses considerably in excess of those used by man to produce subjective effects. Because most studies of drug effects on performance are conducted on laboratory animals and because it is known that species differ widely in their sensitivity to pharmacologic compounds, it was considered advisable to develop a procedure which would permit comparison of drug dose-levels producing only changes in subjective experience with those required to produce performance decrements. In the present study, laboratory primates are being used to develop this procedure.

The drug employed in the study is heroin, administered intravenously. The procedure takes advantage of the fact that if an animal is working for food and a stimulus is presented indicating that electric shock is about to be delivered, there is an abrupt disruption in on-going performance. This procedure is known as conditioned suppression. In the present study, placebo, as well as varying doses of heroin, are being administered via chronic venous catheters. Placebos never signal that shock is to be presented, but an administration of heroin is always followed two minutes later by electric shock. If the animal can discriminate the difference between a placebo and a given dose of heroin, it can reliably anticipate the presentation of shock, and the characteristic anticipatory disruption of on-going behavior can be observed. After demonstrating the feasibility of the technique, different doses of heroin ranging from 1.56 to 200 microg/kg were exposed. Heroin was reliably discriminated from saline at doses as low as 6.25 microg/kg. Furthermore, the discriminability of this dose of heroin did not change over months of repeated testing. The procedure was then modified to explore the comparability of the stimulus characteristics of morphine and heroin. In general it was found, that morphine was not discriminated from heroin, if morphine was presented to the organism at approximately 4 times the concentration of heroin. However, the response to morphine and heroin is not similar in all respects. The differences between morphine and heroin are presently being examined using this technique.

The pharmacological basis of amphetamine-induced hyperkinesia:  
Effects of lesions of catecholamine-containing cell groups in  
the brainstem.

Studies attempting to elucidate the pharmacological actions of amphetamine in inducing locomotor activity have implicated both the catecholamine neurotransmitters norepinephrine and dopamine, as well as serotonin. Perhaps the greatest interest has been paid to the role of the catecholamines due to evidence that pretreatment with  $\alpha$ -methyl paratyrosine, which inhibits catecholamine synthesis, blocks the increases in motor activity in rats caused by amphetamine.

Studies attempting to establish a primacy for either norepinephrine or dopamine in the mediation of the amphetamine locomotor response have produced somewhat conflicting results, but a majority of these studies have implicated dopamine and there have been suggestions that amphetamine effects are mediated by specific dopaminergic pathways. The present studies were undertaken in order to study the effects of electrolytic lesions selectively destroying either noradrenergic or dopaminergic neuro-

transmitter pathways upon the locomotor activity and response to amphetamine in the rat.

Experiment 1 examined the effects of lesions of the nucleus locus coeruleus, which is the origin of noradrenergic fibers that innervate the cerebellum, hippocampus, and all parts of the cerebral cortex. Two groups of four rats each were prepared, one with bilateral lesions of the locus coeruleus, the other sham-operated controls. After recovery from surgery, the animals were given five daily, 30 minute activity tests in photo-beam activity chambers. Subsequently, both groups were tested under 1.0 mg/kg of amphetamine sulfate, a dose that reliably produces hyperkinesia in normal rats. The results of these tests showed that there were no differences between the lesioned and sham rats either in baseline activity levels or in amphetamine-induced activity. Both groups showed normal elevated activity under 1.0 mg/kg of amphetamine. Neurochemical analyses of the brains of the rats revealed that the lesions of the locus coeruleus produced depletions of norepinephrine in the cortex ranging from 86% to 28% with a mean of 57%, relative to the sham controls.

Experiment 2 examined the effects of lesions of the ventral tegmental area dorsolateral to the interpeduncular nucleus, an area of dopaminergic cell groups that send axons to the nucleus accumbens and olfactory tubercle areas of the forebrain, both of which have been implicated in lesion-produced activity changes. Ten lesioned and ten sham-lesioned control animals were prepared and activity tests were conducted as in Experiment 1, with the exception that these groups received tests at various doses of amphetamine, ranging from 0.25 mg/kg to 4.0 mg/kg. The results of these tests showed that the lesioned animals exhibited a long-lasting enhancement of baseline activity, and that there was no decrement in the amphetamine-induced activity response. In fact, there was a non-significant trend in the data suggesting that the lesions of the dopamine-containing cell groups may enhance, rather than attenuate, the response to amphetamine. The chemical analysis of the brains of the lesioned animals revealed that the average depletion of dopamine in the accumbens-tubercle area was 80% relative to the sham-lesioned controls, while the depletion of the corpus striatum was 45%. In addition, it was found that norepinephrine levels in these areas were 38%, and 35% respectively, lower than those of controls. Hippocampal serotonin was also measured, and was shown to be 49% lower than that of the controls.

