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# RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

## Including

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

### ANNUAL PROGRESS REPORT

1 July 1975 - 30 June 1976

## VOLUME I

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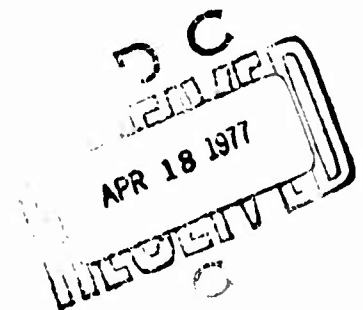
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## VOLUME I

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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

INCLUDING

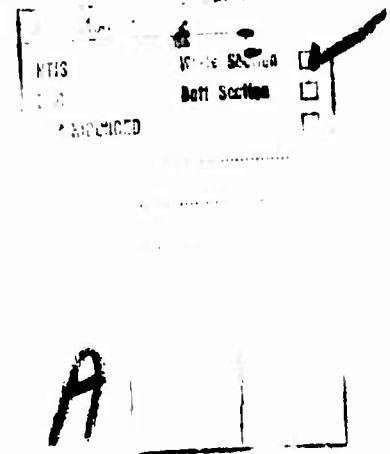
BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY,  
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(Projects, tasks, and work units  
are listed in Table of Contents)

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1 July 1975 - 30 June 1976

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.

The FY 7T report will be included in the FY 1977 Annual Progress Report.

## 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.

## 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 Resources, National Academy of Sciences - National Research Council.

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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 SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL <sup>a</sup>	
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RESPONSIBLE INDIVIDUAL Joy, Robert J. T., COL, MC				NAME <sup>a</sup> Hall, Robert D., MAJ, VC			
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<p>23. (U) Better knowledge of the owl monkey is needed to improve its use as a laboratory resource. Physiologic data are either unavailable or unconfirmed. Biologic and physiologic data will be obtained to optimize colony management procedures, recognize disease states, and successfully breed and raise aotus for studies of falciparum malaria, for which it is the best nonhuman primate host. The Army conducts the lead national effort in malaria drug development research which is dependent on an adequate supply of these animals infected with human malaria strains for drug testing.</p> <p>24. (U) Physiologic data will be obtained from infants to fully mature adults to include the following measurements: hematologic, serum biochemical and enzyme, estrous cycle, gestation period, and growth and development patterns. Standard procedures will be used.</p> <p>25. (U) 76 01 - 76 06 Management procedures modified during the past year include better caging and standardization of diet to assure adequate nutrition, reduce waste, and detect anorexia. Environmental stress was reduced by using one caretaker for routine husbandry and by preliminary utilization of a hormonal assay to detect pregnancy. Rectal temperatures exhibited a wide range of normalcy and was affected by physical and chemical restraint. Changes in body weight as great as 10 per cent occurred over a 2-3 day period. Rectal temperature and body weight changes are, therefore, of limited diagnostic value. Hematologic and serum biochemical measurements confirm and extend existing reports. Karyotyping results should increase breeding efficiency and permit selection of the best animals for malaria research. Vaginal cytology indicate the estrous cycle is approximately 14 days, while other observations approximate the gestation period at 18 weeks. The chronological sequence of dental eruptions provides a method for estimating the age of infant and juvenile aotus. Studies continue. For Technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$39,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>							

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 095 Investigations of the Owl Monkey as a Laboratory Animal

Investigators.

Principal: Robert D. Hall, MAJ, VC

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George H. Wyckoff, Jr., LTC, VC, M. J. Reardon, MAJ, VC,  
L. Crye, SP4, A. L. Abston, SP4.

Description.

The South American "owl" monkey, Aotus trivirgatus, is the most suitable sub-human primate which is susceptible to infection and disease with malaria parasites pathogenic to man. During the Vietnam war, malaria was the single most important infectious disease subverting the health and effectiveness of the soldier. A military requirement to continue research on better methods of chemoprophylaxis and treatment is of the highest order, since malaria is endemic in most of Asia, Africa, and South and Central America. Since 1965, the aotus monkey has been an essential part of the WRAIR malaria research program, approximately 200 monkeys per year were used for this purpose. However, since 1974, the South American countries of origin for this monkey have prohibited their export. Additionally, the aotus is not available from commercial breeding source because none exist. Obviously, the continuance of a successful malaria research program depends, in part, upon establishing a local source of suitable aotus monkeys. From January 1974 through June 1975, 13 aotus infants were born and raised at the WRAIR from 8 of 14 aotus pairs, indicating that the aotus can be conceived, born and raised successfully in captivity. However, many colony management practices, such as caging, temperature and humidity control, lighting, restraint, and diet have been extrapolated from practices successfully used with other species of sub-human primates, or have been designed to approximate the tropical habitat of the aotus, without evidence that either method is optimum for the successful operation of a closed colony of aotus monkeys. Additionally nothing is known about specific biological/biochemical measurements which are essential for recognizing disease states, promoting maximum breeding efficiency, and providing an animal particularly suited for malaria research. The WRAIR now has 233 aotus monkeys, 100 males and 131 females. While most have already been infected with malaria and are of limited use for malaria research,

they are potentially valuable as breeding stock. This is the first report describing management procedures, and the development of biologic and physiologic measurements which will assist in successful development of a closed colony of aotus monkeys for use in malaria research.

#### 1. Colony Management.

Some aspects of caging, environmental control, restraint and diet which have been used and discarded as inadequate are described below in relation to current practices.

No attempt has been made, nor is one considered necessary, to design a cage particularly suited for housing aotus monkeys. Each of 4 cages within a rack currently used measures 31" x 30" x 36" (15.6 ft<sup>3</sup>), is less confining than the cage (9.5 ft<sup>3</sup>) commonly used for rhesus monkeys, and is more manageable and efficient than the large baboon cage (27.8 ft<sup>3</sup>). Metal perches within each cage and grid floors preclude the animal's tails from being soiled by fecal waste or being bitten by animals in adjacent cages, two characteristics commonly associated with use of the rhesus cages. Wire panels over the top of closely approximated racks form an enclosed corridor between rows of cages which facilitates the atraumatic capture of escaped animals. No modification of these cages is anticipated.

Ideal conditions of temperature, humidity, and circadian light have not been evaluated for the aotus. Originally, attempts were made to approximate the high ambient temperatures (78 F to 85 F) and relative humidity (80% to 95%) of the animal's native habitat. Accommodating the animal's nocturnal habits, red filters were placed over fluorescent light bulbs to reduce the intensity of artificial light during the work day. The continued good health of the animals with the relative humidity between 50% and 70% suggests that colonized animals may not require environmental conditions different from those required to maintain other laboratory animals in good health. However, rapid, albeit transient, decreases in ambient temperature which sometimes occur during the winter months, are not as well tolerated in the aotus compared with other monkeys. This poor tolerance to cold may be related in part to the constantly high ambient temperature in which the aotus are housed. Thus far, there have been no attempts to evaluate alternative, suitable conditions of ambient heat and light.

Current and previous observations demonstrated clearly that the aotus, particularly when pregnant, has a poor tolerance for handling

or other forms of environmental stress. Physical examinations given to 8 pregnant aotus received from another institution resulted in 7 abortions. During 1975, vaginal smears were obtained Monday thru Saturday of each week for 40 weeks from 28 aotus monkeys, (including 7 which became pregnant) in an effort to understand the estrus cycle. These manipulations resulted in 5 abortions and irregular estrus cycling for most of the females. During 1974, the 5 mothers which aborted had produced and successfully raised babies when handling was reduced to a minimum. Urine collected from under each cage is now being used to successfully assess pregnancy without handling the animal. During the periodic (Mon, Wed, Fri) measurement of the body weights of 2 aotus over a 3 month period, it was observed that the 2 weights increased or decreased simultaneously in 36 of 40 measurements, suggesting environmental rather than host influences. Paired decreases in weights occurred 11 times (64%) over the weekend and only 6 times (35%) during the week. This unusual frequency of weight reduction over the weekend was attributed to the practice of using a number of different animal caretakers for weekend duty as contrasted with using only one particular man Monday thru Friday. Currently, only one man cares for the aotus monkeys 7 days per week. Weight measurements have not been taken since this management change to evaluate the effect on weekend weight loss. Chemical restraint, Ketamine HCL at a dosage rate of 10 mgm/kg intra-muscularly, is used more frequently with aotus than with other monkeys to reduce the stress of handling. Generally, painful procedures, e.g. bleeding, or procedures lasting longer than 3 min to 5 min require the use of chemical restraint.

Very little is known about the natural diet or nutritional requirements of the aotus monkey. Other animal facilities, including our own, have successfully used commercial monkey diets supplemented with a variety of fresh fruits, artificial vitamins and protein concentrates. The aotus diet currently fed represents a subjective attempt to satisfy all possible nutritional requirements until more definitive data are available. The basic diet was Purina monkey chow 25 (Ralston Purina Co). Other diets fed as a supplement included Marmoset Diet (Riviana Foods Inc) and "liquid" diet, a mixture of baby cereal, orange juice, raw eggs, a high calorie pet supplement, and vitamins B, C, and D. Fresh apples and oranges supplemented these diets. The complexity of this dietary regimen assured that without strict accountability of feeding, monkeys would be overfed, underfed, and poorly fed. Additionally, anorexia, the most useful prodromal sign of illness, is as life-threatening with the aotus as with human infants. A feed trial was designed using 44 pairs of monkeys to assess the optimum number of monkey chow biscuits to feed

when the latter was supplemented on Friday thru Monday with fruit (1/2 apple or orange), on Wednesdays with 1 1/3 oz of Marmoset Diet, and on Tuesdays and Thursdays with 75 ml of "liquid" diet per animal. Each pair of monkeys ate approximately 12, 11, and 8 biscuits per day, respectively, when supplemented with fruit, Marmoset Diet, and liquid diet. Most food was eaten at night. Use of the above described feeding regimen has reduced the wastage of food and provided a specific method for the early detection of anorexia.

## 2. Biologic/Physiologic Measurements.

A number of biologic and biochemical measurements were conducted over the past year to assist early disease recognition, facilitate breeding efficiency, and to detect the most suitable animals for malaria research.

In other species, a sudden and dramatic weight loss is one of the most useful criteria for detecting disease condition. To assess the value of this measurement for aotus, 2 male animals were weighed Monday, Wednesday and Friday of each week for 3 months (41 measurements). The average weights of the two animals + one standard deviation were, respectively,  $927 \text{ g} \pm 52 \text{ g}$  and  $897 \text{ g} \pm 24 \text{ g}$ . The average changes in body weight between the 2 - 3 day measurement periods for the 2 animals, respectively, were  $40 \text{ g} \pm 29 \text{ g}$ , and  $24 \text{ g} \pm 21 \text{ g}$ . These results confirmed previous observations that weight changes of 10% or more of the body weight occurred frequently with aotus monkeys and could not be correlated with ill health. However as previously described, 90% of the measurements showed both animals increasing or decreasing their weight simultaneously, suggesting that environmental factors were the cause.

A sudden rise or fall in rectal temperature is clinically useful in other species as an early diagnostic aid and to establish prognoses. The expected range of "normal" values for the rectal temperature of the aotus monkey was not known. Consequently, rectal temperatures were obtained from 27 adult, female aotus monkeys on 8 consecutive Mondays using a thermistor (Tele-Thermometer, Yellow Springs Instrument Co) placed 3 cm into the rectum. On two occasions, chemical restraint was used as previously described. The mean rectal temperature was one degree lower with chemical restraint (100.8 F) than with physical restraint (101.8 F) and individual measurements ranged from 99.0 F to 104.1 F. These measurements indicate that the "normal" aotus monkey exhibits a wide range of rectal temperature which differs with physical versus chemical restraint and which limits its usefulness as a diagnostic aid. Consequently, to reduce the stress of handling, rectal temperatures will not be routinely used with aotus



monkeys to monitor their clinical status.

Knowledge of the dental eruption sequence of animals provides a useful basis for estimating age. Accordingly monthly dental examinations were made on 9 juvenile aotus, 7 to 30 months old, and twice weekly examination on two newborns followed by biweekly examination after deciduous teeth had erupted. The sequence of eruption for deciduous teeth is shown in Table 1. The aotus are born toothless. Within one week after birth, deciduous incisors begin to erupt; all deciduous teeth appear to be present by the end of the 6th week. The eruption sequence of deciduous teeth is  $I_1$ ,  $I_2$ , C,  $P_1$ ,  $P_2$ , and  $P_3$ . The eruption sequence of permanent teeth, starting and ending at approximately 4 and 16 months of age, respectively, is:  $M_1$ ,  $M_2$ ,  $I_1$ ,  $I_2$ ,  $M_3$ ,  $P_3$ ,  $P_2$ ,  $P_1$  and C (Tables 2 and 2a). Generally, mandibular teeth erupt before maxillary teeth. A few more observations should complete this scheme for estimating the age of juvenile aotus on the basis of dental eruption sequence.

Selected hematologic measurements were made by standard methods from blood collected once from 166 healthy adult aotus monkeys (Table 3), and serum biochemical measurements were made by standard methods from 76 of these same animals (Table 4). The mean and range of hematologic values are similar to those described by Wellde<sup>3</sup> and Porter.<sup>4</sup> Insufficient data do not permit interpretation of the high eosinophilia observed by Wellde<sup>3</sup> and ourselves in a number of animals. Except for minor differences for the ranges of serum albumin and SGOT measurements, our results are comparable to those described by Wellde.<sup>3</sup>

Karyotypic differentiation of aotus monkeys is not only of taxonomic interest to primatologists or helpful in assessing geographic origin but it bears specifically upon the WRAIR's attempt to successfully establish a breeding colony of animals particularly susceptible to malaria. In the largest sample of aotus monkeys (330) examined to date, Ma<sup>5</sup> reported finding 4 phenotypes and 7 karyotypes, with chromosome numbers varying from  $2n = 46$  to  $2n = 54$ . Normally, matings between animals with different chromosome numbers do not result in viable offspring. In fact, aotus with equal chromosome numbers of different types which preclude forming equivalent pairs, may not be able to mate successfully.<sup>6</sup> The failure of Aotus trivirgatus ( $2n = 54$ ) to mate successfully with Aotus griseimembra may represent this situation. Reportedly, a mutation of Aotus griseimembra ( $2n = 54$ ) resulted in offspring with  $2n = 52$  as a result of fusion of chromosome pairs B13 and B14.<sup>5</sup> Mating two aotus with  $2n = 52$  and  $2n = 54$ , respectively apparently produces hybrid offspring ( $2n = 53$ ) with 3 unpaired chromosomes and the same amount of

genetic material as each parent. Ma5 found 130 of the 330 animals she examined were hybrids ( $2n = 53$ ). Mendelian genetics indicates only 10 of 64 zygotes formed from hybrid matings would contain the normal amount of genetic material. Since monkeys with more or less genetic material than normal have not been reported, it is likely that such zygotes are nonviable. Mating a hybrid ( $2n = 53$ ) with a ( $2n = 54$ ) karyotype would increase the probability of a viable zygote to one in four. Obviously, knowing the karyotype of breeding animals is essential for selecting mating pairs most likely to produce viable offspring. Additionally, Aotus griseimembra has been shown to be more susceptible to the human strains of Plasmodium sp. than Aotus trivirgatus.<sup>5</sup> Consequently, blood samples from 105 aotus have been submitted to the New England Regional Primate Center for karyotypic examination. A preliminary report on 50 specimens shows the following karyotypic spectrum: 13 Aotus trivirgatus ( $2n = 54$ ); 22 Aotus griseimembra ( $2n = 54$ ); 5 Aotus griseimembra ( $2n = 52$ ); 9 hybrids ( $2n = 53$ ); and one male aotus ( $2n = 49$ ) with a translocated chromosome. The latter animal is phenotypically similar to Aotus trivirgatus, but reportedly originates from Bolivia rather than Brazil.<sup>5</sup>

Efficient management of a breeding colony is predicated, in part, upon the ability to detect estrus and knowledge of the length of the estrous cycle. In many species of sub-human primates, genital swelling and vaginal blood (menses) are associated with estrus. Estrus is not associated with these phenomena in the aotus, and there are no reports describing its estrous cycles. Consequently, the vaginal cytology of 28 aotus was examined 6 days a week from July 1975 up to the present time. Fourteen animals remained paired with males they had been with for 18 months previously, and 14 were individually caged. Vaginal smears were obtained on calcium alginate swabs, rolled across labelled glass slides, fixed with aerosolized ethanol, stained with Papanicolaou stain, and permanently mounted. In the aotus, like other sub-human primates, the number of exfoliated epithelial cells increased to a maximum at estrus, then gradually declined. However, at estrus the aotus showed a predominance of more primitive intermediate and transitional type epithelial cells, unlike the anucleated squamous cells of the rhesus and other old World monkeys. Although the stress of handling caused the absence of or incomplete ovulatory cycles in most of the females, 13 complete cycles were identified, with a mean duration of  $14 \pm 4$  days. Seven animals became pregnant; two produced live babies, and five aborted, presumably because of excessive handling. Data derived from this study are particularly valuable in relation to the deleterious effect of handling on estrus and gestation. This information is essential for future breeding

studies. However, untoward reactions associated with obtaining vaginal smears and the limited information obtained therefrom would seem to preclude its use in any breeding program.

The length of gestation has not been described for the aotus. The pregnancy of two animals which conceived during the study of vaginal cytology was monitored by palpation, radiographs, and the presence and amount of urinary chorionic gonadotropin (CG) using a Sub-human Primate Pregnancy Test Kit (Ortho Diag Inc., Ramitan, NJ). Starting at the eighth and continuing through the fifth week before parturition, CG was detected in the urine of pregnant aotus. The earliest detection of pregnancy by palpation occurred approximately 2 weeks after the last peak estrus by vaginal cytology, and 16 1/2 weeks before parturition. Radiographically, the fetus measured 6.5 cm x 4.5 cm 8 weeks before parturition. Faint but spotty calcification was evident 6 weeks before parturition, and was distinct one week later. These observations indicated that the gestation period for the aotus monkey is approximately 18 weeks.

#### Summary.

Colony management procedures of aotus monkeys have been modified as a result of observations during the past year. A more suitable cage is now used; the components and amount of daily diet have been standardized to assure adequate nutrition, reduce waste, and detect anorexia; environmental stress has been reduced by utilizing only one caretaker for routine daily husbandry, and by preliminary utilization of a hormonal assay to detect pregnancy in lieu of physical methods. Rectal temperature showed a wide range of normalcy and was affected by physical and chemical restraint. Changes in body weight as great as 10% occurred in apparently normal monkeys over a 2 - 3 day period. Rectal temperature and body weight changes are, therefore, of limited diagnostic value. Selected hematologic and serum biochemical measurements have been made which confirm and extend existing reports. Karyotyping is in progress; results are expected to increase breeding efficiency and permit selection of the most suitable animals for malaria research. Continuing studies on vaginal cytology indicate the estrous cycle is approximately 14 days while other observations approximated the gestation period to be 18 weeks. A general method for estimating the age of infant and juvenile aotus was developed around the chronological sequence of dental eruption. Studies continue.

Table 1: Chronological eruption of deciduous teeth in weeks for two aotus monkeys

Animal Number	Age in weeks	1st Incisor		2nd Incisor		Canine		1st Premolar		2nd Premolar		3rd Premolar	
		MX <sup>1</sup>	MD <sup>2</sup>	MX	MD	MX	MD	MX	MD	MX	MD	MX	MD
44	Birth to 7	1-2	1-2	3-3½	1-2	3-3½	3-3½	3-3½	3-3½	3-3½	3-3½	4-6	4-6
43	2 to 7	1-2	1-2	2½-3	1-2	2½-3½	3-3½	3½-4	3½-4	3½-4	3½-4	4-5	3½-4

1. Maxillary

2. Mandibular

Table 2: Chronological eruption of permanent maxillary teeth in months for 11 aotus monkeys

Animal No.	Age in months	1st Incisor	2nd Incisor	Canine	1st Premolar	2nd Premolar	3rd Premolar	1st Molar	2nd Molar	3rd Molar
29	30									
31	17 to 22			17-18						
32	16 to 20			15-16						19-20
34	13 to 17			13-14						
35	13 to 16			15-17						
36	11 to 15			13-15						12-13
37	10 to 15			13-15	11-12	11-12	8-11			10-11
38	10 to 16			14-16	12-13	NO <sup>1</sup>	NO			10-11
40	7 to 13	10-11	11-12		13-	13-	12-13		7-8	11-12
43	0.5 to 7							4-5		
44	Birth to 7							4-5		

1. Not observed

☒ Teeth erupted before examination

☐ Teeth not erupted

Table 2a: Chronological eruption of permanent mandibular teeth in months for 11 aotus monkeys

Animal No.	Age in months	1st Incisor	2nd Incisor	Canine	1st Premolar	2nd Premolar	3rd Premolar	1st Molar	2nd Molar	3rd Molar
29	30									
31	17 to 22									
32	16 to 20									
34	13 to 17			14-15						
35	13 to 16			15-16						
36	11 to 15			13-14	11-12	11-12				
37	10 to 15			12-13	11-13	11-12				
38	10 to 16			14-16	12-13	12-13				
40	7 to 13	NO <sup>1</sup>	NO		13-	13-	9-11			9-10
43	0.5 to 7							4-5	6-7	6-
44	Birth to 7							4-5		

<sup>1</sup>.Not observed

Teeth erupted before examination

Teeth not erupted

Table 3: Selected hematologic values of 166 Aotus trivirgatus

	Mean	Standard deviation	Range
RBC( $\times 10^6/\text{mm}^3$ )	5.39	0.75	3.30-7.19
PCV(%)	46	5.3	31-60
Hemoglobin(g%)	16.4	2.0	11.5-20.6
MCV( $\mu^3$ )	86.2	7.3	63.1-106.1
MCHC(%)	35.6	2.8	30.2-42.3
WBC( $\times 10^3/\text{mm}^3$ )	12.09	3.89	5.4-28.5
Neutrophiles/ $\text{mm}^3$	3864	2444	364-14,535
Lymphocytes/ $\text{mm}^3$	7127	2549	2295-16,000
Eosinophiles/ $\text{mm}^3$	828	814	0-3696
Monocytes/ $\text{mm}^3$	110	167	0-895
Basophiles/ $\text{mm}^3$	161	162	0-948

Table 4: Selected serum biochemical values of 76 Aotus trivirgatus

	Mean	Standard deviation	Range
BUN(mgm%)	13	6	5-45
Total protein (g%)	7.5	0.5	6.5-8.8
Albumin(g%)	4.1	0.5	2.6-5.0
Globulin(g%)	3.4	0.7	1.7-4.9
A/G ratio	1.30	0.42	0.53-2.94
SGOT <sup>1</sup>	142	63	10-280
SGPT <sup>1</sup>	39	30	6-250
Na <sup>+</sup> (mEq/l)	146	5	114-153
K <sup>+</sup> (MEq/l)	3.1	0.4	2.2-3.9

<sup>1</sup>. Reitman-Frankel units

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 095 Investigations of the Owl Monkey as a Laboratory Animal

Literature Cited:

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION*		2 DATE OF SUMMARY*		REPORT CONTROL SYMBOL	
				DA OB 6504		76 06 30		DD DRP,2:(AR)636	
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		61101A		3A161101A91C		00		114	
11A PRIMARY									
11B CONTRIBUTING									
11C CONTRIBUTING									
12 TITLE (Precede with Security Classification Code)*									
(U) Neurophysiological Control of Antibody Response									
13 SCIENTIFIC AND TECHNOLOGICAL AREAS*									
002400 Bioengineering 016200 Stress Physiology									
14 START DATE			15 ESTIMATED COMPLETION DATE			16 FUNDING AGENCY		17 PERFORMANCE METHOD	
72 04			76 06			DA		C. In-House	
18 CONTRACT GRANT									
A. DATES/EFFECTIVE NA EXPIRATION									
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19 RESPONSIBLE DOD ORGANIZATION									
NAME* Walter Reed Army Institute of Research									
ADDRESS* Washington, D. C. 20012									
RESPONSIBLE INDIVIDUAL									
NAME: JOY, COL R.J.T.									
TELEPHONE 202-576-3551									
20 GENERAL USE									
Foreign intelligence not considered									
21 PERFORMING ORGANIZATION									
NAME* Walter Reed Army Institute of Research									
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)									
NAME* Spector, N.H., Ph.D.									
TELEPHONE 202-576-3457									
SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]									
ASSOCIATE INVESTIGATORS									
NAME: Koob, G. F., CPT									
NAME: Martin, G. E., Ph.D.									
22 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.)									
<p>(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.</p> <p>24. (U) The disciplines and research techniques of neurophysiology, immunology, and parasitology are used.</p> <p>25. (U) Experiments continued during this period on the interactions between lesions of the hypothalamic and related brain regions and the body's immunologic response to parasitic (malaria) and viral (Newcastle's Disease) infections. Although such brain lesions may alter antibody and interferon production by statistical tests, the results must be interpreted cautiously, for extensive control procedures are needed and the minimal level of response needed for biological effect is unclear.</p> <p>Work was continued during this period on a long-term temperature recording system for continuous monitoring of body temperature in rats during and after malarial infection; this system was used to search for the previously reported hyperthermia in rats which follows the disappearance of malarial parasitemia.</p> <p>Work in this unit was terminated at end of the three-year allotment of Institute ILIR funds and was continued as part of work unit 033 Anatomic and Physiological Correlates of Brain Function in Stress and Disease. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 01 July 75 - 30 June 76.</p>									

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DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

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 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.

PROGRESS

Effects of Hypothalamic Lesions on Antibody Production

Male rats previously injected with pertussis vaccine and ovalbumin received either large or small lesions bilaterally in the anterior hypothalamus/preoptic area; or they received one of several sham operations. Statistically significant changes in antibody titer were subsequently seen in the lesioned animals, as determined with Bing's hemagglutination method, but the magnitude of the changes may have been too small to alter the animals' resistance to disease.

Malaria: Long-Term Body Temperature Recordings

In an effort to obtain detailed data on the various components of the febrile response in malaria, a system for continuous recording of body temperature in the free-moving rat was completed and a manuscript submitted for publication. See the report on Anatomical and Physiological Correlates of Brain Function in Stress and Disease, June 1976.

Research in this work unit was incorporated into work unit 033, Anatomical and Physiological Correlates of Brain Function in Stress and Disease on 30 January 1976 and was terminated on 30 June 1976.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 114 Neurophysiological control of antibody response

Literature Cited.

Publications:

1. Spector, N.H., Cannon, L.T., Diggs, C.L., Morrison, J.E., and Koob, G.F.: Hypothalamic lesions: effects on immunological responses. The Physiologist 18: 401, 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)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 DISB'N INSTR <sup>a</sup>	8B SPECIFIC DATA: CONTRACTOR ACCESS	9 LEVEL OF SUM
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B. CONTRIBUTING							
C. CONTRIBUTING							
11 TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Behavioral Scheduling in Psychosomatic Disease							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</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	76 06		DA		C. In-House		
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE N/A				PRECEDING			
B. NUMBER *				FISCAL YEAR		C. FUNDS (In thousands)	
C. TYPE				75		1 55	
D. KIND OF AWARD				76		2 69	
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME * Walter Reed Army Institute of Research				NAME * Walter Reed Army Institute of Research			
ADDRESS * Washington, D.C. 20012				ADDRESS * Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: JOY, COL R. J. T.				NAME *MANNING, CPT, F. J.			
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21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: HURSH, CPT S. R.			
				NAME: CUTHBERT, CPT B. N.			
22 KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup>							
(U) Psychosomatic Disease; (U) Autonomic Dysfunction;							
(U) Operant Conditioning; (U) Stress							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text 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-derived 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 organism as well as the continuous updating of appropriate operant technology as a prerequisite for the exploration of potentially productive methods of treatment.							
25. (U) 75 07 - 76 06 A previous study showing profound behavioral disruption in the monkey by sudden extension of daily shock avoidance sessions from one to 12 hrs was shown to be a function of the abruptness of the shift rather than the 12 hr schedule itself. Disruption of behavior by signalled and unsignalled food and shock is accompanied by distinctive changes in heart rate and blood pressure. Hardware and software have been developed with which to further analyze these changes, as well as tonic cardiovascular effects in subjects reinforced for elevations in blood pressure. Division reorganization requires termination, but further work will be pursued in work unit 071, task 01, project 3A161102B71P. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

\*Available to contractors upon originator's approval

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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 115 Behavioral scheduling in psychosomatic  
disease

Investigators:

Principal: CPT Frederick J. Manning, MSC

Associate: CPT Bruce Cuthbert, MSC;  
MAJ Frank J. Sodetz, MSC.

Description.

The role played by behavioral or psychological factors in the production of disease and/or recovery is one of the least understood and most complex aspects of military medicine. Exposure to the extremes of emotional and performance demands associated with mission accomplishment and survival produces changes in the organization of the internal physiology of the soldier. It has been established that repeated or prolonged exposure of the behaving organism to psychological stress can result in enduring alterations in physiology of the kind presumed to be significant in the etiology of a number of psychosomatic diseases such as essential hypertension or ulcer. Studies are conducted to determine the impact of psychological stimuli and various performance demands on cardiovascular and other visceral systems as models for extending competence in this area to permit integration of the results of our stress research programs with some of the medical and psychiatric sequelae of combat stress and exhaustion.

Deliberate induction of pathology obviously precludes the use of human subjects, so this work unit depends heavily on animal model systems as a substitute. Findings with such systems also serve as a guide to correctional studies of human psychosomatic disease by work units such as those of the Departments of Experimental Psychophysiology and Military Psychiatry.

Behavioral and cardiovascular response to the interaction of respondent conditioning and operant contingencies.

Experiments originally begun in 1972 to investigate interactions of behavioral contingencies and cardiovascular function in rhesus monkeys were resumed. These experiments were designed to shed light on the behavioral and physiological changes which result when respondent conditioning

procedures are superimposed upon ongoing operant baselines. It is hoped that the results will add to our knowledge of the organization of behavior and of the cardiovascular system, and the interrelationships between the two, in response to environmental events.

One experiment involves the presentation of distinctly signalled food and shock during a session in which the monkey is working for food on a DRL 60-second schedule. Prior results showed that the pre-shock stimulus reliably elicited large increments in blood pressure (e.g., consistent increases of 50 mm or more in one subject) which persisted over days until the shock was omitted. Heart rate was inconsistent: two monkeys showed decreased heart rate (HR) during the shock CS, while a third speeded HR considerably. Interestingly, the rate of lever-pressing corresponded to HR change for these three animals. The third monkey increased his rate of lever-pressing during the CS, while the two animals who slowed HR suppressed responding considerably while awaiting the shock. These results are in accord with Obrist's cardiac-somatic coupling hypothesis, in which changes in heart rate and bodily activity are held to be linked centrally under most conditions. Changes in response to the pre-food stimulus in these DRL animals were less pronounced. Blood pressure in two of the animals decreased slightly, while a third showed small increases which were far smaller than those seen in anticipation of shock. The HR change and alterations in lever-pressing rate were both inconsistent.

Markedly different responses to a pre-food stimulus were observed when this respondent procedure was administered to animals working on a Sidman avoidance baseline. Here blood pressure consistently showed moderate to large increases in anticipation of food in all three animals studied to date. Heart rate responses were less stable. One monkey exhibited heart rate increases throughout the 100-second food CS; in two others heart rate increased markedly during the first 20 seconds and was inconsistent for the remainder of the CS. Changes in responding were not tied to HR as for the DRL animals: the monkeys who increased rate actually suppressed lever-pressing considerably, while of the other two animals one increased responding and the other decreased. One monkey working on Sidman avoidance was given a CS followed by shock. Blood pressure and HR both increased considerably during the pre-shock tone, and lever-pressing was also increased in rate during the CS. As with the pre-shock CS in the DRL experiment, HR and lever-pressing thus changed together.

The results to date clearly indicate that the anticipation of different environmental stimuli (e.g., shock versus



food) can exert markedly different effects on the physiological functioning of the organism. In addition, the anticipatory response to the same stimulus may vary depending on the nature of the background task. Superimposed on these effects are also individual differences both in autonomic activity and lever-pressing behavior.

The present replication of these procedures is intended to bolster confidence in the generality of the results by increasing the sample size, and also to attempt to resolve some of the inconsistencies in the data as they stand. To the latter end, a number of steps have been taken. First, the capability to measure cardiac output has been added with the help of the Cardiorespiratory Department. Because blood pressure is not a primary variable but represents the product of total peripheral resistance and cardiac output, it is anticipated that recording the latter variable in addition to pressure will give a more comprehensive understanding of the changes which occur.

Measures of somatic activity are also being used to test more fully the cardiac-somatic linkage hypothesis in this paradigm. While HR and lever-pressing did not invariably change together in the available data, many other occurrences of somatic activity might have gone undetected. Therefore, an accelerometer has been obtained to detect movements in the chair, and EMG electrodes will be implanted to measure alterations in muscle tension. Recordings from these variables may account for some of the apparent inconsistencies from animal to animal in the present results.

Finally, new instrumentation have developed to measure physiological change in five minute blocks for selected periods of up to 75 minutes before and after, as well as during, the sessions. This should provide information on the reactions to the imposition of different operant schedules, and again shed light on individual differences in physiological change. The one monkey so measured to date (a second died in surgery) has shown consistent increments in blood pressure and heart rate during DRL sessions, the latter variable generally increasing over the course of the session to increases of 60 beats per minute and more. To the extent that ceiling effects limit the upper levels of physiological functioning, individual differences in response to the session per se may influence change with respect to a CS during the session.

These considerations point up the complexity of the interactions between operant behavior, other skeletal

muscle behavior, and autonomic activity. However, an analysis of these relationships in the present paradigms should contribute to the understanding of the ways in which environmental events affect the overall functioning of the organism.

Reorganization of the Division of Neuropsychiatry requires that future work in this area be pursued within a more comprehensive work unit entitled "Behavioral Variables in Autonomic Function and Disease in Military Personnel," Project Number 3A161102B71P, Task Area 01, Work Unit 072.



Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT  
RESEARCH

Task 00 In-house Laboratory Independent Research

Work Unit 115 Behavioral scheduling in psychosomatic  
disease

Literature Cited.

Publications:

1. Natelson, B.H., Holaday, J.W., Meyerhoff, J.,  
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cortisol, and glucose: relation to light onset and be-  
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409-415.

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)636	
3 DATE PREV SUMMARY 75 07 01	4 KIND OF SUMMARY U	5 SUMMARY SCT <sup>a</sup> U	6 WORK SECURITY <sup>a</sup> U	7 REGRADING <sup>a</sup> NA	8A ORIGIN INSTR <sup>a</sup> NL	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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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) 75 07 - 76 06 This work unit was terminated as a result of the reorganization of the Division of Neuropsychiatry. Work was continued on the examination of sympathetic nervous system functional abnormalities related to the causal state. Development of multiphasic, non-invasive cardiovascular monitoring technology made substantial progress. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 75 - 30 JUN 76.							

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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.

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Description

The physiology of the autonomic nervous system (ANS), the conscious regulation of ANS activity and the participation of the ANS in disease states are of major importance to military medicine. Investigations focus on: (a) the applicability of operant and respondent conditioning principles to chronic clinical syndromes, such as hypertensive cardiovascular disease, and to situationally induced stress states, such as battle fatigue; and (b) autonomic dysfunction in chronic pain states such as causalgia and related states induced by traumatic nerve injury. The development of non-invasive monitoring techniques for measuring cardiac and peripheral vascular activity was a necessary condition for the study of ANS activity in health and disease.

The progress report is organized as follows: '1) Clinical studies on causalgia. (2) Quantitative photoplethysmography. (3) Interpretation of the photoplethysmograph signal. (4) Causalgia. Measurement of sympathetic nervous system activity in affected patients and controls. (5) Monitoring of cardiovascular responses. This work unit is terminated, however, the research is being continued under work unit 039, Military Stress: Health, Performance, and Injury Factors.

Progress

1. Clinical Studies on Causalgia

The term causalgia was first used by Silas Weir Mitchell as a convenient and descriptive term for the burning pain which occurred after a nerve wound (1,2). In two treatises, he presented his detailed clinical studies on ninety-four cases of nerve wounds sustained during the American Civil War. Twenty-four of these cases had burning pain and dysesthesia in the affected area as a consequence of the injury. The pain varied in intensity from a trivial burning to a state of torture. In general, the patients were portrayed as emotional wrecks whose energies were devoted to minimizing environmental stimuli which might increase the constant pain.

After World War II, several reports were published which summarized the clinical findings and therapeutic results in 397 patients with causalgia (3-10). It is clear from these reports that the term causalgia was used not in reference to a symptom which occurred after nerve injury

but instead as a clinical entity. The undefined criteria for diagnosis generally included a penetrating missile injury to a major peripheral nerve, burning pain, and dysesthesia in the affected area. Local autonomic nervous system abnormalities were seen in an unrecorded number of these patients. It was noted that the skin temperature, color and sweating could be clinically greater or less than that of the unaffected extremity. Sympathetic ganglionectomy proved to be the best therapeutic procedure for relief of pain.

The presence of autonomic abnormalities in the affected extremity and the success of sympathectomy for pain relief suggested that this pain syndrome was due to abnormal sympathetic nervous system hyperactivity (11,12). In addition, it was suggested that causalgia represents only one variant of a more general clinical entity called reflex sympathetic dystrophy. It was implied that the initiating trauma produced a local reflex increase in sympathetic nervous system activity which resulted in the burning pain, dysesthesia, and dystrophic skin changes. No experimental evidence was given to confirm this hypothesis. In fact, the clinical material available suggested that this hypothesis was inappropriate because patients with causalgia had been described who had evidence of decreased SNS activity in the affected region.

The increased availability of medical care and use of surgical procedures has brought attention to some of the unfortunate consequences of surgery. A rare complication is a clinical syndrome indistinguishable from that of causalgia (13-15). In addition, patients with a similar pain syndrome have been described after IM injections, blunt trauma, and bone fractures.

A review of the case reports of causalgia published in English suggested that causalgia could be best defined as a post-traumatic pain syndrome and not as a disease entity. It appears that the diagnosis is generally made on the basis of four criteria: (1) steady, often severe burning pain; (2) dysesthesia in the affected area such that most if not all cutaneous stimuli to the affected area are perceived as pain; (3) the occurrence of both the pain and dysesthesia after either an iatrogenic or environmental traumatic event with presumed nerve injury; and (4) severe behavioral changes in the affected individual such that the person's entire behavior is dominated by the pain syndrome. These criteria appear both adequate and sufficient to encompass the reported cases. Bonica has suggested that the diagnosis of causalgia should only be made when the four criteria above are present with evidence of local sympathetic nervous system hyperactivity (11). Sunderland has suggested that the diagnosis be confined to trauma involving only major peripheral nerves (16). These suggestions appear unreasonable since they would exclude a large number of the reported cases on the basis of associative findings instead of the dominant characteristics (burning pain and dysesthesia following trauma).

During the last three years, fifteen patients with causalgia diagnosed according to the criteria above have been examined by this investigator (LTC Tahmouh). The patients were seen in conjunction with the Department of Neurosurgery of WRAMC and the Department of Neurology of NNMC. The post-traumatic pain syndrome occurred after a missile injury in five patients, after surgical procedures in six patients, and after closed or open bone injuries in four patients. The severity of the pain syndrome and degree of behavioral change were remarkably similar in this clinical group. The pain and dysesthesia were the major clinical abnormalities, and other consequences from the initiating trauma were ignored by the patient. Nine patients had a plethoric appearance in the affected region when compared visually to the homologous extremity, five patients had no abnormal appearance and one patient had a pale, shiny appearance. Only three patients had marked sweating in the affected region. The response to pain-relieving procedures appeared independent of the initiating factor and the presence of autonomic nervous system abnormalities. The duration of the pain syndrome appeared to be the primary determinant of therapeutic result. Five patients with pain of less than six months duration obtained excellent pain relief with four of the patients back to full activity. Two of these patients had virtually spontaneous pain relief, two had pain relief after use of propranolol, and one had pain relief after sympathectomy. Eight patients with pain of greater than 12 months duration were moderately improved after a number of procedures but had residual problems and were unable to resume full activity. Two patients with pain of greater than 12 months duration had a number of operative procedures including sympathectomy without pain relief.

The clinical studies suggest that the four criteria for the diagnosis of causalgia presented above are adequate and permit diagnosis of a homogeneous population of patients with pain. Patient selection on the basis of initiating factor or ANS abnormalities would only arbitrarily exclude a portion of this homogeneous population. Although the amount of pain relief obtained after several procedures varied widely in this group, the determining factor appears to be the duration of the pain prior to therapy and not the initiating factor or presence of ANS abnormalities.

This comprehensive review of the literature and prospective patient follow-up was necessary to provide adequate definition of the clinical problem. It is hoped that this definition of diagnostic criteria will result in earlier diagnosis and a decrease in the number of chronic intractable cases. The material presented is being prepared as a review article on causalgia to be submitted to the journal, Pain.

Continuation of these clinical studies is planned to define the scope of the problem in relation to a combat population at risk. A retrospective epidemiological study of causalgia in soldiers at risk

during the Vietnam War is planned in cooperation with the Department of Psychiatry. In addition, identified cases will be followed through Army and Veteran Hospital records to estimate the frequency of chronic residual deficits.

## 2. Quantitative Photoplethysmography

A non-traumatic, simple, inexpensive and reliable technique for the measurement of peripheral blood volume and blood flow would be invaluable to the clinician and scientist. Photoplethysmography is the only available technique which could meet the above criteria. It involves the measurement of relative changes in peripheral vascular activity based on the differential optical properties of blood and tissue and changes in the optical properties of blood due to changes in the orientation of red blood cells secondary to pulsatile flow. Photoplethysmography also permits the measurement of blood volume and blood flow in a specific vascular bed. Since the cutaneous vascular bed is under sympathetic nervous system regulation, measurement of cutaneous blood volume and blood flow provides an indirect measurement of sympathetic nervous system activity.

The requirements for quantitative photoplethysmography are as follows: (1) a linear transducer whose output is related solely to light intensity; (2) an application procedure which is reproducible and produces minimal distortion at the measurement site; and (3) a system calibration. Work during the last year has culminated in fulfillment of all the requirements necessary for quantitative photoplethysmography (Pub. 1, 2), and this technique has been employed in the indirect measurement of sympathetic nervous system activity in patients with causalgia and matched controls.

## 3. Interpretation of the Photoplethysmograph Signal

The relationship between infrared backscattered light, cutaneous blood content and blood flow has been qualitatively determined by integrating information from several disciplines. Theoretical and experimental results from the fields of optics, fluid physics and human anatomy-physiology serve as the basis for our present interpretation of the photoplethysmograph signal. Studies with an in vitro system support and supplement this body of data.

The measurement sites of interest for quantitative photoplethysmography are superficial blood vessels and the distal ventral surface of fingers and toes. A first-order optical model of the measurement site over superficial blood vessels would be a horizontal cylinder of blood covered by a partially reflecting surface (i.e., a ground-glass tube). An optical model which takes into account the major characteristics of cutaneous tissue of the ventral surface of fingers and toes is much more complex. Three layers must be considered: (1) the epidermis, (2) the dermis, and (3) the subcutaneous tissue. The major component of the epidermis is the stratum corneum. This is a thick (approximately 0.5 mm), dead and relatively homogenous layer.