The results of Experiment 1 showed that animals with lesions of the locus coeruleus behaved similarly to non-lesioned animals in activity testing, with respect to both normal exploratory activity levels and amphetamine-induced hyperactivity. While these

results do not rule out a role of norepinephrine in the amphetamine activity response, they do suggest that the integrity of the dorsal noradrenergic pathway originating from the locus coeruleus is not essential for this behavioral effect of amphetamine. The results of Experiment 2 showed that brainstem lesions that significantly depleted the forebrain of dopamine, norepinephrine, and perhaps serotonin as well, produced elevated activity levels but failed to abolish the hyperkinetic response to amphetamine. This was somewhat surprising in view of the popularity of some recent theories proposing that the effects of amphetamine in producing hyperactivity are mediated by catecholamine-containing pathways. However, the region of the ventral tegmentum is clearly involved in the control of locomotor behavior, probably in an inhibitory manner. The results of these studies suggest that the pharmacological basis of amphetamine effects may not be attributed either to a specific catecholamine neurotransmitter or to a specific neural pathways associated with them.

Behavioral Contrast and THC. A recent hypothesis that THC increases the aversiveness of non-reinforcement was tested by assessing the effects of THC on behavioral contrast. That is, three monkeys were trained to lever-press for food reinforcements on a multiple VI 30 sec schedule. When performance was reliable, the schedule was changed to multiple VI 30 sec Extinction. Responding, of course, declines during the extinction component of such a schedule, but in the VI component responding typically increases to levels above those of the baseline VI conditions. Some investigators feel that this paradoxical increase during the unchanged component is related to the aversiveness of the altered component. All three subjects in this experiment were tested for such increased responding three times, twice with saline (S) pretreatment and once with THC pretreatment, in the order S, THC, S. No consistent difference was observed during the THC phase of the experiment, thus providing no support for the hypothesis.

Tolerance to the Effects of THC on Spaced Responding in Rats.

In a preliminary study to determine appropriate dose levels and schedule parameters, rats were given lever press training under one of two DRL schedule of reinforcement: DRL 10 seconds, under which only interresponse times >10 sec produced food pellets, and DRL 30 sec. When performance had stabilized, THC was given via intragastric syringe. Effects on the DRL 10 sec were equivocal except at doses above 12 mg/kg, which depressed response rates. Under the DRL 30 sec contingency, doses below 6 mg/kg produced enhanced responding, and doses above this depressed responding. Both effects diminished when dosing was continued on a daily basis, i.e. tolerance was observed.

Subsequent experiments have been and will be addressed to the nature of this prominent tolerance. We have suggested elsewhere that this tolerance is predominantly a response to the disadvantageous effects of the drug rather than to the drug itself. That is, the changes in the animals behavior subsequent to initial exposure to THC are mostly a function of the Law of Effect, and are only marginally related to the presumably "pharmacological" tolerance responsible for such things as increased LD<sub>50</sub> after repeated exposure.

In one experiment designed to test this proposal, we are training rats to lever-press under a DRL 30 contingency. Daily lever-press sessions are then suspended for 12 days. During this time half the subjects are given daily doses of either THC (4 mg/kg) or vehicle placebo. On day 13 through 18 rats are tested for lever-pressing 3 hrs after intragastric dose of THC. To date we have not seen any difference between groups in the rate-enhancing effect of THC, or on the diminution of this effect over a series of 5 daily tests.