The dermis is a mixture of loose connective tissue in which the cutaneous capillaries, venules and arterioles are located. It varies in thickness from 0.2 - 4 mm. The subcutaneous tissue consists primarily of fat cells, sweat glands and nerve fibers (17).

One approach of modeling the optical behavior of this measurement site is to treat each layer as an optically homogenous sheet. With this approximation and given the optical properties of each sheet, the total reflectivity of the tissues can be determined (18). For a three-layer system, the fraction of backscattered to incident light is given by:

$$R = R_1 + \frac{T_1^2 [R_2 + R_3 (T_2^2 - R_2^2)]}{1 - R_2 (R_3 + R_1) - R_1 R_3 (T_2^2 - R_2^2)} \quad (\text{equation 1})$$

where  $R_1$  through  $R_3$  are the reflectances of the three layers and  $T_1$  through  $T_3$  are the transmittances of the layers.

Hardy (19) determined quantitatively the near infrared transmittance and reflectance for various thicknesses of excised, bloodless human skin. He found that skin pigmentation had little effect on these properties at near infrared wavelengths. Table 1 presents a summary of his results for white breast skin of various thickness illuminated by near infrared radiation.

Table 1. Near infrared transmittance and reflectance of white breast skin (19)

<u>Thickness (mm)</u>	<u>% Transmittance</u>	<u>% Reflectance</u>
0.43	60	10
0.67	40	14
0.84	32	16
1.60	17	22

The optical properties of blood have been extensively studied (20, 21), and the near infrared transmittance and reflectance of various slab thicknesses experimentally determined (Table 2).

Table 2. Near infrared transmittance and reflectance of blood (21)

<u>Thickness (mm)</u>	<u>% Transmittance</u>	<u>% Reflectance</u>
0.45	58	16
0.65	52	22
0.85	46	25
1.70	30	34



When the optical properties of skin and blood are compared for similar thicknesses it appears that there is more absorption of near infrared radiation by skin than blood. By inspection of equation 1 it is evident that the total reflected light from the skin depends on the transmittance and reflectance characteristics of each layer in a complex manner.

In addition to the reflected light intensity due to tissue content, we have also observed a time variation in the amount of backscattered light. Since it has been experimentally determined that this variation is correlated with the pulsatile arterial blood flow (Pub. 2), we believe that the time dependent nature of the backscattered light intensity is due to tissue blood flow. Since pulsatile blood flow occurs only in the arterial system of the circulation (22), we further believe that the backscattered light variation is a function of arteriolar and not capillary or venule blood flow.

How does arteriolar blood flow produce the variations in backscattered light intensity with time? To answer this question, we considered the details of fluid flow.

Since plasma behaves as a Newtonian fluid, the determination of its flow characteristics through a rigid tube is relatively simple. The velocity profile will be parabolic with maximum velocity at the axis of the tube and zero velocity at the walls. Shear ( $dv/dr$ ), the rate of change of velocity ( $V$ ) with respect to distance ( $r$ ) from the tube axis, is given by.

$$\frac{dV}{dr} = \frac{4F}{\pi R_0^4} \quad (\text{equation 2})$$

where

$dV/dr$  = shear

$F$  = volume flow

$R_0$  = tube radius

$r$  = distance from tube axis

The presence of shear complicates the motion of red blood cells suspended in plasma. There is both a translational and a rotational force (torque) on the RBCs. Since torque is a function of both orientation and shear, the rotation of a single RBC is not uniform. As an illustration of the consequences of non-uniform rotation, consider the example of an ellipsoidal particle (a hydrodynamic approximation to an RBC). The probability function for the angle ( $\theta$ ) between the axis of symmetry of the ellipsoid and the direction perpendicular to flow in a constant shear is:



$$P(\phi) = \frac{r_e}{2\pi(r_e^2 \cos^2 \phi + \sin^2 \phi)} \quad (\text{equation 3})$$

where  $r_e$  is the axis ratio of the ellipsoid (23).

When a number of RBCs are suspended in plasma, the cell-cell and cell-fluid interactions further complicate the characteristics of blood flow. The parabolic velocity distribution described for plasma no longer accurately describes the flow of whole blood.

High-speed cinematography shows that the blood cells follow a helical spiral while rotating about their long and short axes and that considerable distortion in the shape of the cell takes place during flow. These films also show that the cells are not uniformly distributed about the cross-section of the tube but tend to crowd about the axis (25).

Having described the characteristics of blood flow, it is necessary to determine which characteristics are responsible for the variations in backscattered light intensity. There are four possibilities: (1) breakdown of cell aggregates and rouleau by the shear gradient; (2) deformation of the cells; (3) axial accumulation of the cells and; (4) reorientation of the cells.

Results from in vitro studies relating blood flow to backscattered light revealed similarities between our findings and those of Dellimore and Gosling (26). During studies of the fluctuations in blood conductivity produced by pulsatile blood flow, these authors found that: (1) with increasing hematocrit, conductivity became more sensitive to changes in flow rate; (2) conductivity changes were related to shear, not velocity; (3) there is a phase lag between a change in the shear and a change in the conductivity; and (4) the use of "sphered" blood cells eliminated the conductivity variations with flow. Based on the experimental data and some additional theory, they concluded that a reorientation of the cells is the only possible mechanism for the variation in conductivity with flow and that the average orientation is a function of shear.

Ross (27) has made theoretical calculations relating the optical properties of a red blood cell to its orientation. These calculations show a significant change in the backscattering characteristics of a single cell with different orientations. Given that the orientation of red blood cells can affect the amount of backscattered light, it appears probable that changes in red blood cell orientation produce both the variation in conductivity and backscattered light with flow.

The remaining problem is to determine how orientation varies with shear. From equation (3), it is seen that orientation should be independent of shear, which contradicts experimental evidence. However, equation (3) is only for a single, unrestrained cell. At normal hematocrits, interactions between cells prevent such a free rotation. Therefore two mechanisms are at work: (1) the reorientation of the cells due to shear, and (2) the disorientation due to cell interactions. This system is at a dynamic equilibrium for a given shear and changing the shear alters the equilibrium between oriented and non-oriented cells. This situation can be described quasi-thermodynamically by an orientation parameter which can be experimentally determined (28).

In order to test both the sensitivity of reflected light intensity (R) to changes in blood content and to study the effect of changes in blood flow shear on the time variation in the amount of backscattered light, an in vitro system was designed which permitted study of steady and pulsatile blood flow. For a given pyrex cell diameter, a linear relationship was found between hematocrit and reflected light intensity. Therefore, if the tissue components at the measurement site are similar, differences in reflected light intensity reflect differences in RBC content. That the time variant backscattered light signal is due to pulsatile blood flow was established as follows: (1) steady and pulsatile flow of hemolyzed blood produced no change in reflected light intensity; (2) steady flow of whole blood produced only a transient on-off change in reflected intensity; and (3) pulsatile blood flow produced pulsatile changes in reflected light intensity similar to that obtained from human subjects. Since the pulsatile changes in backscattered light continued as long as blood was present in the pyrex cell even though saline was being pumped into the system, the time variant light signal could not be due to a direct effect of the pump on RBCs but instead was due to a secondary effect. Since the amplitude of the time variant light signal was found to be dependent on hematocrit, volume flow rate and tube radius (factors which also determine the shear), it appears reasonable to suggest that this signal is dependent on shear.

In summary, the time variant backscattered light signal is related to pulsatile blood flow which occurs in the arterial tree but not the capillaries or venules. The amplitude of this signal is related to volume blood flow, vessel radius and hematocrit through their influence on shear. If vessel radius and hematocrit are constant, then the time variant signal is related to volume blood flow. Changes in this signal following sympathectomy are probably related to both a change in vessel radius and volume blood flow. As discussed earlier, changes in shear result in changes in the orientation of RBCs with a resultant change in the amount of backscattered light. Therefore, both the changes in conductivity and backscattered light seen with pulsatile blood flow appear to be due to the same mechanism.

The present work has clearly established the qualitative relationships between the photoplethysmograph signals and physiological variables. Extension of this work is planned to develop quantitative relationships between the backscattered light and both tissue blood content and blood flow.

#### 4. Causalgia. Measurements of Sympathetic Nervous System Activity in Patients and Matched Controls

The presence of abnormalities of skin temperature, sweating and color in the affected area of patients with causalgia has been repeatedly noted since the first descriptions by Silas Weir Mitchell (1, 2). Measurements of these variables in patients with causalgia have not been performed prior to this study (Pub. 3). An understanding of the technical and methodological problems involved in obtaining these measures is necessary in order to interpret their significance.

The measurement of skin temperature (ST) can be reliably performed with one of a number of commercial thermistors. However, the level of sympathetic nervous system activity is only one of several major factors which influence skin temperature (29). Skin blood flow, body heat production, sweating, environmental temperature and air circulation are known factors which affect skin temperature. Sympathectomy produces an immediate increase in skin temperature probably secondary to a local increase in cutaneous blood flow.

Skin conductance (SC) measurements have been used as a reliable index of sweat gland activity and indirectly as an index of sympathetic nervous system (SNS) activity. The SNS provides the sole nervous system innervation to these glands.

The descriptions of causalgia in association with a plethoric appearance to the affected area suggest that the cutaneous blood volume (CBV) is increased. The reports of skin temperature asymmetries suggested that cutaneous blood flow (CBF) may also be abnormal in the affected area. Since the dominant hypothesis concerning the pathophysiology of causalgia is based on decreased cutaneous blood flow with resultant ischemic pain and trophic skin changes, measurements of both cutaneous blood volume and blood flow were necessary to test this hypothesis. Due to the absence of adequate measuring techniques, it was necessary to devote a major effort to the development of quantitative photoplethysmography (Sec. 2) and the interpretation of the photoplethysmograph signal (Sec. 3).

The sympathetic nervous system innervates the smooth muscle of arteriolar walls, and influences cutaneous blood flow by changing the resistance of the arteriolar vessels through changes in vessel diameter (29). The SNS may also innervate the small venules and thus influence cutaneous blood volume.

Nine patients with causalgia and matched controls had measurements of skin conductance, temperature, blood volume pulse and blood volume performed on the affected and non-affected extremities. The clinical characteristics of the patients with causalgia are presented in Table 1. The controls were matched to the patients by age and sex, and an "affected" and "non-affected" extremity was assigned to each control on the basis of his matched patient's clinical condition.

Continuous measurements were performed during each of two 30-minute sessions which were repeated in an AB or ABBA design over two or four days, respectively. All transducers were switched from limb to limb between sessions on each day. Therefore, each physiological measurement was recorded for equal times on each limb by each transducer. This design permits statistical analysis to differentiate limb from transducer dominance.

The qualitative temporal characteristics of representative data for each measure are presented in Graphs 1 - 4. One reading was taken at the end of each five-minute period for skin conductance, temperature and blood volume. The average of five blood volume pulses was taken at the end of each five-minute period. Skin temperature (Graph 1) remains relatively stable during sessions, and when variations occur, the pattern is similar for the two extremities. Skin conductance (Graph 2) varies dramatically during a session, and the variations are also similar for the two extremities. This suggests that the dominant control of both ST and SC occurs at a central and not peripheral site. In addition, SC asymmetries between the limbs are clearly evident which are consistent within a day but vary across days. These findings are not consistent with the idea that a right and left spinal cord center exists with one center fixed at a different level than the other (30). It suggests that a more dynamic interaction occurs in the central nervous system which may result in SC limb asymmetries but that these asymmetries are variable and not fixed. Estimation of limb dominance requires multiple measurement days.

Skin blood volume pulse or blood flow (Graph 3) varies dramatically within a session. Skin blood volume (Graph 4) remains relatively unchanged during a session and asymmetries between the limbs are generally consistent within and across days.

Since inspection of the graphs revealed no period X limb interaction and a probability test revealed no session X limb interaction, the data were collapsed into day means. Tables 2 -5 present the day means for skin temperature, skin conductance, skin blood volume pulse and skin blood volume. In Table 6, the mean limb differences (algebraic sum of the differences between affected and non-affected limb means across days) are presented for patients and controls.

Since this study was designed to test the hypothesis that causalgia is associated with sympathetic nervous system hyperactivity in the affected area, it was necessary that the measurements permit

ordinal but not ratio comparisons. The measurements of skin conductance and temperature permit interval comparisons but the measurement of skin blood volume and blood flow can be compared only in an ordinal manner. Since only an ordinal relationship of sympathetic nervous system activity to each of these measures is known, the hypothesis that SNS hyperactivity is associated with causalgia was examined in terms of non-parametric statistics.

When the absolute value of each measure was compared between the causalgia and control groups, it was found that only skin conductance was significantly different with the causalgia group lower than the controls. When the mean limb differences were compared, only significant differences in skin temperature were found.

The results of this study clearly indicate that sympathetic nervous system hyperactivity is not consistently present in the affected limb of patients with causalgia. Autonomic abnormalities are present more often in patients and the asymmetries between limbs are of greater magnitude but the direction is not consistent. It appears that the autonomic abnormalities present in causalgia are associated phenomenon and do not play a significant role in the pathophysiology of this post-traumatic pain syndrome.

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

Non-invasive recording of beat-by-beat cardiovascular system behavior has been the subject of continued technological development. Within a single constant-environment chamber, an array of monitoring devices are applied to human subjects which detect electrophysiologic signals. Analogue signals derived from such monitoring include 1) Electrocardiogram; 2) Phonocardiogram and low-frequency Vibrocardiogram; 3) Carotid and Radial Pulse Wave; 4) Left Brachial sphygmomanometric blood press; 5) Digital (Infrared) Photoplethysmography; 6) Limb and Thoracic Impedance plethysmography; 7) Chest displacement Respirometry. Construction of hardware for digital processing and recording of these data has been completed. For each heartbeat, events or intervals defined by each of the signals are referenced to a time base with 1 millisecond resolution. For pulsatile wave-forms, the integral, peaks and troughs, and peak 1st derivative of waves are determined and recorded. The net output of digital processing consists, for each beat, of some 30 parameters recorded, involving systolic cardiac intervals, pulse propagation time, and pulse wave contour analysis. Analysis of these parameters and their complex interaction provides indices of cardiovascular behavior not previously applied together in psychophysiologic research.

A protocol proposal is pending approval which will permit application of this monitoring system to human subjects. Procedures involving stimulation of subject with (1) Cold pressor tests; (2) Valsalva maneuver (3) Mental Arithmetic; (4) Postural change, (5) Isometric exercise; and

(6) Isotomic exercise will provide normative data defining sensitivity and accuracy of the apparatus, mechanistic analysis of observed cardiovascular responses, and development of analytic software techniques applicable to the complex volume of data recorded.

TABLE 1. CLINICAL CHARACTERISTICS OF CAUSALGIA PATIENTS

<u>PATIENTS</u>	<u>AGE, SEX</u>	<u>PRECIPITATING FACTOR</u>	<u>DURATION OF PAIN</u>	<u>PREVIOUS TREATMENT</u>	<u>AUTONOMIC ABNORMALITIES IN AFFECTED EXTREMITY</u>
Case 1 (P)	49, F	Transverse Carpal Ligament Release	18 Months	Analgesics, P.T.	None
Case 2 (E)	60, F	Cervical Laminectomy	3 Years	Analgesics, P.T.	Cold, Plethoric
Case 3 (G)	45, F	Fasciotomy of Hand	4 Months	Narcotics, P.T.	Cold, Plethoric, Trophic changes
Case 4 (R)	51, F	Lumbar Laminectomy	12 Months	Narcotics, P.T., Nerve Exploration, Sympathetic Blocks	None
Case 5 (DR)	40, F	Multiple I&D	10 Months	Sympathetic Blocks	None
Case 6 (W)	25, M	Shrapnel Injury	7 Years	Analgesics, Nerve Exploration, Sympathetic Blocks	Sweaty
Case 7 (Y)	35, M	Compound Fracture of Foot	4 Years	Sympathetic Blocks	Warm, Dry, Plethoric
Case 8 (F)	34, M	Gunshot Wound	7 Years	Stellate Ganglionectomy	Trophic changes
Case 9	47, M	Gunshot Wound	3 Years	Stellate Ganglionectomy, Thalamotomy	Cold, Dry, Constricted, Trophic changes

TABLE 2. MEAN SKIN TEMPERATURE VALUES (°F) FOR PATIENTS AND CONTROLS

PATIENT	DAY 1	DAY 2	DAY 3	DAY 4	CONTROL	DAY 1	DAY 2	DAY 3	DAY 4
Case 1 (P) AL NL	90.0 90.0	85.0 85.2			Control 1 (N) AL NL	87.5 88.0	87.8 87.8		
Case 2 (E) AL NL	83.0 87.5	84.5 87.0			Control 2 (S) AL NL	91.2 91.8	90.2 90.6		
Case 3 (G) AL NL	89.9 93.6	90.5 93.7	91.6 94.0	90.5 93.7	Control 3 (B) AL NL	86.5 88.5	93.3 93.9	88.5 90.4	88.8 90.6
Case 4 (R) AL NL	80.0 78.5	80.9 79.6			Control 4 (Z) AL NL	76.8 77.0	85.2 85.2		
Case 5(DR) AL NL	90.2 88.2	88.4 87.1			Control 5 (M) AL NL	83.0 83.0	90.0 90.0		
Case 6 (W) AL NL	93.9 92.9	93.3 92.8	93.5 93.2	93.4 93.7	Control 6 (G) AL NL	94.4 94.4	94.1 94.5	94.2 94.5	94.2 94.8
Case 7 (Y) AL NL	90.0 78.5	84.7 74.9	88.0 79.8	85.2 78.1	Control 7 (W) AL NL	75.2 75.2	76.0 77.2	79.1 78.7	80.0 80.1
Case 8 (F) AL NL	92.4 93.1	93.3 94.1	93.9 94.2	92.9 93.6	Control 8 (T) AL NL	94.2 94.3	93.9 92.4	94.3 94.3	93.3 93.4
Case 9 (C) AL NL	89.0 94.2	87.2 92.5			Control 9 (J) AL NL	85.6 86.4	88.0 88.0		



TABLE 3. MEAN SKIN CONDUCTANCE VALUES ( $\mu\text{M}$ ) FOR PATIENTS AND CONTROLS

PATIENT	DAY 1	DAY 2	DAY 3	DAY 4	CONTROL	DAY 1	DAY 2	DAY 3	DAY 4
Case 1 (P) AL NL	5.1 3.4	4.5 4.8			Control 1 (N) AL NL	3.5 3.0	2.7 2.4		
Case 3 (G) AL NL	0.5 1.0	0.8 0.7	0.6 0.2	0.7 0.3	Control 3 (B) AL NL	1.9 2.2	1.6 1.4	1.8 1.0	1.0 1.6
Case 4 (R) AL NL	1.2 0.9	0.5 0.2			Control 4 (Z) AL NL	1.6 2.2	1.9 3.4		
Case 6 (W) AL NL	3.2 1.1	1.0 0.6	1.1 0.9	1.7 0.5	Control 6 (G) AL NL	2.7 2.8	2.7 2.1	2.1 2.0	1.9 1.6
Case 7 (Y) AL NL	2.5 4.6	0.9 3.3	1.7 2.3	0.5 2.5	Control 7 (W) AL NL	3.2 2.7	6.0 3.8	5.3 3.0	2.6 3.4
Case 8 (F) AL NL	2.8 2.0	0.5 1.0	2.2 3.4	0.9 1.6	Control 8 (T) AL NL	2.5 2.5	2.1 3.5	3.4 5.2	1.4 1.2
Case 9 (C) AL NL	0.0 2.3	0.0 3.1			Control 9 (?) AL NL	4.2 3.0	5.3 4.5		

TABLE 4. MEAN SKIN BLOOD VOLUME PULSE VALUES (mV) FOR PATIENTS AND CONTROLS

PATIENT	DAY 1		DAY 2		DAY 3		DAY 4		CONTROL		DAY 1		DAY 2		DAY 3		DAY 4	
	AL	NL	AL	NL	AL	NL	AL	NL	AL	NL	AL	NL	AL	NL	AL	NL	AL	NL
Case 1 (P)	26.4	21.8	14.4	9.3					Control 1 (N)		15.0	13.4	6.4	9.2				
Case 2 (E)	5.8	6.6	5.9	9.2					Control 2 (S)		9.4	11.0	5.8	7.4				
Case 3 (G)	2.5	10.5	2.5	21.1	2.5	19.9	2.5	15.4	Control 3 (B)		5.3	10.8	13.7	25.6	5.6	23.8	11.6	14.0
Case 4 (R)	1.9	1.3	3.0	4.0					Control 4 (Z)		0.6	0.6	1.6	1.4				
Case 5 (DR)	10.0	9.5	3.7	4.2					Control 5 (M)		6.8	7.4	7.2	5.8				
Case 6 (W)	7.5	3.6	7.0	4.0	8.6	7.3	5.6	9.4	Control 6 (G)		14.7	16.2	9.5	15.3	14.9	12.3	12.7	18.4
Case 7 (Y)	18.6	6.4	19.0	6.9	33.4	10.6	22.1	19.0	Control 7 (W)		2.3	1.8	3.8	2.2	3.8	2.7	4.7	5.7
Case 8 (F)	12.7	4.8	7.0	4.5	7.7	2.8	4.1	5.6	Control 8 (T)		10.0	7.6	7.5	9.0	10.1	12.3	7.0	8.6
Case 9 (C)	13.6	18.0	7.0	14.4					Control 9 (J)		3.0	2.8	5.0	5.9				

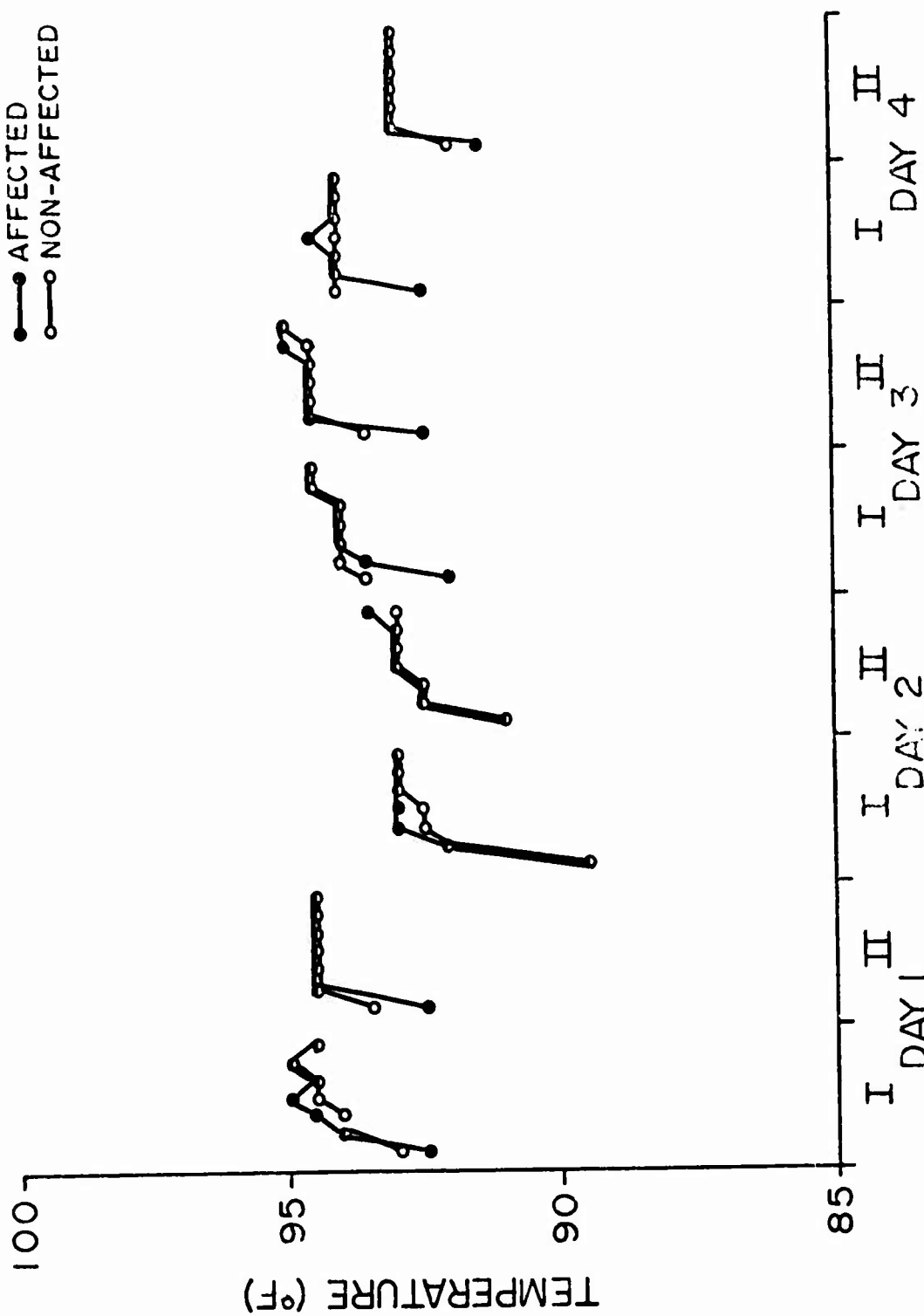
TABLE 5. MEAN SKIN BLOOD VOLUME VALUES (V) FOR PATIENTS AND CONTROLS

<u>PATIENT</u>	<u>DAY 1</u>	<u>DAY 2</u>	<u>DAY 3</u>	<u>DAY 4</u>	<u>CONTROL</u>		<u>DAY 1</u>	<u>DAY 2</u>	<u>DAY 3</u>	<u>DAY 4</u>
Case 6 (W) AL	2.01	1.99	1.83	1.66	Control 6 (G) AL	AL	1.82	1.67	1.72	1.66
NL	1.80	1.86	1.78	1.60	NL	NL	1.63	1.51	1.58	1.50
Case 7 (Y) AL	1.73	2.07	2.31	2.16	Control 7 (W) AL	AL	2.20	2.24	2.09	2.26
NL	3.03	2.47	2.64	3.53	NL	NL	2.00	2.05	1.97	2.31
Case 8 (F) AL	2.20	1.89	2.09	2.00	Control 8 (T) AL	AL	2.24	2.33	2.15	1.81
NL	1.39	1.42	1.57	1.46	NL	NL	1.99	1.94	2.18	1.85

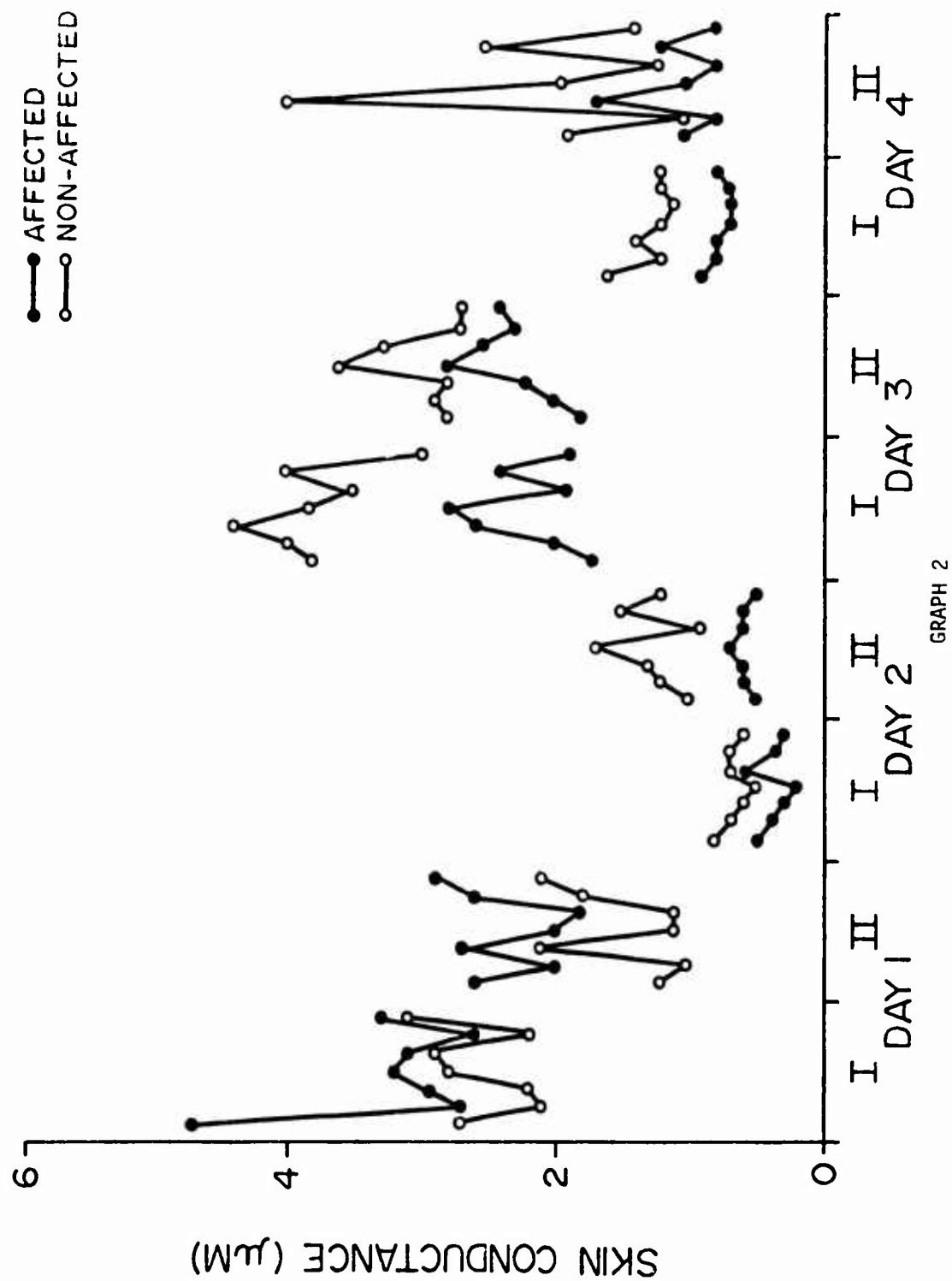
TABLE 6. MEAN LIMB DIFFERENCES FOR PATIENTS AND CONTROLS

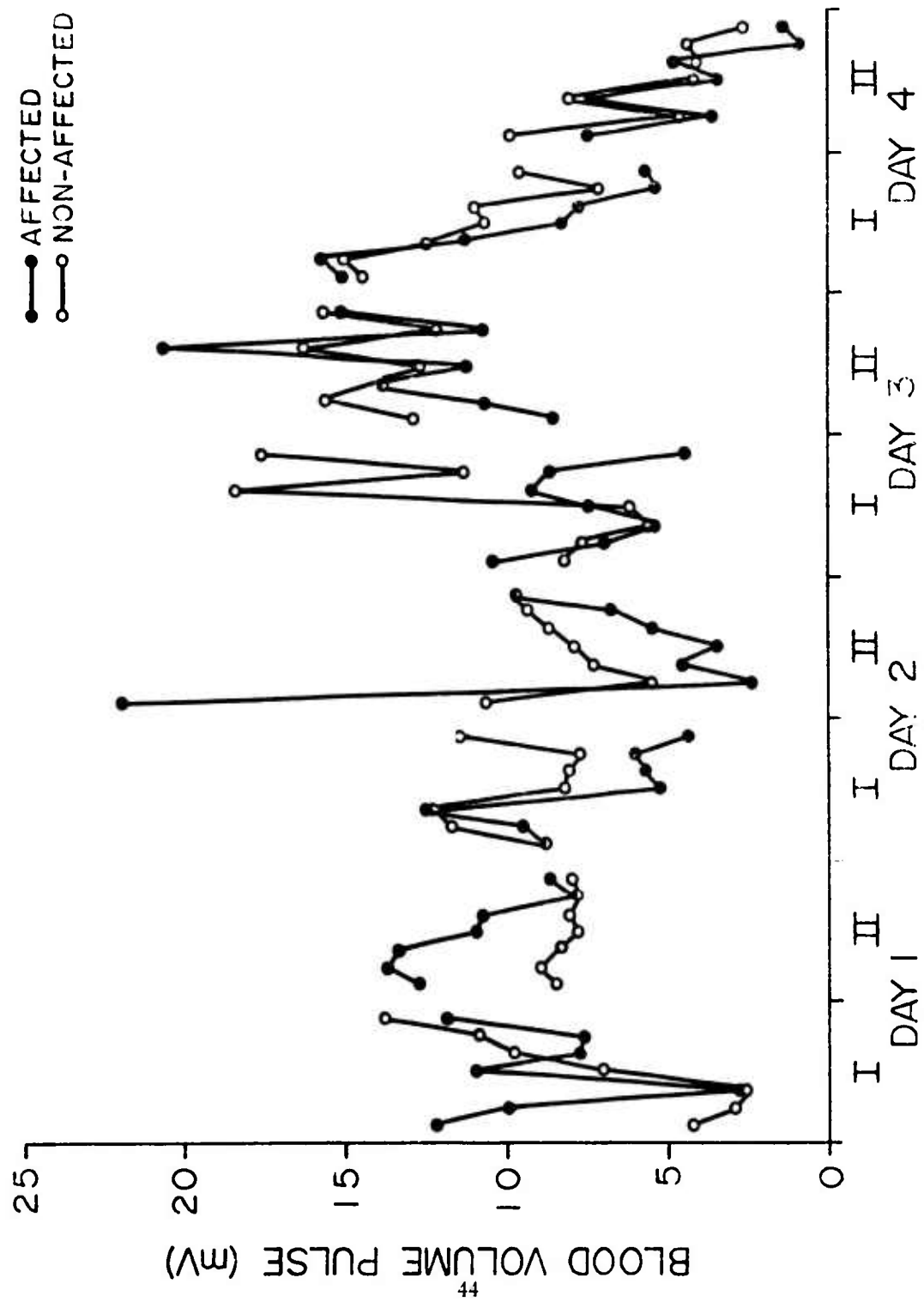
PATIENT	TEM ( $^{\circ}$ F)	SC ( $\mu$ M)	BVP (mV)	BV (V)	CONTROL	TEM ( $^{\circ}$ F)	SC ( $\mu$ M)	BVP (mV)	BV (V)
Case 1 (P)	-0.1	0.7	4.8		Control 1 (N)	-0.2	0.4	-0.6	
Case 2 (E)	-3.5		-2.0		Control 2 (S)	-0.5		-1.6	
Case 3 (G)	-3.1	0.1	-14.2		Control 3 (B)	-1.6	0.0	-9.5	
Case 4 (R)	1.4	0.3	-0.2		Control 4 (Z)	-0.1	-1.0	0.1	
Case 5 (DR)	1.6		0.0		Control 5 (M)	0.0		0.4	
Case 6 (W)	0.4	1.0	1.1	0.11	Control 6 (G)	-0.3	0.2	-2.6	0.16
Case 7 (Y)	9.2	-1.8	12.6	-0.85	Control 7 (W)	-0.2	1.0	0.6	0.12
Case 8 (F)	-0.6	-0.4	3.4	0.58	Control 8 (T)	0.3	-0.7	-0.7	0.14
Case 9 (C)	-5.2	-2.7	-5.9		Control 9 (J)	-0.4	1.0	-0.4	

●—● AFFECTED  
○—○ NON-AFFECTED

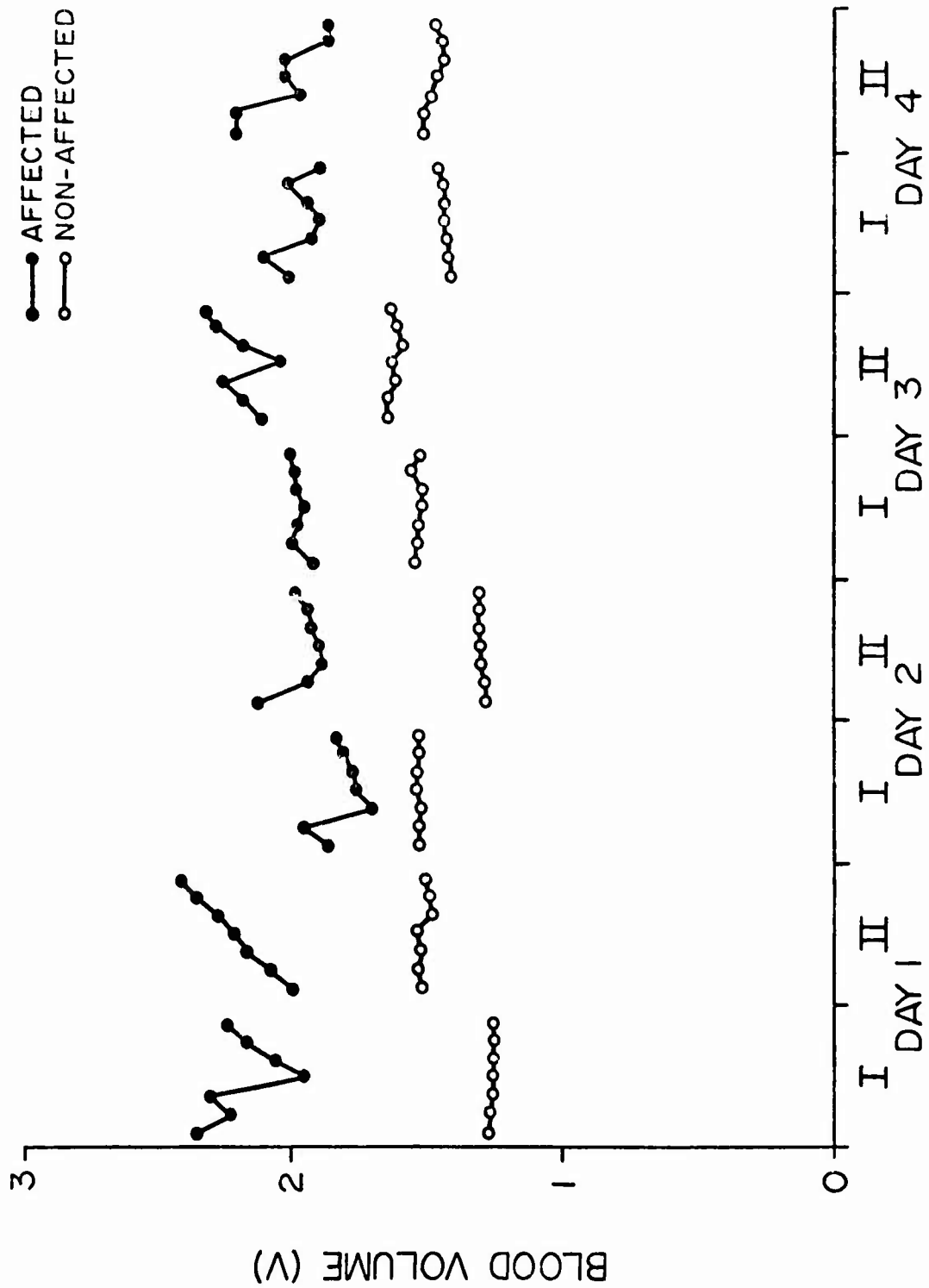


GRAPH 1





GRAPH 3



GRAPH 4



Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 116 Autoregulation of autonomic response

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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-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 DISB'N INSTR <sup>a</sup>	8B SPECIFIC DATA - CONTRACTOR ACCESS	9 LEVEL OF SUM
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10 NO / CODES <sup>a</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>a</sup>							
(U) Antigenic Components of the Cell Wall of Neisseria meningitidis							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
010100 Microbiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R. J. T.				NAME: Russell, COL P. K.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3756			
22 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: DA			
23 KEYWORDS (Precede EACH with Security Classification Code) (U) Cell wall; (U) Protein; (U) Lipopolysaccharide; (U) Polysaccharide; (U) Antibodies; (U) Meningitis; (U) Human Volunteers							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 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 immuno-adsorbent techniques. Cellular immune responses will be measured by in vitro techniques							
25 (U) 75 07 - 76 06 A new candidate group B vaccine consisting of high molecular weight group B polysaccharide complexed to serotype II outer membrane protein has been prepared for testing in animals and humans. The presence of bactericidal activity in acute sera from recruits with systemic meningococcal disease has been demonstrated by selective removal of serum IgA which blocks the lytic activity of IgG and IgM. This suggests that host susceptibility may depend upon the serum level of specific IgA blocking antibody. A solid phase radioimmunoassay (SPRIA) was developed which permits quantitation of human antibody to meningococcal serotype protein and lipopolysaccharide (LPS) antigens. Antigen-specific protein and LPS serotyping of 90 strains of meningococci was performed using a new SPRIA inhibition procedure. A significant correlation was found between the occurrence of type II protein and type 3, 7 LPS. A significant antibody response to the type 3 LPS determinant was demonstrated in recruits with systemic meningococcal disease due to type II epidemic strains. Human antibodies specific for the group C polysaccharide were shown to cooperate with lymphocytes and monocytes in complement independent killing of group C meningococci. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75-30 Jun 76. Support in the amount of \$45,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

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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.

Principal: Malcolm S. Artenstein, M.D.\*

LTC J. McLeod Griffiss, MC

Associate: MAJ George H. Lowell, MC; Wendell D. Zollinger, Ph.D.;  
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MSG Adam D. Druzd.

I. Studies of the immunochemistry and serology of Neisseria meningitidis outer membrane complex (OMC) antigens.

Previously completed studies have identified protein antigens within the OMC of Neisseria meningitidis which confer distinct serotype specificity within and across capsular serogroups (1-5). Two of these serotype determinants, II and IV, appear to confer epidemic potential to meningococci of serogroups B, C and Y, with type II currently being the more important (6-7). The type II determinant, however, has not proved useful as a vaccine in limited human immunogenicity trials. Consequently, increased effort has been directed at understanding the third major surface antigen of the meningococci, its lipopolysaccharide.

Two approaches, utilizing different methodologies developed during the preceding year, were adopted during this reporting period. In the first, a modified Farr-type primary radioactive antigen binding assay (RABA) (8) was used to investigate LPS determinants and to demonstrate that most type II meningococci of serogroup B, C and Y possess a common LPS determinant to which humans respond during the course of infection. The second approach combined previously described LPS and protein (1) serotyping systems into one serological assay: inhibition of a solid phase radioimmunoassay (SPRIA) by specific antigens.

A. A lipopolysaccharide (LPS) antigen common to type II epidemic strains of Neisseria meningitidis and the immune response thereto during infection in recruits.

Epidemic strains of N. meningitidis, of serogroups other than A, are of two distinct serotypes: II and IV. Whereas the antigenic determinant responsible for type II specificity has been extensively studied and shown to be an OMC protein (4,5), relatively little is known of the antigenic array of the outer membrane lipopolysaccharide or LPS of meningococci of this or other serotypes (8,9).

\* Died 9 March 1976.

A group B, type III meningococcus, designated as strain 135B, was initially chosen for study since its LPS was reported to lack galactose and was thought to be structurally analogous to that of a galactose-4-epimerase deficient, or Rc mutant of an enteric bacillus (10). It was anticipated, therefore, that antisera raised to this strain would be immunologically active against most meningococci due to cross reaction with common LPS core structures.