#### Sex differences and hormonal interactions with behavioral effects on THC.

Pirch and his colleagues have reported on sex differences in some responses of rats to THC. We plan to extend these studies to the sensitive operant tasks which we have already investigated in some detail. Preliminary findings at one dose level suggest that the performance of female rats bar-pressing for food pellets on a VI 30" schedule is indeed more sensitive to THC than that of males. Gonadectomy and/or hormone administration appear as possible avenues of investigation should full dose-response curves confirm these data.

#### Heroin as an aversive reinforcer.

The effectiveness of the drug state induced by heroin hydrochloride in supporting taste aversion learning was studied. Groups of five rats each were injected either intraperitoneally or intravenously with various dose levels of heroin after consuming 4 ml of 0.1% saccharin solution. A control group was injected via both routes of administration with comparable volumes of saline. Taste aversion learning was assessed 48 hrs later with 20-min 1-bottle saccharin ingestion tests.

The saline-injected control rats drank a mean of 17.6 ml of saccharin solution during the post-injection tests. The three groups of rats injected intraperitoneally with either 2.5, or 10.0 mg/kg

of heroin consumed 14.5, 13.8, and 6.9 ml. respectively, demonstrating an increasing level of aversion for the saccharin as heroin dose level increased. The three groups injected intravenously with either 0.5, 2.5, or 5.0 mg/kg of heroin consumed 17.7, 12.1, and 17.3 ml, respectively, showing that only the intermediate dose of 2.5 mg/kg was effective in reducing saccharin consumption.

These results indicate that injections of heroin at dose levels of 10.0 mg/kg IP and 2.5 mg/kg IV will produce avoidance learning within a taste aversion paradigm. Surprisingly, animals injected with 5.0 mg/kg IV did not form aversions. It may be suggested that intravenous administration of heroin at this dose, which invariably produced immediate convulsions and which proved fatal to two out of seven rats injected, may have produced a state of immediate anesthetization that interfered with conditioning.

#### Acute and chronic effects on THC on a complex behavioral task.

Rats performed in a two-lever chamber under a complex schedule of reinforcement. A different fixed-interval schedule was programmed on each lever, but no exteroceptive stimuli were present to indicate to the rat which schedule was in effect at any given time. This particular behavioral task was chosen as a baseline for the study of THC because an animal could greatly increase or decrease its overall response rate on either or both levers without substantially changing its overall frequency of reinforcement. This was possible because the delivery of reinforcement was based on a single response on the appropriate lever after a period of time had elapsed. Whether a rat responded on either lever or sat quietly in the chamber during the time interval had no effect on the frequency of reinforcement as long as one response was made after the time elapsed. This particular feature of the procedure helped to separate the effects of the drug from the general behavioral effects of reducing the frequency of reinforcement. This procedure also allowed the concurrent measurement of two operants (responding on each of the levers) which were both reinforced with the same reinforcer (milk), but which were independent of one another.

In the acute-dosing phase of the experiment, rats received THC once a week. Doses were doubled and presented in an ascending order. Presumably, tolerance to the effects of THC developed more rapidly than the doses were increased, as decreases in response rates were not seen until very high doses (32 and 64 mg/kg). When doses were quadrupled and given in an ascending order, 5 rats out of the 5 showed almost total suppression of responding on both levers at 8 mg/kg.

### Acute effects of ethanol on a complex behavioral task.

Rats performed in a second two-lever chamber under the same complex schedule described above for the THC rats. Ethanol was administered every three to five days. Seven different doses (.5 g/kg to 3.5 g/kg) were given. There were three administrations of each dose. Monotonically decreasing dose-response curves for both levers were obtained in all 6 rats. However, as in many published reports of the effects of ethanol on various types of fixed-interval schedules, there was almost no change in the animals ability to perform appropriately under the schedule as measured by the patterning of responses over time. In other words, even though response rates decreased, the fixed-interval scallops remained intact.

### The effects of THC and ethanol given in combination.

Several sets of dose-response curves have been determined. Using the group of animals from the acute ethanol study, two different doses of ethanol were given chronically and an ascending series of THC doses were administered acutely. The effects of the THC were much larger (more suppression of responding) when the doses were given in combination with the ethanol than when the THC was administered alone. It is important to note that all animals were tolerant to the dose of ethanol that was used in the combination. The animals that were receiving the larger dose of ethanol (2.5 g/kg) showed total suppression of responding at a lower dose of THC than the rats in the other group that received 2.0 g/kg of ethanol. Using the set of animals from the chronic THC study, 16 mg/kg of THC was administered in combination with an ascending series of doses of ethanol. Even though all the rats were tolerant to the THC, the THC greatly potentiated the effects of the ethanol at every dose.