Accordingly, antisera was raised in rabbits to the intact strain, 135B, and its bactericidal activity determined in a radioactive bactericidal assay against 42 strains of meningococci of groups A, B, C and Y and of serotypes II, II-IV, III, IV and V (Table 1). Contrary to expectations, there was a clear difference in susceptibility of strains, which appeared related to their serotype. Type II and II-IV strains were largely susceptible, while type IV strains were largely insusceptible, type III and V strains were uniformly sensitive.

The net surface charge of bacteria is largely determined by the sum of the charges of the polysaccharide moieties at their surface. Since "smooth", unencapsulated bacteria have a complete complement of oligosaccharide units within their LPS, their net surface charge differs from their rough mutants which are deficient in these oligosaccharides (11).

In order to determine if the net surface charge of strain 135B was significantly different from that of a standard group B strain, the intact 135B was subjected to aqueous biphasic partition in a polyethylene glycol/dextran T500 phase system suspended in 0.01M phosphate buffer of pH 6.8 (11), before and after digestion of the overlying group B capsular polysaccharide by neuraminidase, and compared with the prototype group B, type II strain, 99M (Fig. 1). Before digestion of the capsule both group B strains partitioned similarly into the polyethylene glycol (PEG) rich phase, suggesting a similar net surface charge; after digestion of the capsule strain 99M was excluded from both phases, but 135B continued to partition primarily into the PEG-rich phase. A gonococcus, treated identically and shown for comparison, was excluded from both phases in a fashion similar to 99M. This datum suggested a dissimilarity in net surface charge of the polysaccharide moieties of the outer membrane LPS of the two group B strains.

The LPS was next extracted by the hot phenol water method from strain 135B and from strain 126E, the prototype group C, type III strain, the KDO:dry weight ratios of the two products were 1.0 and 4.7 respectively, suggesting a greater monomer size for the 135B LPS than for that of 126E.

When the two LPS preparations, intrinsically radiolabelled with  $^{14}\text{C}$  acetate, were chromatographed over 15M of Sephadex 6-50 superfine in 3% deoxycholate with cytochrome C co-chromatographed as a molecular size marker (Fig. 2), the 135B LPS monomer was found to be somewhat

Table 1. Sensitivity of 42 meningococci to bactericidal anti-body in 135B antisera - by group and type.

Serotype	Serogroup	Nr	Sens <sup>1</sup> (%)	Resis (%)
II	C	10	8	2
	B	3	3	0
	Y	5	3	2
	Total	18	14 (78)	4
II-IV	C	3	2	1
	B	1	1	0
	Y	5	4	1
	Total	9	7 (78)	2
II or II-IV		27	21 (78)	6
III V	3A,2B,1C	6	6	0
	2Y	2	2	0
III or V		8	8 (100)	0
IV	B	1	1	0
	C	2	0	2
	Y	4	1	3
	Total	7	2	5 (71)

<sup>1</sup>  $6 \times 10^6$  meningococci killed by a standard 1:50 dilution of rabbit antisera raised to two injections of  $1 \times 10^6$  organisms (strain 135B) at a six week interval.

Fig. 1

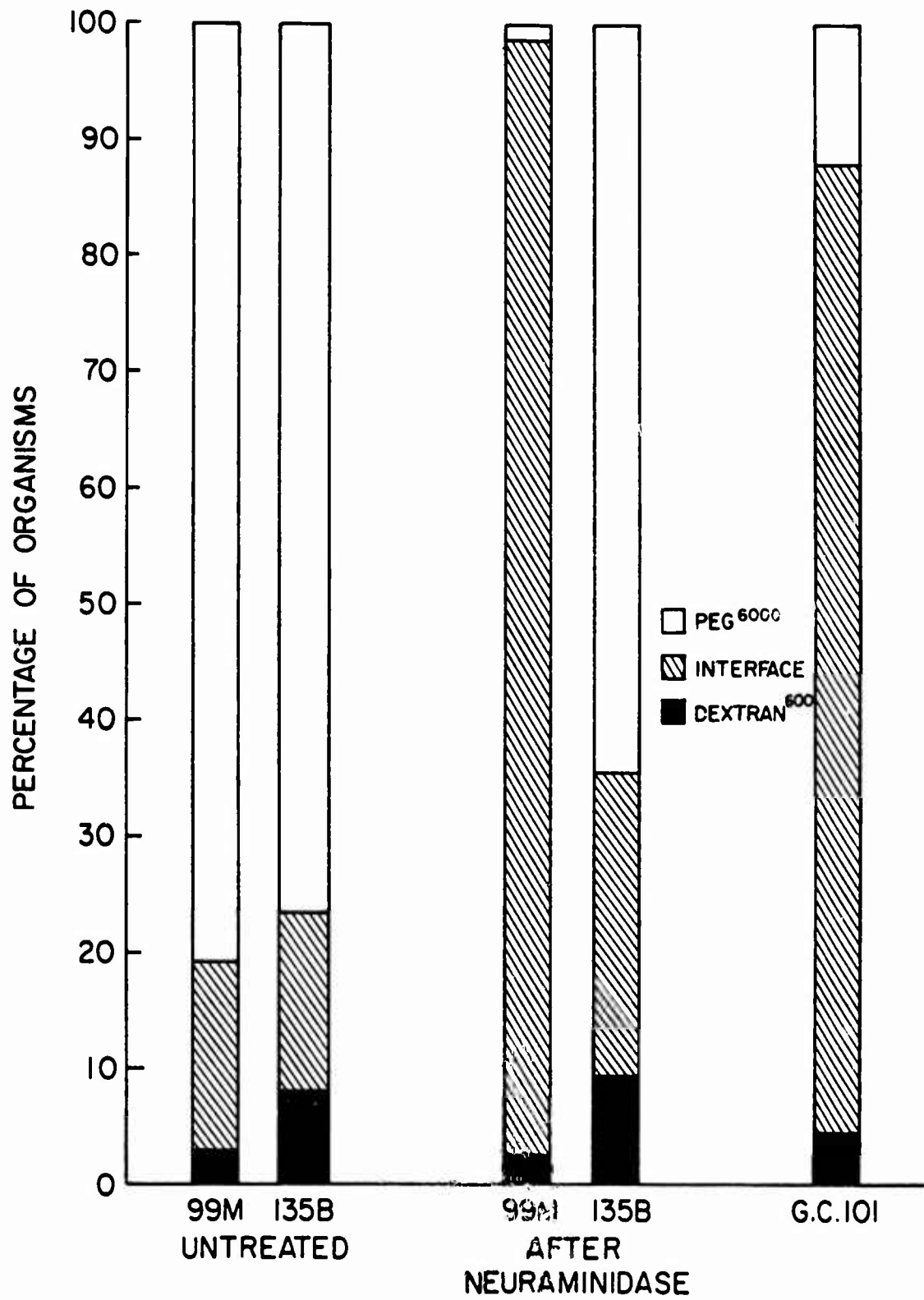
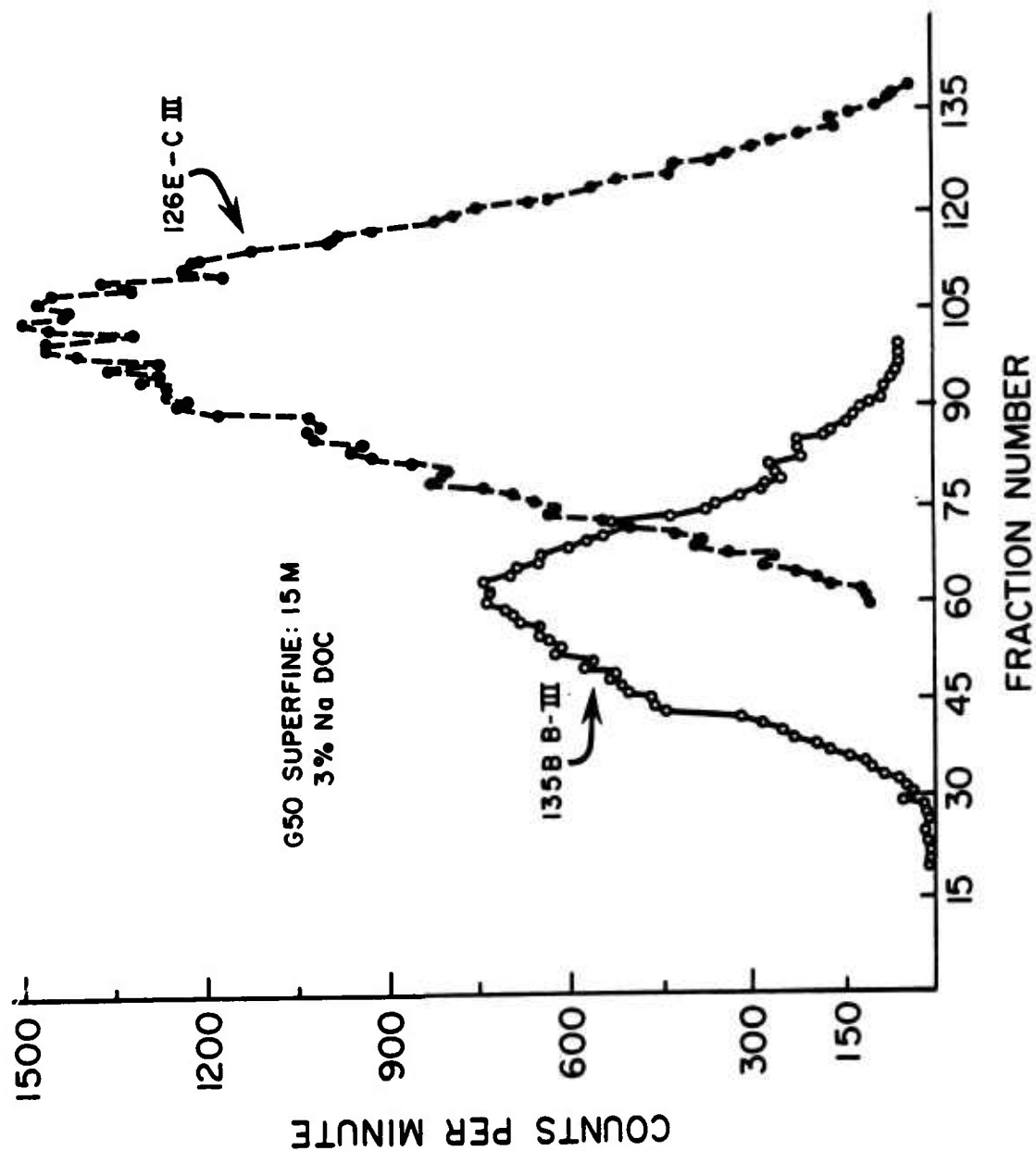




Fig. 2



larger, confirming the KDO:dry weight ratios. Both LPS monomers were between 11,500 and 12,000 daltons, similar to the monomer size of smooth enteric LPS's (8).

When the binding capacity of antisera raised to the intact strain 135B was determined for individual fractions across this LPS elution profile, in a modification of the Farr assay (8), it was found to vary for individual fractions (Fig. 3). This has previously been shown to indicate co-existence of relevant and null antigenic determinants within a single LPS structure (8).

Confirmation of this antigenic heterogeneity was obtained by comparing the binding profile of antisera raised in a single rabbit, #560, with that of a pooled antisera raised in five separate rabbits, for increasing amounts of native LPS, and of the pooled fractions from the chromatographed LPS, which evidenced 100% binding by antisera from rabbit #560. In Fig. 4 it can be seen that sera #560 incompletely bound the native LPS, but completely bound up to 750 nanograms of the 100% fractions of the chromatographed LPS; while pooling of individual rabbit sera increased the binding of the native LPS by combining antibody populations from individual rabbits who responded to different LPS determinants when presented with the intact organism.

Taken together, these data are inconsistent with the hypothesis that 135B LPS is "rough" but, rather, demonstrate a degree of antigenic complexity associated with smooth LPS. Partial analysis of this complexity was accomplished by inhibition of the Farr assay.

Binding of three separate radiolabelled LPS preparations from strain 135B by pooled anti-135B antisera was inhibited by a single non-radio-labelled preparation. Inhibition of binding of two of the radiolabelled preparations was similar, while inhibition of binding of the third preparation was markedly less efficient (Fig. 5), indicating antigenic variations between preparations (12). All four preparations, however, were found to contain galactose, glucose and glucosamine, as well as KDO and heptose, by gas-liquid chromatography (10,13).

Since the pooled anti-135B LPS appeared to be bactericidal for type II and III meningococci, but not type IV, phenol water extracted LPS from group C prototype strains of type II (138I), type III (126E) and type IV (118V) was used to inhibit binding of the 135B LPS by its pooled homologous antisera. LPS from the type II and type III strains each inhibited 30-40% of the homologous binding, while LPS from the type IV prototype strain was ineffective. When the type II and III LPS's were combined their inhibitory effect was additive and equaled that of the homologous LPS (Fig. 6).

Thus, it was shown that 135B LPS contains at least two definable antigenic determinants, one associated with type III strains and one associated with type II strains, which appear to explain the specificity

Fig. 3

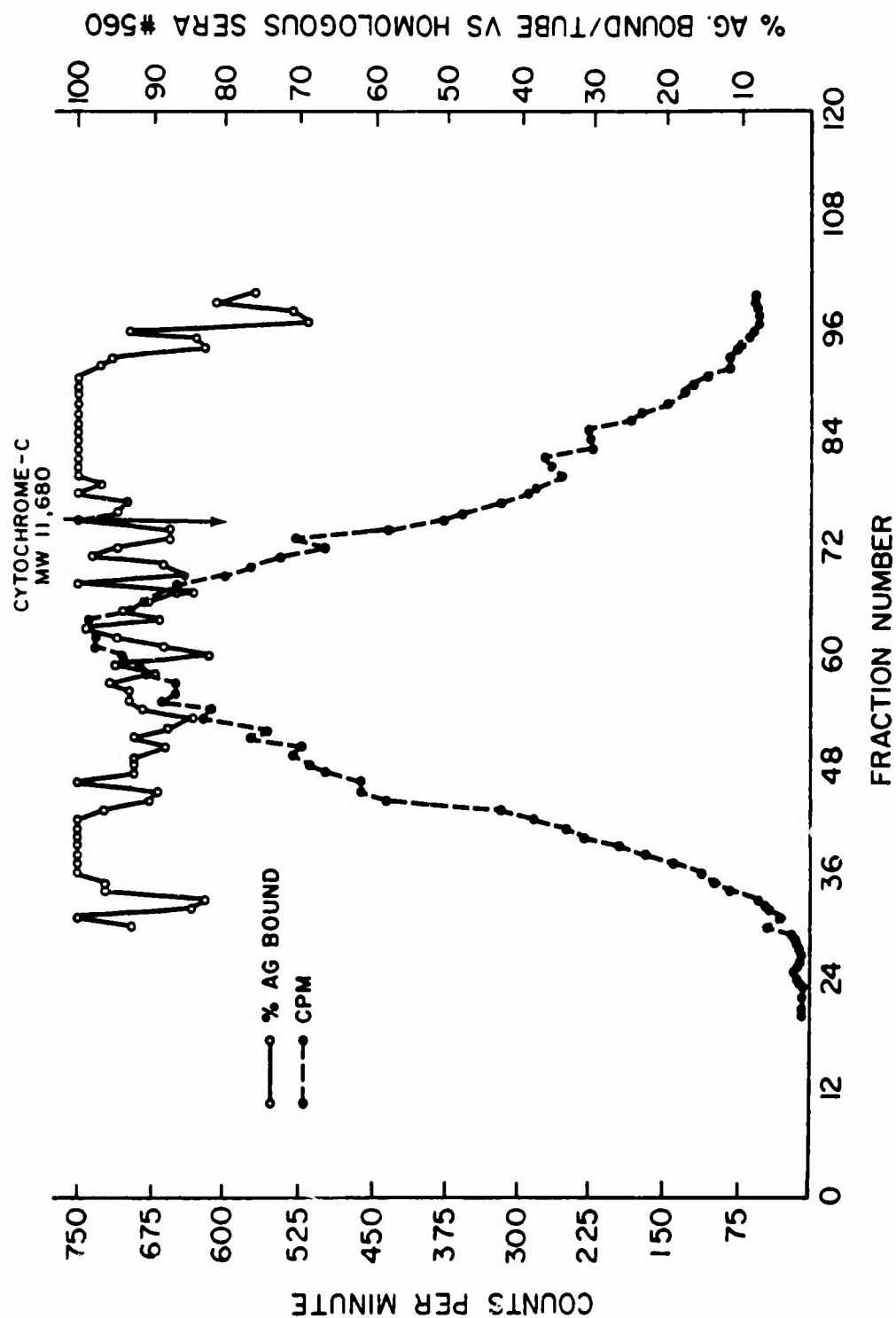


Fig. 4

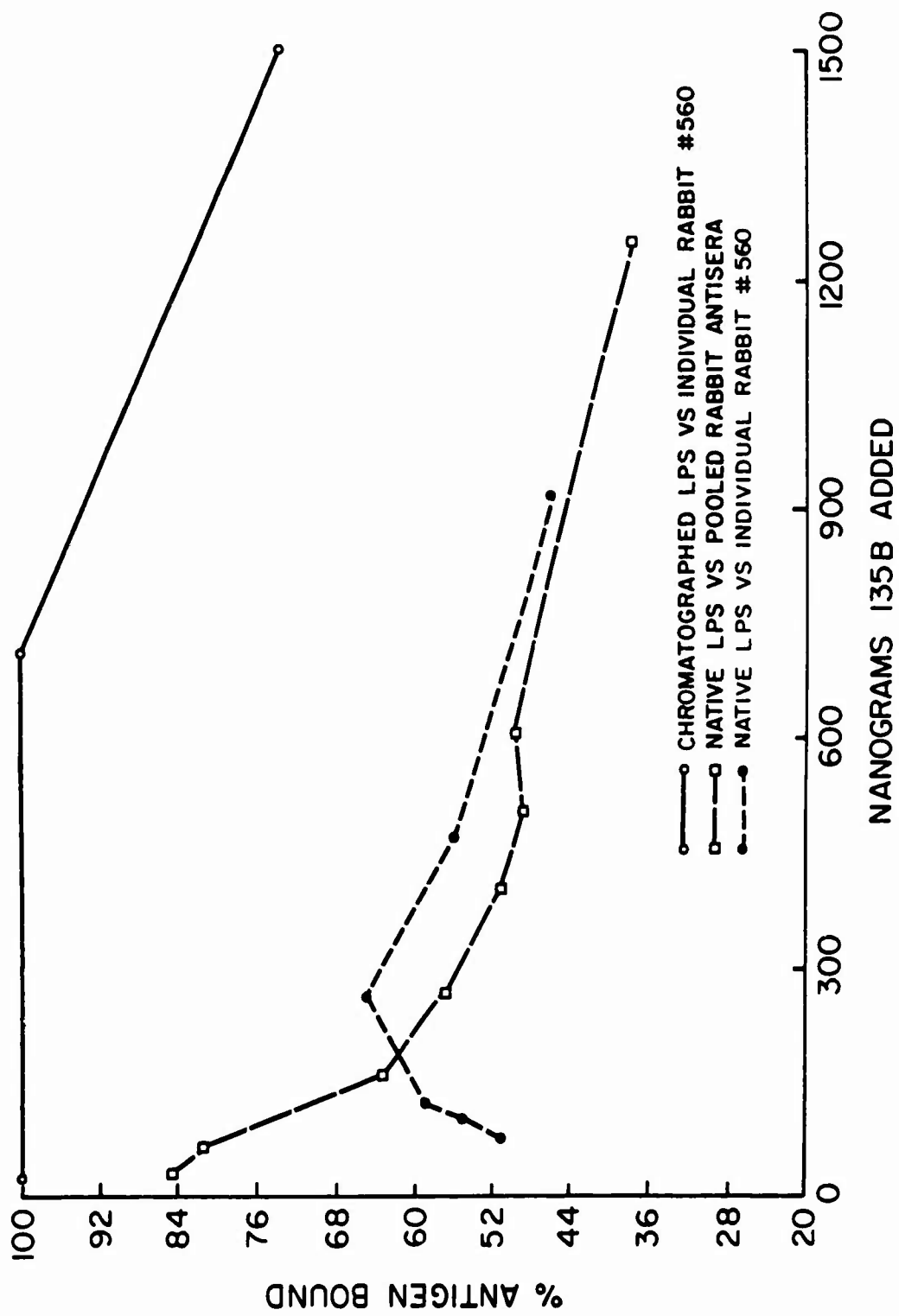


Fig. 5

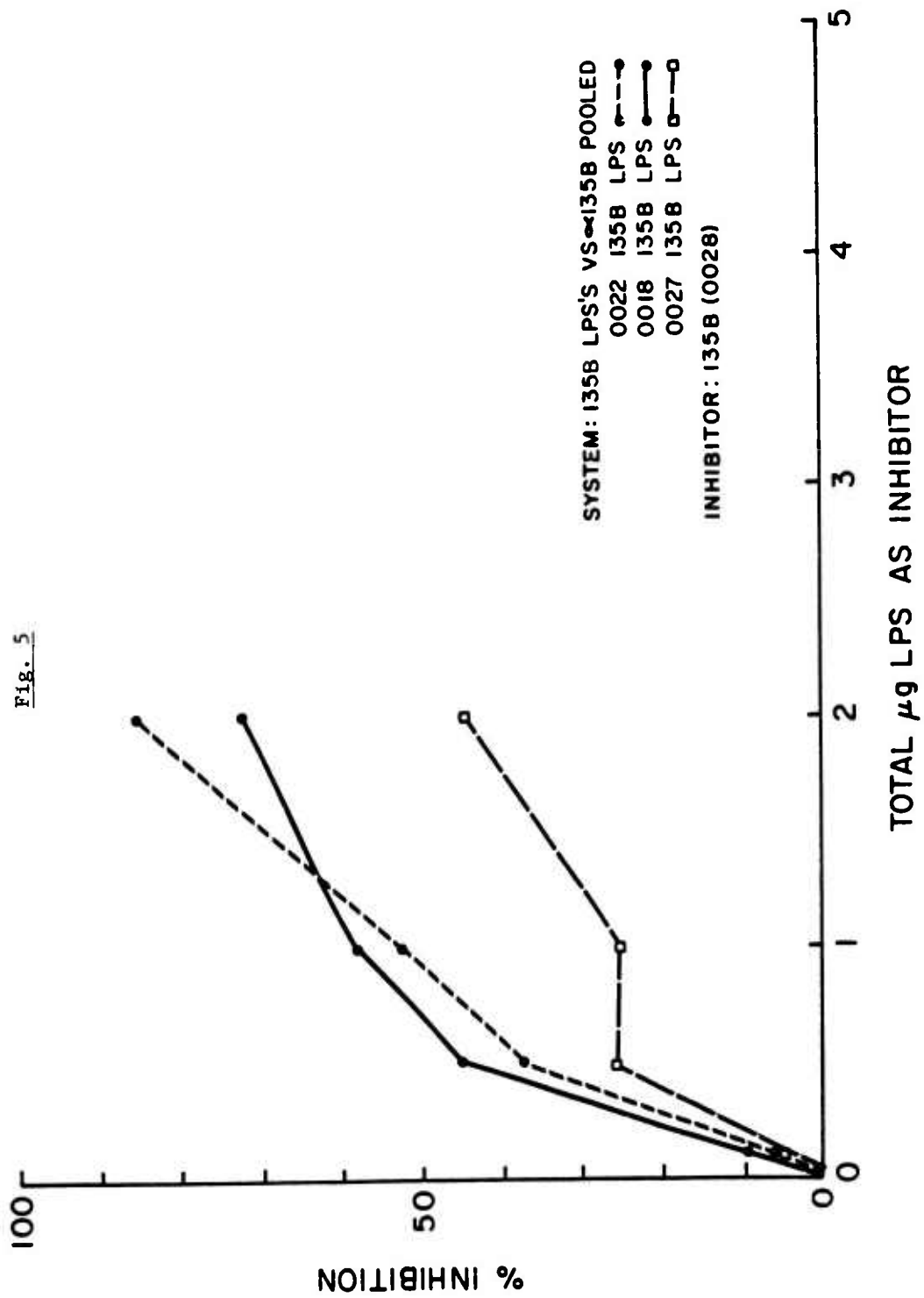
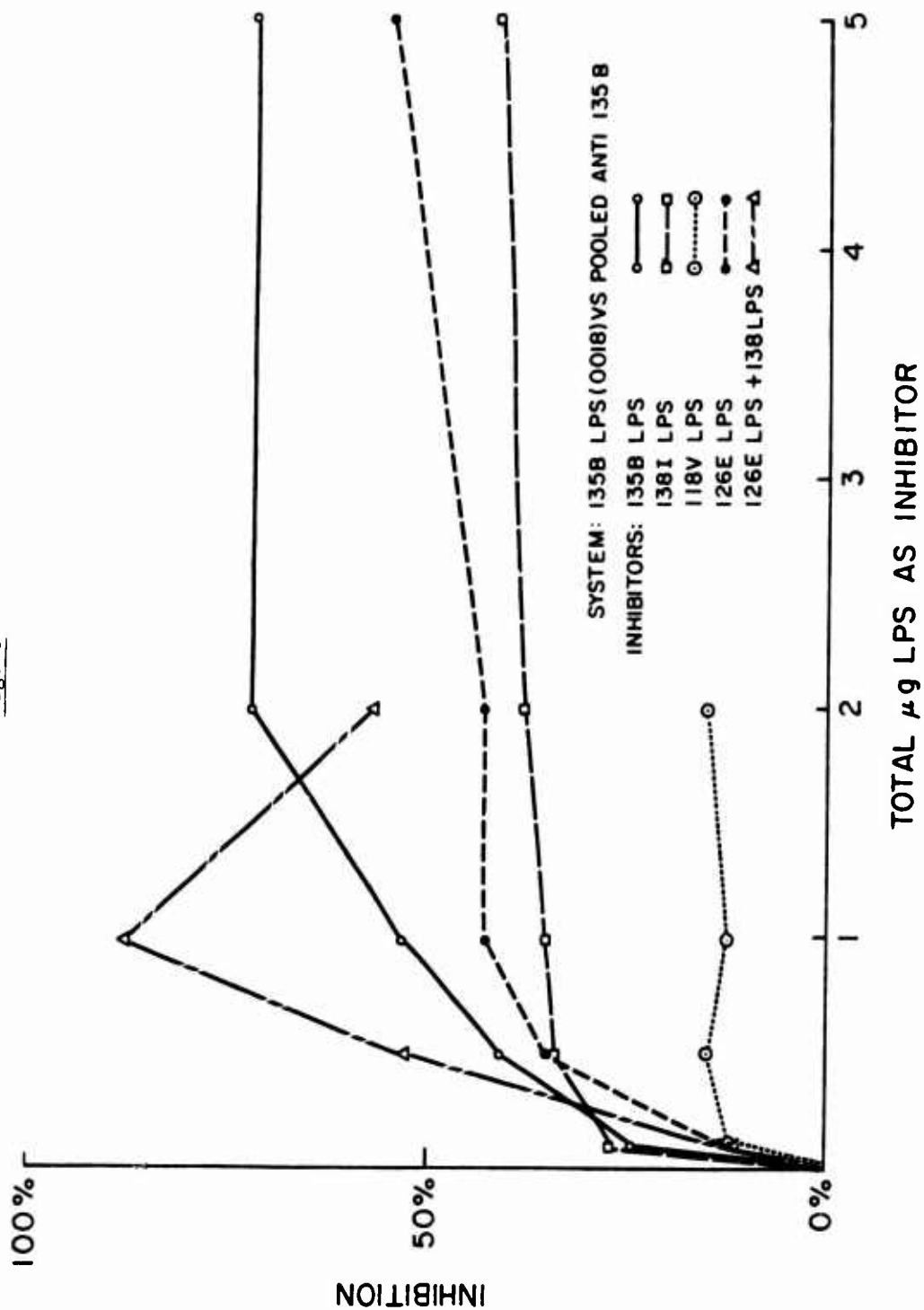


Fig. 6



of 135B antisera for strains of these two serotypes in the bactericidal assay. Whether type V strains, which are also killed by 135B antisera, possess yet a third LPS determinant or a determinant shared with type II or III strains, or both, remains to be determined.

An immunoabsorbent, prepared by covalent coupling of 135B LPS to an epoxy derivative of Sepharose, removed antibody from normal human sera as well as rabbit antisera, which, after elution with 0.05M glycine hydrochloride of pH 2.5, was bactericidal against the homologous strain. The exact antigenic specificity of this antibody is under active investigation.

The demonstration that the LPS of a group B, type III, meningococcus possesses at least two distinct antigenic determinants, associated with type II and type III meningococci respectively, antibodies against which appear to be bactericidal for strains of these two serotypes, led to an investigation of the immunological response to these antigenic determinants during the course of natural infection in military recruits.

The binding capacity of acute and convalescent sera from 59 recruit patients, infected with meningococci of various serotypes, for 33 nanograms of 135B LPS was measured utilizing the Farr assay (8).

All lipopolysaccharides used were hot phenol water extracts, containing no detectable nucleic acid or capsular polysaccharide and less than 1% protein combination. Meningococci were typed according to the schema of Gold et al (1) with slight modification and were of four serotypes: II, II-IV, IV, and V, and three serogroups: B, C, and Y.

The 59 sets of sera were drawn on the day of hospitalization or day of diagnosis and at intervals of approximately 7, 10, 21 and 30 days from military recruits with meningococcal disease between Oct. 68 and Jun 72. There were 38 cases from Ft. Dix, NJ, 8 from Ft. Leonard Wood, MO, 11 from Ft. Lewis, WA, and one each from Walter Reed and Ft. Knox, KY. Thirty-eight cases were of serogroup C, 12 of serogroup B, and 9 group Y. Of these isolates 32 were type II, 8 type II-IV, 17 type IV, and 2 type V (Table 2).

In Fig. 7 the mean rise in binding capacity by type II and II-IV case sera, expressed as nanograms of 135B LPS bound, is shown for sera drawn at each time period after infection. For graphic clarity, sera were grouped; i.e., sera drawn on days 6-9 were grouped at 7.5 days and for days 10-14 as day 12. The parentheses indicate the number of sera tested for that time period. This plot shows that types II and II-IV case sera demonstrated patterns of binding capacity similar enough to be grouped together, showing a rise of about 4 nanograms by day 7.

When the binding capacity by type II and II-IV case sera was compared to that of type V and type IV case sera (Fig. 8), type II and

Table 2. Location, time, serogroup and serotype of 59  
infecting strains of Neisseria meningitidis.

Army Area (time)	Sero- group	Serotype				TOTAL
		II	II-IV	IV	V	
Ft. Dix, NJ (11/69-6/71)	B	7		4		11
	C	19	3	1		23
	Y	1		2	1	4
		27	3	7	1	38
Ft. L. Wood, MO (12/70-9/71)	C	4	1			5
	Y		2	1		3
		4	3	1		8
Ft. Lewis, WA (10/68-4/69) WRAMC, Ft. Knox, KY (6/72-3/71)	B		1			1
	C	1	1	8		10
	Y	1			1	2
		2	2	8	1	13
TOTAL		33	8	16	2	59



Fig. 7

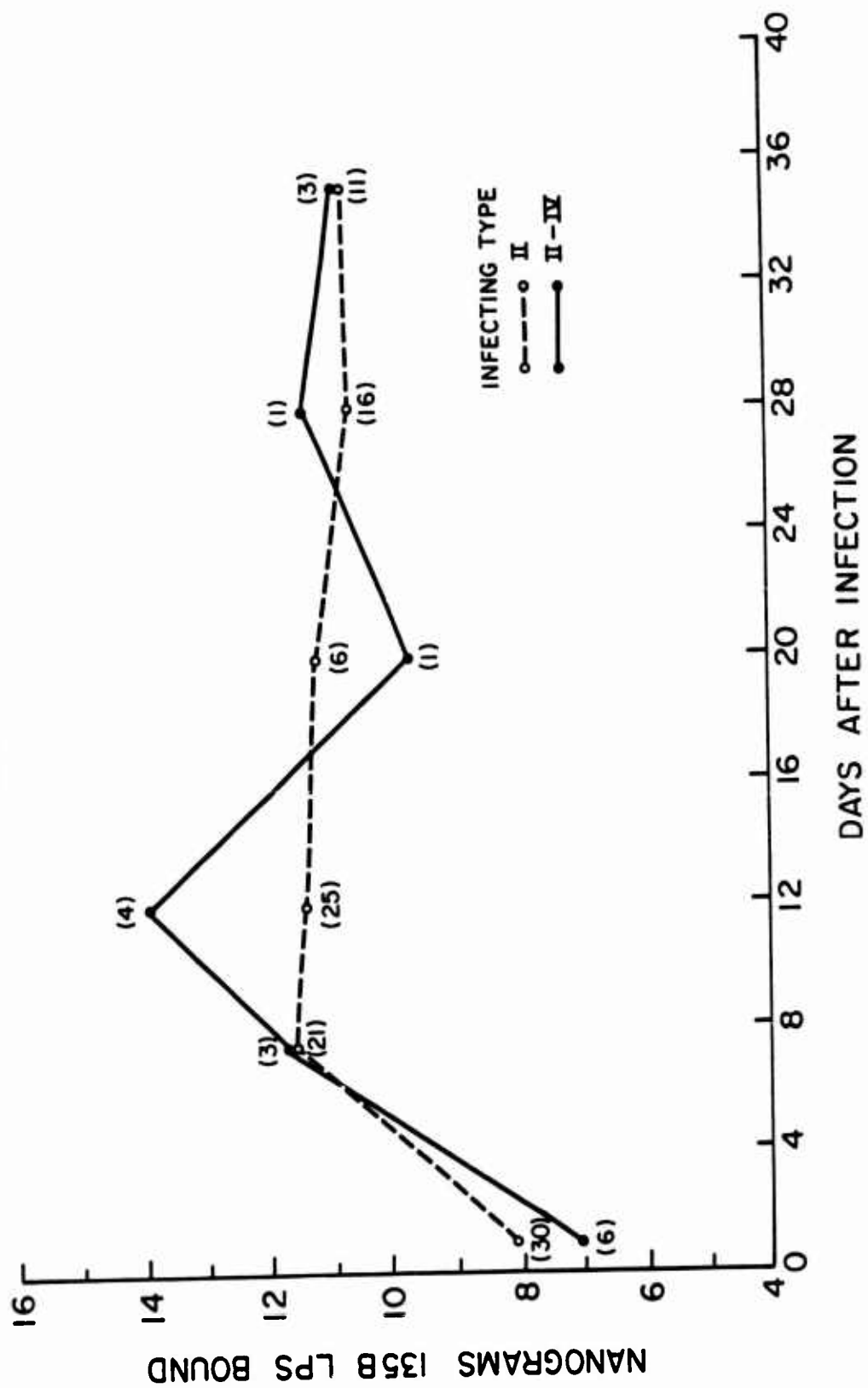
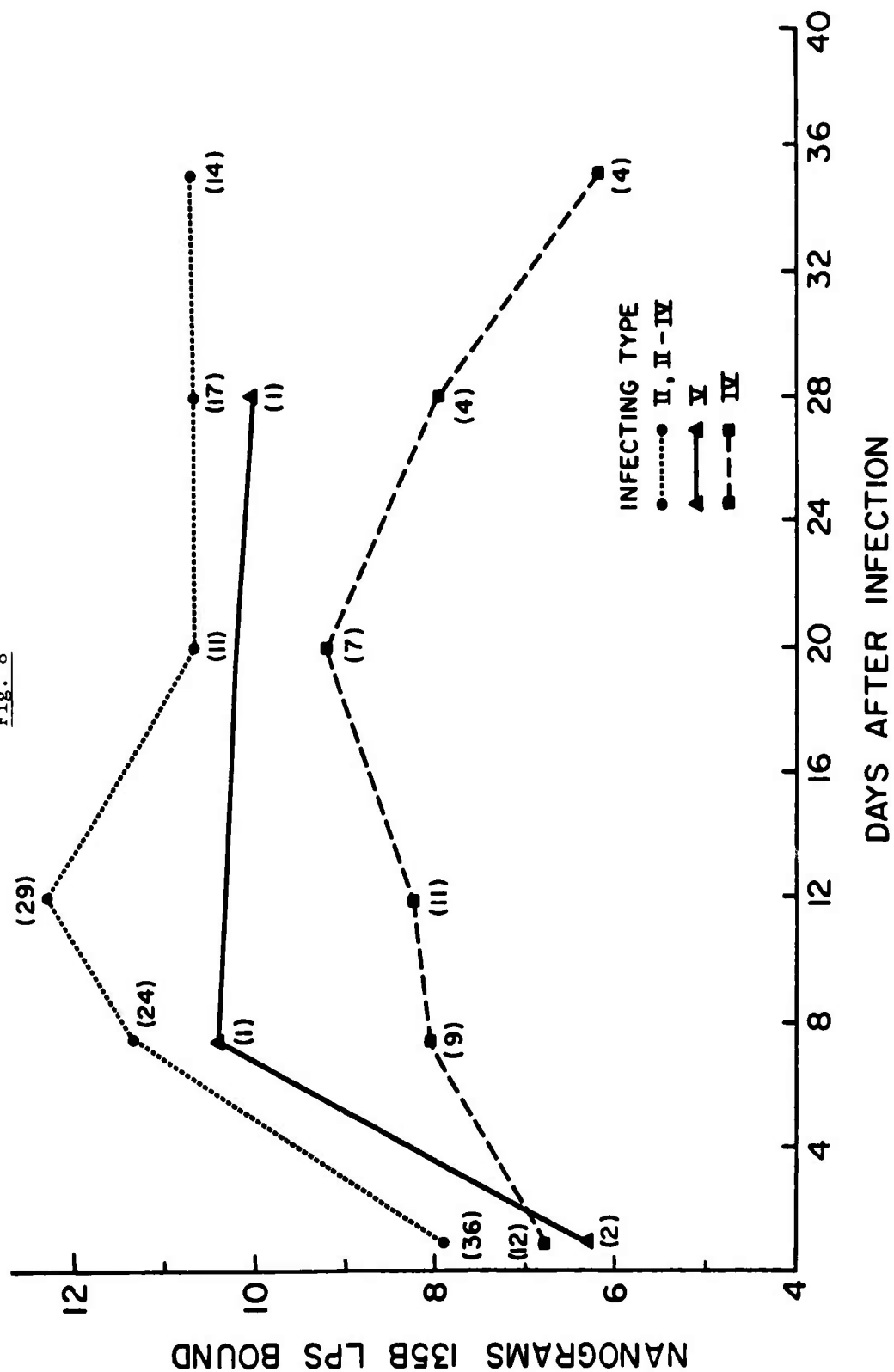


Fig. 8



I-IV cases were found to rise, on the average, 4 nanograms by day 12, while type IV case sera rose only a mean of 1.0 nanogram by day 12. Type V cases demonstrated a marked increase in binding, however, the sample size is quite small.

When the patterns of rise in binding capacity were analyzed by the serogroup of the infecting strain (Fig. 9), type II and II-IV case sera were found to demonstrate a rise in binding capacity by day 14 regardless of the serogroups of the infecting strain. Group C cases had a mean rise of 5 nanograms, somewhat greater than group B cases which rose only 3 nanograms. Type IV cases, on the other hand, failed to show a rise of greater than 1.0 nanograms, regardless of the serogroup of the infecting strain (Fig. 10).

Thus, regardless of serogroup, type II and II-IV cases demonstrated a mean rise of about 4.5 nanograms, with B cases rising the least, 3 nanograms, and Y cases rising the most, 6 nanograms, while type IV cases rose only about one nanogram, with B cases demonstrating the greatest rise.

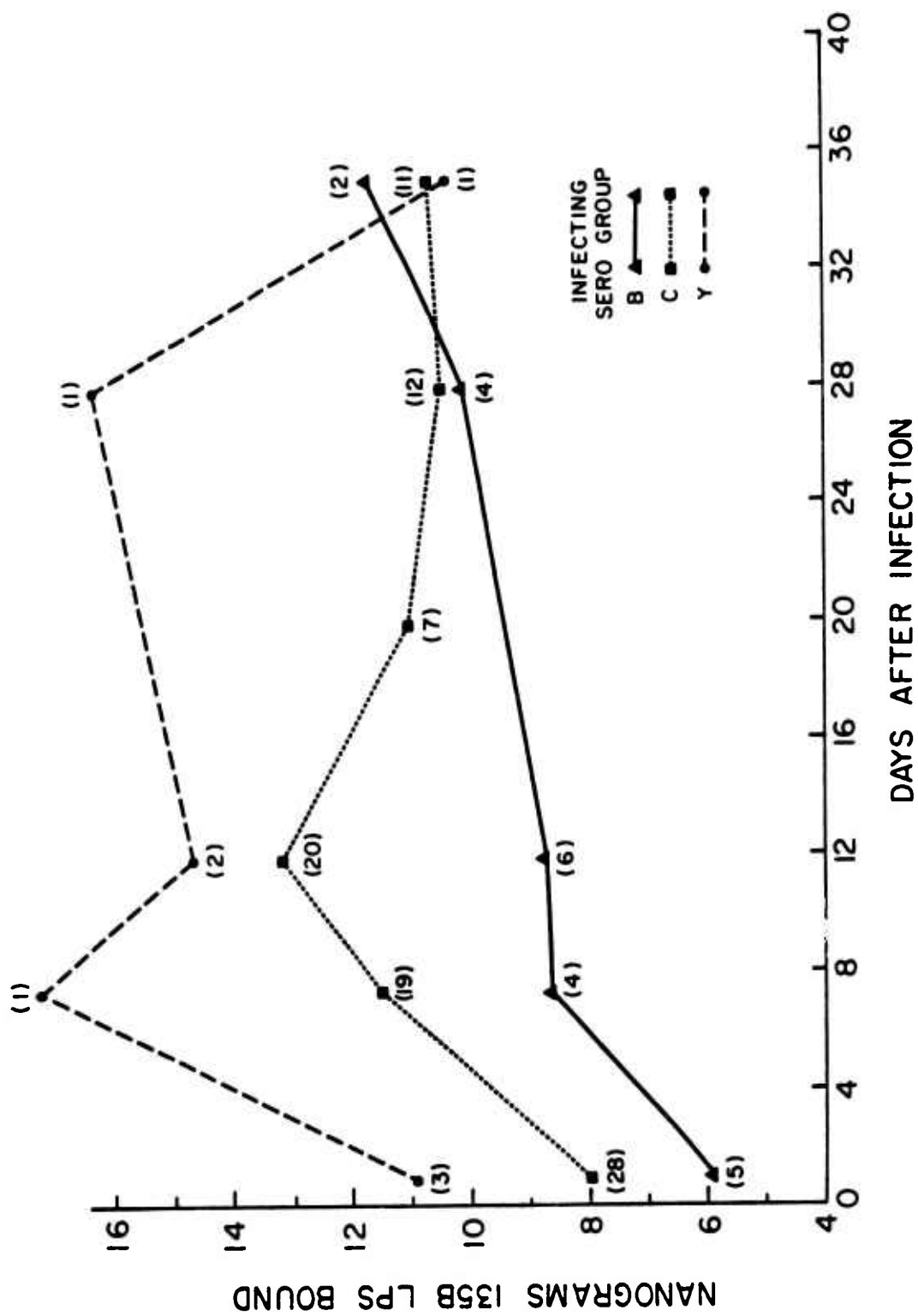
On this basis we selected a 2 nanogram rise as a cut off and examined those cases for which there was an acute serum and a convalescent serum by day 14. Twenty-six of the 33 type II and II-IV cases from which these two sera were available demonstrated a rise, whereas only 4 of 11 type IV cases demonstrated such a rise. This difference was significant by  $\chi^2$  analysis ( $p = <.005$ ). When sets of acute and 21 day sera were included, three additional type II cases, for which no 14 day sera were available, demonstrated a 2 nanogram rise in binding capacity (Fig. 11). None of the type IV sera without a rise by 14 days evidence a rise by 21 days. This difference is also statistically significant ( $p = <.001$ ).