### Drug effects on schedules that engender similar response rates by means of different contingencies.

A prevalent notion among behavioral pharmacologists is that the on-going rate of responding is the critical determinant of the effect of a drug. Consequently, many experiments are being carried out in various laboratories where different rates of responding are engendered by adding contingencies to a schedule. For example, it is possible to generate three different rates on a VI 3-min schedule by adding different DRL requirements to the schedule. This has the effect of requiring the animal to space his responses differently (closely vs. far apart). The current practice is to treat these carefully shaped rates of responding as if they are

the same as naturally occurring rates. This experiment was designed as a direct comparison of these two types of schedules. Monkeys work on a multiple schedule with fixed-interval schedules in both components. However, one of the fixed-intervals has the added requirement that the animal must make the reinforced response in a certain time period since his last press (a paced schedule). For example, the monkey may have to wait at least 1 sec but not more than 4 sec in order to be reinforced. The parameter values of the waiting period have been adjusted for each animal so that his non-drug performance looks almost identical under both schedules. Given this baseline, the rate-dependency hypothesis would predict that the drug effect would be identical under both schedules. However, if the contingencies of reinforcement are more important in determining drug effects, then the drug effects might be quite different since it is much harder for the animal to satisfy the rules for reinforcement under the paced schedule.

Two monkeys are currently part way through an amphetamine dose-response curve. Preliminary indications are that the paced schedule is much more depressed under drug than is the regular fixed-interval. This is the expected direction of the data if the contingencies of reinforcement are more critical than the rate in determining the effect of the drug. After the dose-response curve is complete, a dose that produces suppression of both schedules will be administered chronically. We expect to see partial recovery of the unpaced schedule until the frequency of reinforcement is back to baseline. However, we do not expect to find total tolerance. On the other hand, we expect to eventually see almost total tolerance under the paced schedule since that is the only way for the animal to perform and to receive the maximum number of reinforcements in each session.

Differential effects of delta-9-THC on performance by monkeys on two time-based schedules of reinforcement.

Two monkeys were trained on a multiple fixed-interval (FI) 120 sec, differential reinforcement of low-rate (DRL) 120 sec schedule of reinforcement in which the two schedules, each correlated with a distinctive cue, alternated throughout an experimental session. Under chronic daily treatment with delta-9-tetrahydrocannabinol in doses of 3.5 or 7 mg per os for more than 40 consecutive days, performance on the DRL was affected much less dramatically than that on the FI schedule, with the latter showing substantial increases in rate of responding throughout the chronic drug administration periods. Even though reinforcement frequency on the DRL schedule remained suppressed and FI reinforcement frequency was unaffected during chronic drug treatment, DRL performance returned to baseline (i.e. showed tolerance), while FI performance did not.

Serotonergic involvement in the selection and ingestion of a morphine solution by rats.

Albino rats were given a solution of 0.5 mg morphine HCL per cc of a 0.1% saccharin solution as their only source of fluid on two of every three days. On the third day, they were allowed free access to both this solution and one containing only the saccharin. Within a period of 4-6 weeks, most rats overcame a strong initial aversion to morphine and showed a pattern of copious ingestion on non-choice days plus preference for the drug solution on choice days. Naloxone injection elicited the classical withdrawal syndrome though it did not increase morphine intake or preference, and morphine pre-treatment reduced both ingestion and preference. The serotonergic blockers p-chlorophenylalanine (PCPA) and p-chloroamphetamine (PCA) also reduced both intake and preference. Chlorotyrosine, a known metabolite of PCPA which does not affect serotonin levels did not reliably affect preference for morphine solution over the saccharin vehicle, nor did Lilly 110140, a highly specific inhibitor of uptake by serotonin neurons. The latter did however, delay the effect of PCA on morphine preference.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03 Psychiatry

Work Unit 025 Analysis and management of behavior and stress  
in military environments

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