Since 135B LPS has antigenic determinants associated with both type II and type III meningococci, the demonstrated rise in binding capacity could have been directed at either the II-associated or the III-associated determinant, or both.

Since most type II and many type IV meningococci also possess the type III determinant, the data were analyzed according to the presence or absence of co-existing type III. For the 59 cases, 87% of the infecting strains were type III in addition to either II, II-IV, IV, or V. Of those cases which demonstrated a greater than 2 nanogram rise, 70% of the infecting strains were also type III, while of those which did not demonstrate a rise, 75% of the infecting strains were also type III. Thus, serologic response to 135B LPS appeared independent of the type III antigenic determinant. To further demonstrate this, we inhibited the binding of 135B LPS by case sera from four patients infected with type II or type II-IV meningococci with the LPS from prototype group C, type II, III, and IV meningococci. Fig. 12 is representative of results obtained with the four cases. The binding of 135B LPS was inhibited primarily by the homologous LPS and that

# SERO TYPE II, II-IV

Fig. 9



# SERO TYPE IV

Fig. 10

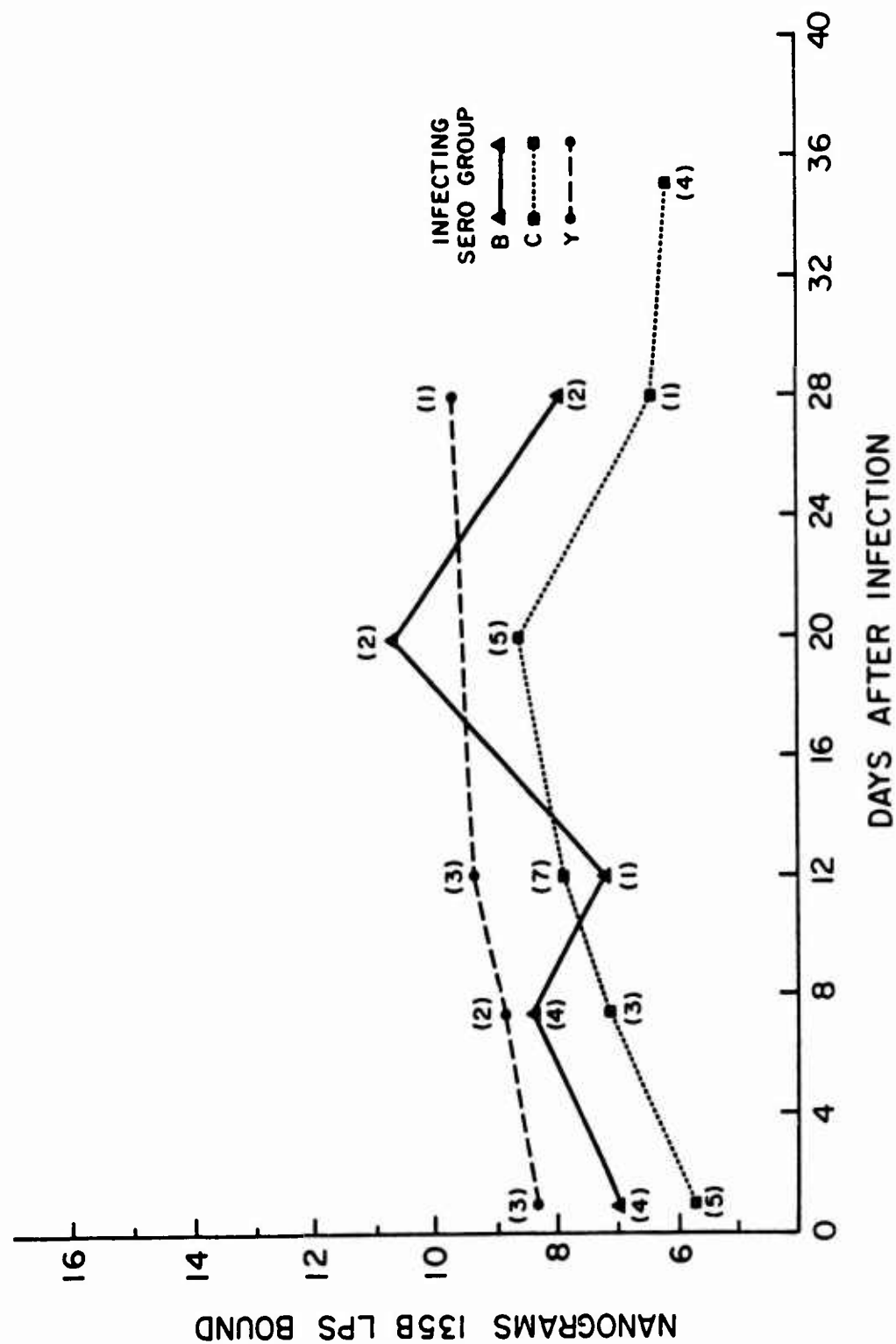


Fig. 11

PERCENTAGE OF CASES  
DEMONSTRATING A RISE  
IN BINDING CAPACITY OF  
GREATER THAN TWO NAN-  
OGRAMS BY DAY 21

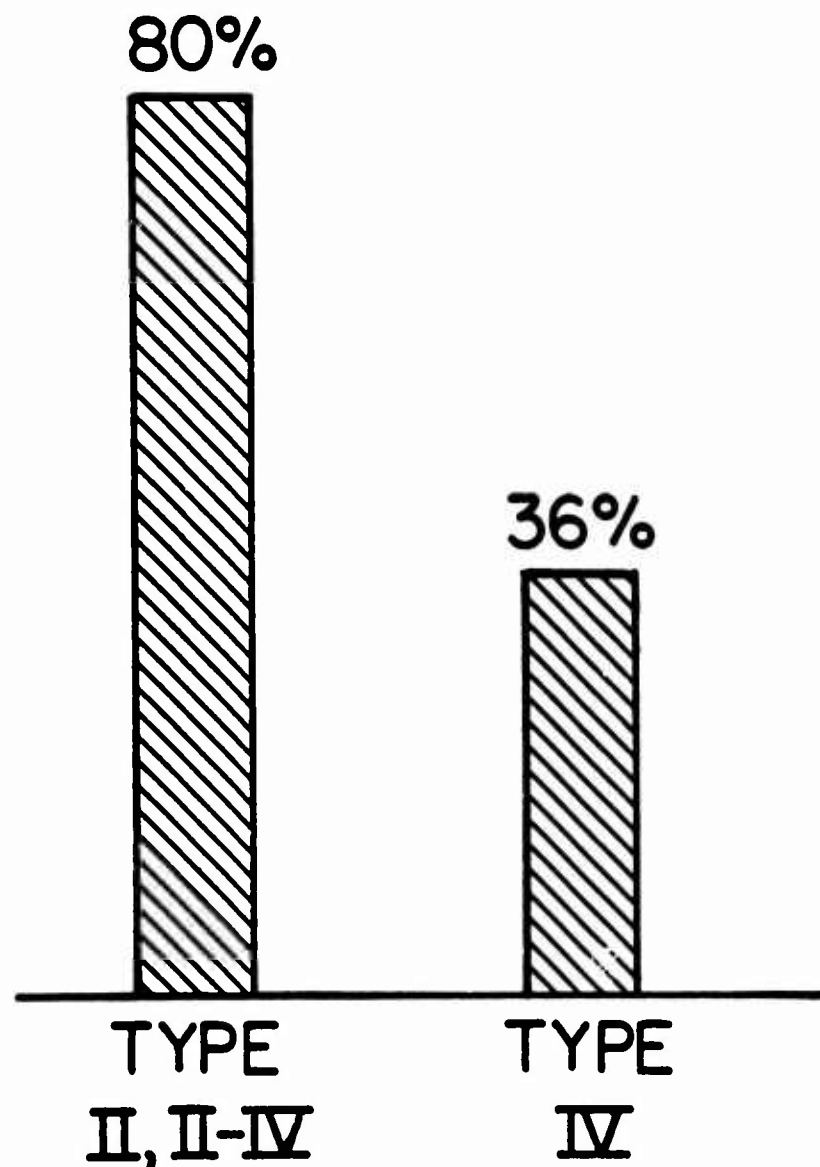
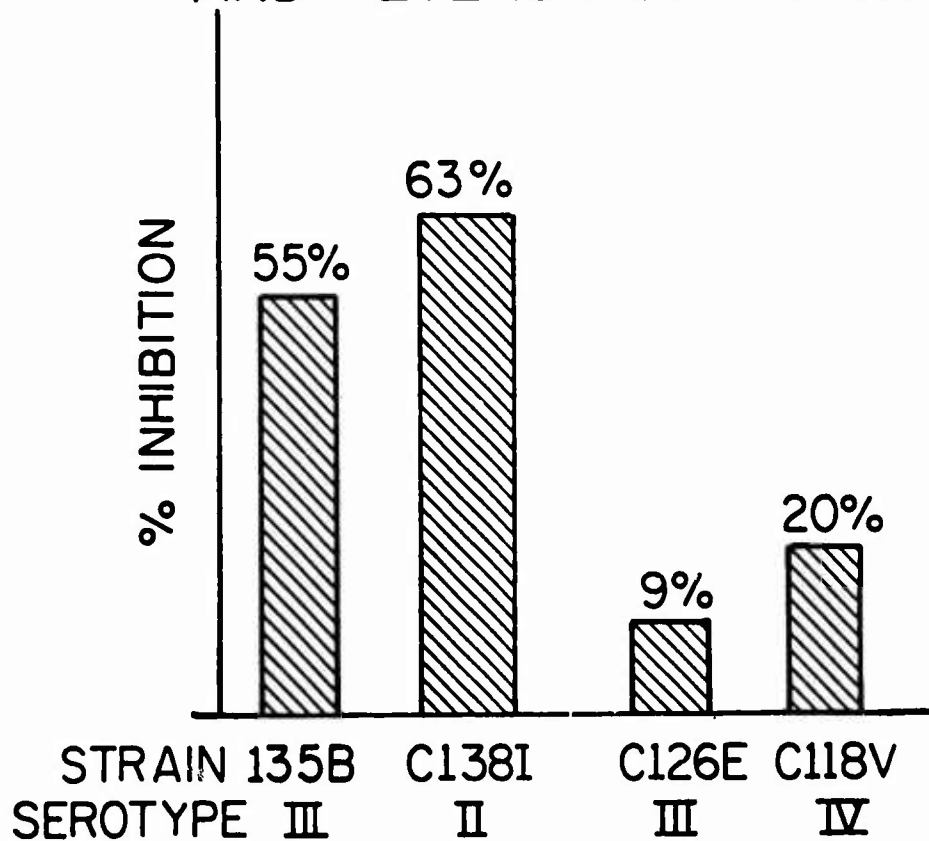


Fig. 12

INHIBITION OF TYPE II-IV  
CASE SERUM BINDING OF  
135BLPS BY HOMOLOGOUS  
AND HETEROLOGOUS LPS'S



extracted from the type II meningococci, strain 138I, while there was only minimal inhibition by LPS from the prototype III and IV strains.

These data demonstrate that patients infected with type II, II-IV, and possibly V, meningococci respond to the LPS determinant associated with serotype II, while those infected with serotype IV meningococci do not.

B. Antigen-specific serotyping of *N. meningitidis* by inhibition of a solid phase radioimmunoassay.

Strains of meningococci are grouped serologically on the basis of their capsular polysaccharides and may be further subdivided on the basis of differences in their noncapsular surface antigens - outer membrane lipopolysaccharides (LPS) and proteins - into serotypes (1,2). This subtyping is commonly performed by testing the capacity of rabbit antisera raised against standard typing strains to kill a given strain (1) or to produce precipitin bands when reacted in agar gels or capillary tubes with antigen extracted from the strain to be typed (2). Although the antigens responsible for the type specificity expressed in these assays are assumed to be the outer membrane proteins, this has only been explicitly demonstrated for the type II antigen (4,5). It is likely that antibodies to the LPS antigens are involved to some extent in these reactions, particularly with the bactericidal serotyping (6). The bactericidal serotyping is further complicated by the requirement for a suitable complement source which does not have intrinsic bactericidal activity for the strains to be typed (6).

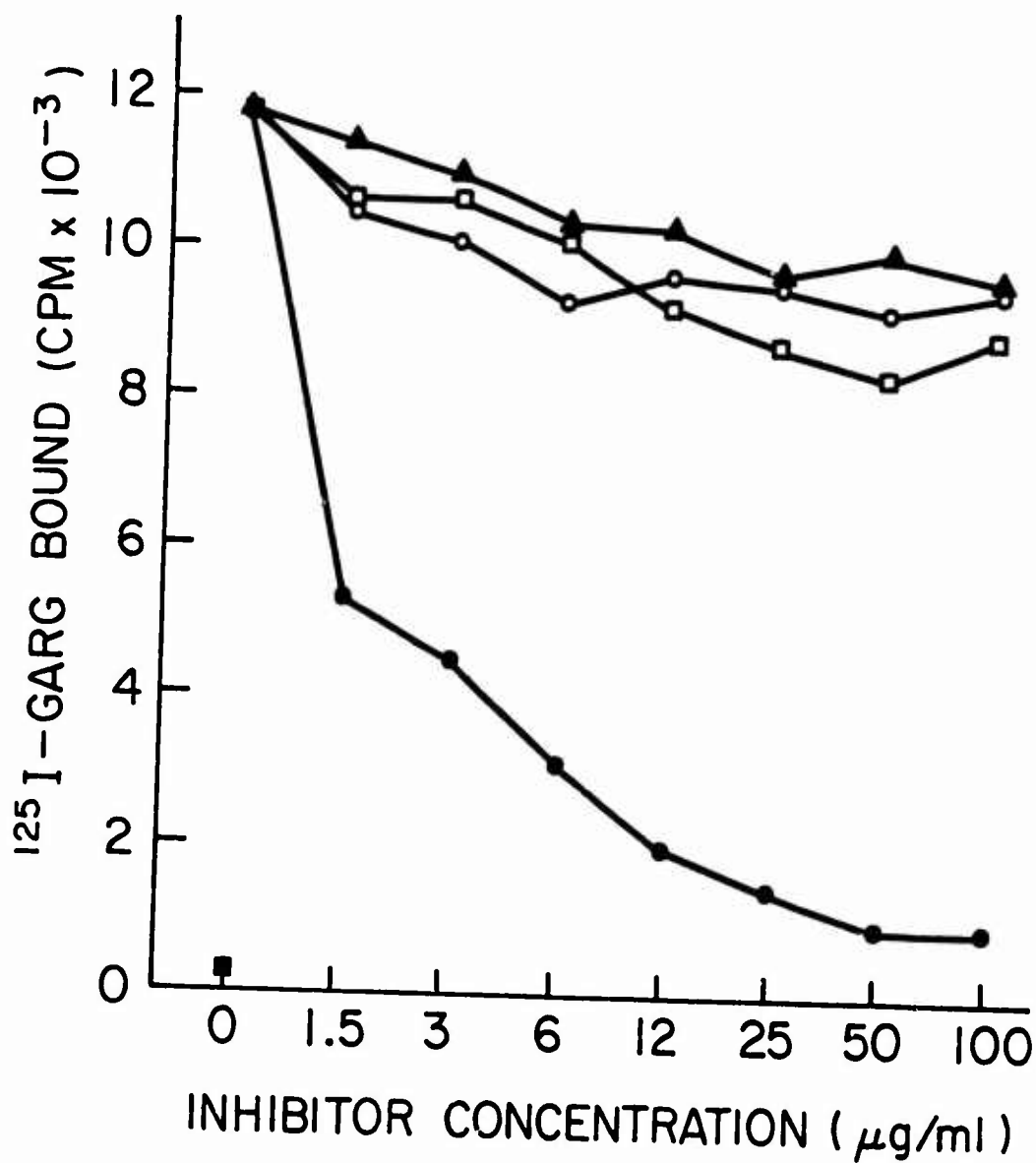
In order to circumvent these and other problems we have developed a serotyping procedure which allows one to specifically determine both the LPS and protein serotypes of meningococcal strains, using the same, simply prepared, antigen extracts. The serotyping is performed in flexible polyvinyl microtiter plates, generally following the procedures and conditions described for the quantitative SPRIA described in a subsequent section of this report. Briefly, the binding of a limiting amount of rabbit type-specific antibody to the homologous antigen, immobilized on the surface of the plate, was inhibited by preincubation with a standard concentration (50 mcg/ml) of antigen (outer membrane complex) from the strain to be typed. The amount of antibody bound was determined, after washing, by incubation with excess <sup>125</sup>I-labeled goat anti-rabbit gamma globulin and counting the amount of <sup>125</sup>I bound per well. The percent inhibition was calculated as:

$$100 - \frac{\text{net CPM bound with inhibitor present}}{\text{net CPM bound in uninhibited control}} \times 100$$

For LPS typing, purified LPS was used as the antigen on the plate; for protein typing the antigen was the outer membrane complex containing both LPS and serotype protein. In the latter case the typing sera were



Fig. 13. Antigen inhibition of the SPRIA. Binding of rabbit anti- $^{138}\text{I}(\text{C})$  antiserum (1:50,000 dilution) to  $^{138}\text{I}(\text{C})$  OMC was inhibited by preincubation for one hour at  $37^\circ\text{C}$  with various concentrations of  $^{138}\text{I}(\text{C})$  OMC ( $\bullet$ ),  $^{35}\text{E}(\text{C})$  OMC ( $\circ$ ),  $^{126}\text{E}(\text{C})$  OMC ( $\square$ ) or  $^{118}\text{V}(\text{C})$  OMC ( $\blacktriangle$ ). The test was then completed using the standard procedure.



absorbed as necessary with LPS-sensitized sheep erythrocytes to remove antibodies to the LPS moiety, and controls were included to insure that maximum inhibition by the homologous LPS and/or capsular polysaccharide was less than 10%. It was not feasible to use purified proteins as the antigen on the plate, since the protein could not be purified free of LPS without denaturation of important protein determinants.

The effect of inhibitor concentration is shown in Fig. 13. Maximum inhibition by both homologous and heterologous antigens was obtained at 20 mcg/ml. Based on experiments of this type, a standard inhibitor concentration of 50 mcg/ml was chosen for routine serotyping.

Sera for protein serotyping were made against both the serotyping strains developed in the Department of Bacterial Diseases (WRAIR) (1,3,6) and those of Frasch and Chapman (2). The protein and LPS serotypes of these prototype strains were reexamined and internally compared in a block inhibition experiment using the combined set of 22 protein typing sera together with the set of homologous antigens. The results of these experiments are given in Table 3. In the protein typing, some WRAIR prototype strains were typable using the antisera against the Frasch and Chapman strains and vice versa. Strong intersystem, reciprocal cross reactions (>70% inhibition) were observed between strains with the following types: 2 and II; 11 and IV or VII; 9 and X. Several weaker one-way cross reactions (>50% but <70% inhibition) were also observed. The strong reciprocal cross reactions were interpreted as being redundant and one serum from each pair was eliminated from the combined system. The sera used for subsequent protein typing are listed in Table 4, along with a suggested protein type designation. The LPS typing sera and type designation are also listed.

Using this system approximately 60 additional strains of meningococci representing all major serogroups were tested to determine their protein and LPS types. All but four strains were typable with the eight LPS typing sera but approximately 30% were nontypable with the 18 protein typing sera. A partial correlation of the protein and LPS types was possible for those strains having protein types P1 through P6 (Table 5). Protein type P2 was associated with LPS type 03 on 19 of 19 strains and with type 07 on 16 of 19 strains. In addition, P4 was associated with 04 in 6 of 6 strains and P5 with 02 in 2 of 2 strains. Protein types P1, P3 and P6, however, were not consistently associated with a single LPS type. Thus, although some significant correlations between protein and LPS types are evident, the two antigens generally must be considered to be independent antigenic markers.

Serotyping by SPRIA inhibition has a number of important advantages over existing methods: a) the results are antigen specific, b) no complement is required, allowing serum sensitive strains to be typed as easily as serum resistant strains, c) the results are

Table 3. Protein and LPS serotypes of meningococcal serotyping strains as determined by SPRIA inhibition.

Strains	Serogroup	Bactericidal serotype*	SPRIA inhibition	
			Protein type <sup>a</sup>	LPS type
<u>WRAIR</u>				
60E	C	I	I	3, (4, 7)
138I	C	II	II :2	3
126E	C	III	III	1, 8, (3)
118V	C	IV, III	IV, III:11	4, 2
35E	C	V	V : (5)	2
32I	C	VI	VI : (5)	2, (4)
89I	C	VII, IV	VII, IV:11	4
190I	B	VIII	VIII	1, 3, 7, (4)
6155	B	X, (I)	X :9	3, 7
6557	B	XI, III	XI	1, 8, (7)
6940	B	XII, III	XII	1
 <u>Frash &amp; Chapman</u>				
M1080	B	1	1	4, 7, (3, 8)
B16B6	B	2	2 :II, VI	2, (3)
M981	B	4	4	5
M992	B	5	5	6
M990	B	6	6 : (X)	3, (7)
M986	B	2, 7	2 :II, VI	3, (7, 2)
M978	B	8, (3, 1)	8	8, 4(7, 3)
M982	B	9	9 :X	7
M1011	B	10, 2	10, 2 :II, VI	3, (7)
M136	B	11	11 :IV, (VII)	4
S3032	B	12	12	(3, 7)

<sup>a</sup> Types appearing after the colon indicate inhibition of sera from the other set. Numbers given in parentheses indicate inhibition between 50 and 70% while numbers not in parentheses indicate >70% inhibition.

Table 4. Sera used for protein and LPS serotyping by SPRIA inhibition.

Protein typing sera	Type	LPS typing sera	Type
60E <sup>a</sup>	P1	126E	01
M986 or 138I	P2	35E	02
126E	P3	6275	03
118V/60E + 118V LPS	P4	89I	04
35E/35E LPS	P5	M981	05
32I/60E + 118V	P6	M992	06
89I/118V	P7	6155	07
190I	P8	M978	08
M982	P9		
M1011/B16B6	P10		
6557	P11		
6940/6940 LPS	P12		
M1080	P13		
M981/M981 LPS	P14		
M992/M992 LPS	P15		
M990/M136	P16		
S-3032	P17		
138I/99M	P18		

<sup>a</sup> Immunizing strain/absorbing strain or antigen.

Table 5. Occurrence of each LPS serotype with WRAIR protein serotypes I-VI in 39 meningococcal strains.

Protein serotype	No. of strains with this type	No. of strains having this LPS serotype							
		01	02	03	04	05	06	07	08
P1	2	0	1	1	1	0	0	1	0
P2	19	1	9	19	2	2	0	16	0
P3	19	4	11	12	3	6	0	11	3
P4	6	1	1	2	6	0	2	3	0
P5	2	0	2	0	0	0	0	0	0
P6	10	0	3	7	2	1	1	7	0

quantitatively expressed as percent inhibition which eliminates subjective judgements of precipitin lines or agglutination patterns, d) both protein and LPS type can be determined in the same system using a single antigen extract, and e) calculation and tabulation of the data can easily be handled by a desk top programmable calculator.

## II. Serum IgA as determinant of susceptibility to meningococcal disease.

Susceptibility to systemic meningococcal disease correlated directly with the absence of strain-specific bactericidal activity normally present in the serum of 80-90% of young adults (14). The demonstration that circulating anti-meningococcal IgA is capable of blocking the complement mediated bacteriolytic activity of anti-meningococcal IgG and IgM (15), led to an investigation of its possible role in producing the susceptible state by abrogating bactericidal activity. The sera of military recruits - a population at greater than expected risk were accordingly studied during two concurrent epidemics.

Sera were drawn on the day of hospitalization, or the day of diagnosis, from all recruits with meningococcal disease at Ft. Dix, NJ and Ft. Leonard Wood, MO during a period of epidemic incidence of predominantly serogroup C meningococcal disease (6). Twenty-eight sera were selected (Table 6) on the basis of adequacy of the serum sample, availability of each homologous infecting strain and to represent patients infected with all three of the causative serogroups and serotypes at several time periods. Thirteen of the infecting meningococci were serogroup C, 8 group Y and 7 group B; 21 were type II or II-IV, 6 type IV and 1 type V. Twenty were isolated at Ft. Dix and 8 at Ft. Leonard Wood.

Serum IgA was removed from an aliquot of each serum by adsorption to goat anti-human IgA,  $\alpha$ -chain specific, the IgG fraction of which had been covalently linked to cyanogen-bromide activated Sepharose (15). Serum IgG was separated by elution from DEAE Sephadex with an 0.018 M phosphate buffer of pH 6.22 (15). IgM was separated by filtration chromatography over an agarose:polyacrylamide gel equilibrated in 0.1 M Tris in 0.15 M NaCl of pH 7.4 (16). Concentrations of immunoglobulins were determined by the Mancini method of radial immunodiffusion. IgG and IgM fractions were free of other classes of Ig as determined by radial immunodiffusion, with a lower limit of sensitivity of <20 mgm/100 ml. for IgG and IgA and of <25 mgm/100 ml. for IgM.

The bactericidal activity of acute serum from each patient, before and after removal of IgA, and of IgG and IgM fractions separated therefrom was tested in a radioactive bactericidal test (17) against each of the homologous infecting strains and titres calculated for killing of  $2.5 \times 10^6$  meningococci. Dilutions of absorbed sera were determined by reference to IgM concentration.

Table 6. Time and location, serogroup and serotype of 28 meningococcal isolates.

Location <sup>1</sup>	No.	Serogroup	Serotype	Time of isolation
Dix	4	C	II (1 II-IV)	April-May 1970
(20)	4	C	II (1 II-IV)	Aug 1970-March 1971
	2	Y	II	Dec 1969; Feb 1971
	2	Y	IV	Aug 1970; Oct 1970
	1	Y	V	Dec 1969
	4	B	II	Sept-Oct 1970
	3	B	IV	May-July 1971
L-W	5	C	II (1 II-IV)	March-May 1971
(8)	2	Y	II-IV	Dec 1970; May 1971
	1	Y	IV	Aug 1971

<sup>1</sup> Dix - Ft. Dix, NJ; L-W - Ft. Leonard Wood, MO

Bactericidal activity was deficient in 24/28 unabsorbed sera (Table 7) using the criteria defined by Goldschneider et al. in the original studies establishing the correlation between susceptibility and bactericidal activity. Immunoglobulin levels of all three classes were within the normal range for all 28 sera. After removal of IgA, bactericidal activity was uniformly present at a mean titer of  $>1:90$ . The mean quantities of IgM and IgG at that dilution which killed  $2.5 \times 10^6$  meningococci were  $<1.76$  and  $<13.5$  mcgms respectively.

IgG separated from 17 of the acute sera was either devoid of bactericidal activity or minimally active (Table 8). The mean quantity of IgG at those dilutions which killed  $2.5 \times 10^6$  meningococci was  $>67.8$  mcgm as compared to  $<13.1$  mcgm of IgG in the whole sera after removal of IgA. This datum strongly suggested that the lytic activity in whole serum was due to IgM.

When the bactericidal capacity of IgM, separated from six sera, was similarly compared with the concentration of IgM in the corresponding whole sera, after removal of IgA (Table 9), good correspondence was found. A mean of  $<0.84$  mcgms of purified IgM effectively killed  $2.5 \times 10^6$  meningococci as compared with a mean of  $<1.14$  mcgm of IgM in the whole sera, after removal of IgA. Thus, the scant bactericidal activity found in 10 of 17 IgG fractions could be accounted for by contaminating quantities of IgM, below the sensitivity of the immunodiffusion plates - or equivalent to  $<2.5$  mcgm.

These six sera were drawn from individuals infected with group B and C, type II and group Y, type V strains. IgM was also separated from three sera, drawn from individuals infected with group Y, type II and IV and group B, type IV strains (Table 9). No bactericidal activity was found in these IgM fractions, due to binding of lytic antibody to the agarose:polyacrylamide gel. The lytic activity from the corresponding whole sera, after removal of IgA, was likewise removed by simple absorption with the unmodified gel. These data suggest that lytic IgM and blocking IgA in this population of military recruits is directed at at least two distinct antigenic determinants - one common to Y II and Y and B IV meningococci, the other common to C and B II and Y V strains. They further suggest that the naturally occurring galactan, agarose, may be cross reactive with the galactose containing endotoxic lipopolysaccharide of Y II, Y IV and B IV meningococci, since galactose is not found in their capsular polysaccharide. A similar cross reaction has been demonstrated between a naturally occurring galactan of gum arabic and the lipopolysaccharide of Salmonella typhimurium, type 5 (18).

Further evidence for a disparity between the antigenic determinants against which lytic IgM was directed was afforded by demonstration of the specificity of the removed IgA (Table 10).

The bactericidal activity of whole sera, before and after removal of IgA, drawn from individuals infected with a C II and Y II meningococci, respectively, was determined against the homologous and a



Table 7. Homologous meningococcal lytic activity<sup>1</sup> and mean Ig levels before and after removal of IgA of 28 acute case sera.

No.	Whole sera <sup>2</sup>				After removal of IgA		
	IgM	IgG	IgA	Titre	Titre <sup>3</sup>	IgM <sup>4</sup>	IgM <sup>4</sup>
20	149.7	1108	176.8	<1:10	>1:90	<1.66	<12.4
1	145.7	1894.5	322.8	1:10		3.4	28.2
3	148.3	2107.4	157.9	<100% at 1:10	>1:86	<1.72	<15.3
Total	149.4	1280.3	180.5	<1:10	>1:85	<1.75	<13.4
4	193.7	1657	194.6	>1:80	>1:106	<1.83	<14.3
Total	155.7	1338.2	182.5		>1:90	<1.76	<13.5

<sup>1</sup> Calculated for kill of  $2.5 \times 10^6$  meningococci

<sup>2</sup> Mean Ig levels in mgm/100 ml

<sup>3</sup> Calculated by reference to IgM level as dilution marker

<sup>4</sup> Mean Ig levels in mcgm/ $2.5 \times 10^6$  meningococci

Table 8. Homologous lytic activity of IgG, separated from 17 acute case sera by DEAE ion-exchange chromatography, compared with IgG levels in dilution of each sera which lysed  $2.5 \times 10^6$  meningococci after removal of IgA.

Strains		IgG level <sup>1</sup>	
Group	Type	DEAE	After removal of IgA
C	II-IV	>61.9	19.3
C	II	>60.0	24.0
C	II	>136.0	14.4
C	II	>33.6	15.9
C	II-IV	<12.7*	<20.0*
C	II	10.8	3.1
C	II	27.7	13.7
C	II	31.2	<4.5
C	II	>278.0	9.85
C	II	>91.3	2.8
Y	IV	39.8	10.5
Y	II	92.1	10.0
Y	V	>95.1	10.4
Y	IV	20.1	15.7
Y	II	62.6	10.1
B	II	31.1	10.9
B	IV	30.9	8.5
B	IV	37.4	18.7
Mean		>67.8	<13.1

<sup>1</sup> In mcgm/ $2.5 \times 10^6$  meningococci

\* Titre of whole serum >1:80

Table 9. Homologous lytic activity of IgM, separated from 8 acute case sera by gel filtration, compared with IgM levels in dilution of each sera which lysed  $2.5 \times 10^6$  meningococci after removal of IgA.

Strains		IgM level <sup>1</sup>	
Group	Type	ACA	After removal of IgA
C	II-IV	1.9	2.15
C	II	0.5	1.37
C	II	1.1	<.69
C	II	0.12	0.4
B	II	<1.1	1.5
Y	V	0.33	0.74
Mean		<0.84	<1.14
Y	II	0.2	.9
Y	IV	0.2	1.03
B	IV	0.2	2.7

<sup>1</sup> In mcgm/ $2.5 \times 10^6$  meningococci

<sup>2</sup> Lytic activity, after removal of IgA, absorbable by unmodified agarose gel.

Table 10. Specificity of removed "blocking" IgA.

	Bactericidal titre <sup>1</sup>			
	Homologous		Heterologous	
	Gp C	Ty II	Gp B	Tp IV
Whole serum	<1:10		<1:10	
After removal of IgA	1:100		<1:19	
	<u>Gp Y   Tp II</u>			
Whole serum	<1:10		N.T. <sup>2</sup>	
After removal of IgA	1:140		1:140	

<sup>1</sup> Determined for killing of  $2.5 \times 10^6$  meningococci

<sup>2</sup> Not tested

heterologous B IV strain. As expected, removal of IgA from the C II sera did not result in bactericidal activity against the B IV strain, while removal of IgA from the Y II sera resulted in identical titres of activity against both homologous and heterologous strains.

These data demonstrate that levels of anti-meningococcal IgA, sufficient to completely abrogate the lytic activity of anti-meningococcal IgM, can be readily achieved in vivo and that during epidemic meningococcal disease such circulating IgA is of similar narrow specificity to that of protective IgM. Furthermore, acquisition of specific, blocking IgA provides an attractive hypothetical explanation for the sudden emergence of epidemic susceptibility to the meningococcus in subpopulations at greater than expected risk of disease.

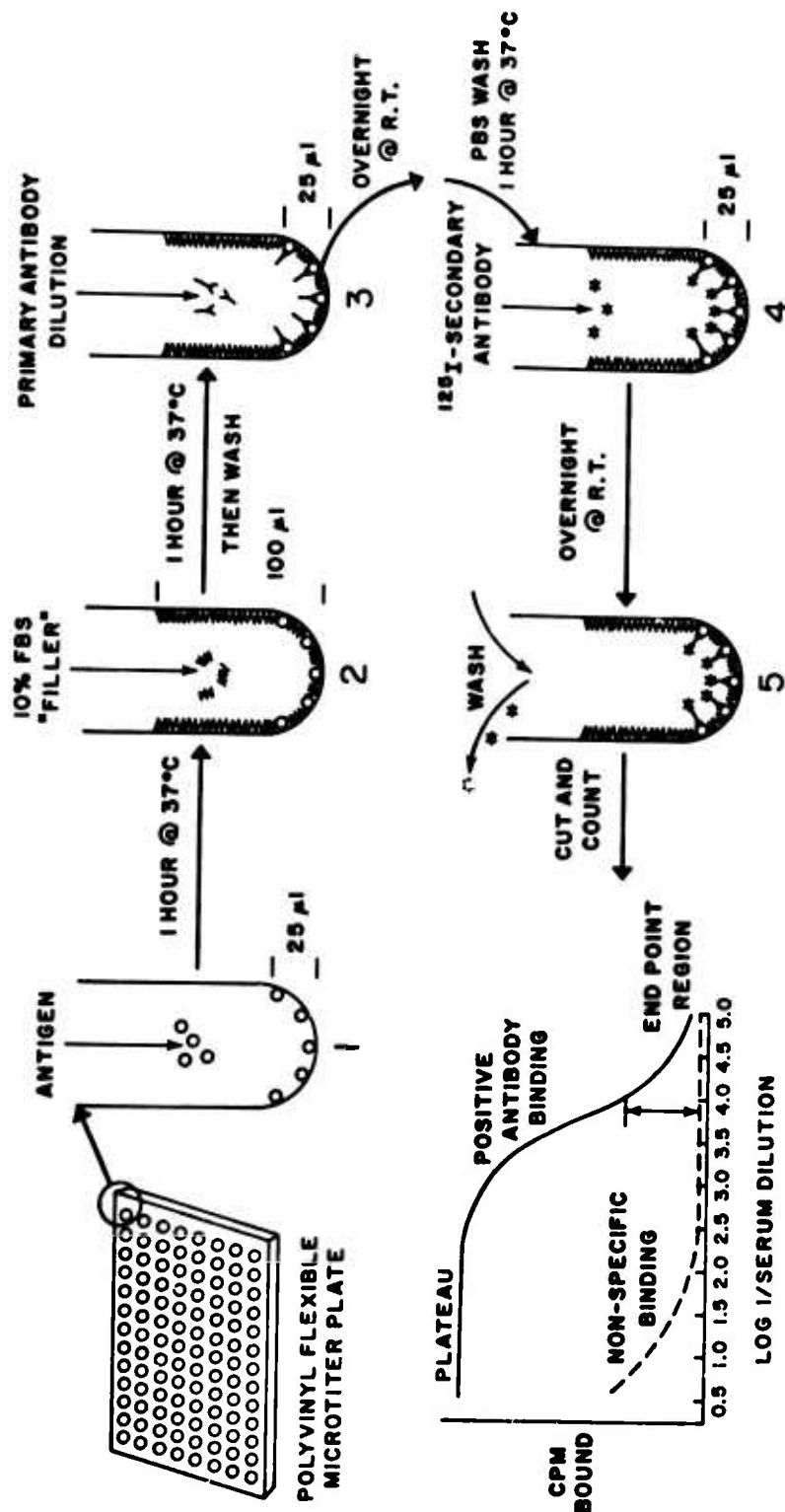
### III. Quantitation of a solid phase radioimmunoassay for antibody to outer membrane antigens of *Neisseria meningitidis* and other bacteria.

Studies of the role of the protein and LPS serotype antigens in human immunity to *N. meningitidis* require an accurate, sensitive assay for antibodies to these antigens. Of several assays considered, the micro SPRIA, described by Rosenthal, Hayashi and Notkins (19,20), appeared most adaptable to our needs. Preliminary tests, however, suggested that several problems had to be resolved. The binding curves obtained exhibited pronounced prozones, lacked quantification, and were otherwise difficult to reproduce and interpret. The studies described here were designed to investigate the binding and complex inter-relationships of the antigen, primary antibodies, and radiolabelled secondary antibodies in order to define conditions which would result in a quantitative assay.

After initial studies to determine optimal conditions the standard procedure shown in Fig. 14 was adopted. The assay is performed in polyvinyl flexible microtiter plates using a volume of 25  $\mu$ l for all reagents except the filler. Antigen was added and allowed to bind for one hour at 37°C and then aspirated and replaced by 100  $\mu$ l of filler containing 10% fetal bovine serum. After another hour at 37° the plate was washed and serial dilutions of the serum to be tested were added. These primary antibodies (PAb) were allowed to bind overnight at room temperature after which the solutions were aspirated and the wells thoroughly washed with PBS. <sup>125</sup>I-labelled antigammaglobulin at a standard concentration of 20 ng active antibody per 25  $\mu$ l was added to each well. The binding of these secondary antibodies (SAb) was again allowed to continue overnight at room temperature, after which the solution was aspirated, the plate washed, and the wells cut into tubes and counted. A plot of CPM of <sup>125</sup>I bound vs. the reciprocal dilution of PAb results in a binding curve of the type shown in Fig. 14. The curve is characterized by a plateau in the region of excess PAb and an

Fig. 14. Schematic of the solid phase radioimmunoassay.

# RADIOIMMUNO ASSAY SCHEMATIC



end point region in the area of excess SAb. Non-specific binding to wells coated with filler only, i.e. no specific antigen, is also shown.

Detailed studies of the kinetics and extent of antigen, PAb and secondary antibody binding were performed. The concentration of antigen required to adequately coat the wells varied for different antigens. LPS was most efficient and capsular polysaccharide was the least efficient of the antigens tested. The effect of antigen concentration on the resultant binding curve is shown in Fig. 15. Wells were coated with meningococcal OMC at concentrations ranging from 0 to 400  $\mu\text{g/ml}$  and each concentration was tested against the same dilution series of primary antibody and the same secondary antibody. Binding of  $^{125}\text{I}$  increased with increasing antigen concentration up to 25  $\mu\text{g/ml}$  beyond which no further increases were observed. At optimal concentrations, the binding of antigen was essentially complete after one hour at  $37^\circ$ . These results emphasize the importance of performing the assay under conditions of antigen excess.

The basis for quantitating the assay became apparent from studies of the relationship between the amount of SAb bound and the amount of PAb bound at different points on the binding curve. Experiments were performed in which partially purified rabbit antibodies to meningococcal outer membrane protein were labelled with  $^{125}\text{I}$  and used as PAb followed by either unlabelled or  $^{125}\text{I}$ -labelled SAb. The results of one such experiment are shown in Fig. 16.

The amount of primary or secondary antibody bound is plotted on a log scale vs. the reciprocal dilution of PAb. Each point is the mean of 4-6 replicas. The amount of PAb bound was found to be directly proportional to the amount added over the entire range tested, indicating that antigen was not limiting. The binding of SAb shows a short plateau where nearly all active SAb is bound and then falls off at higher dilutions of PAb. The ratio of SAb bound to PAb bound increases and then levels off and becomes essentially constant suggesting saturation of the binding sites of the PAb. This plateau is reached at the point where the amount of SAb bound has dropped to about 25-30% of the maximum or plateau level. This region of excess SAb where the amount of SAb bound is directly proportional to the amount of PAb bound was designated the end point region. The direct proportionality between the secondary and primary antibody bound in this region suggested that one might quantitate the assay by determining the constant of proportionality.

In order to determine the amount of specific antibody per ml from the CPM of  $^{125}\text{I}$  bound ( $\text{CPM}_{\text{ep}}$ ) and reciprocal dilution of PAb at the end point ( $D_{\text{ep}}$ ) three relationships must be known: 1) the relationship between the CPM of  $^{125}\text{I}$  bound and the amount of SAb bound, i.e. the specific activity of the SAb (SA), 2) the ratio of SAb bound to PAb bound at the end point ( $R_{\text{ep}}$ ), and 3) the fraction of PAb added per well that is bound at the completion of the assay ( $F_{\text{ep}}$ ). These relationships

Fig. 15. Effect of antigen concentration used for coating the plate on the resultant SPRIA binding curve. Meningococcal strain 138I(C) OMC at 0 (■), 0.1 (■), 0.4 (●), 1.6 (●), 6.3 (Δ), 25 (▲), 100 (▼), or 400 ( + )  $\mu$ g protein/ml was allowed to bind to the plate for 1 hr at 37°C. The assay was performed in duplicate using the standard procedure. The same dilution series of a rabbit antiserum to strain 138I(C) was used as PAb and  $^{125}$ I-labelled goat anti-rabbit gammaglobulin at 20 ng active antibody/25  $\mu$ l as SAb.



Fig. 15

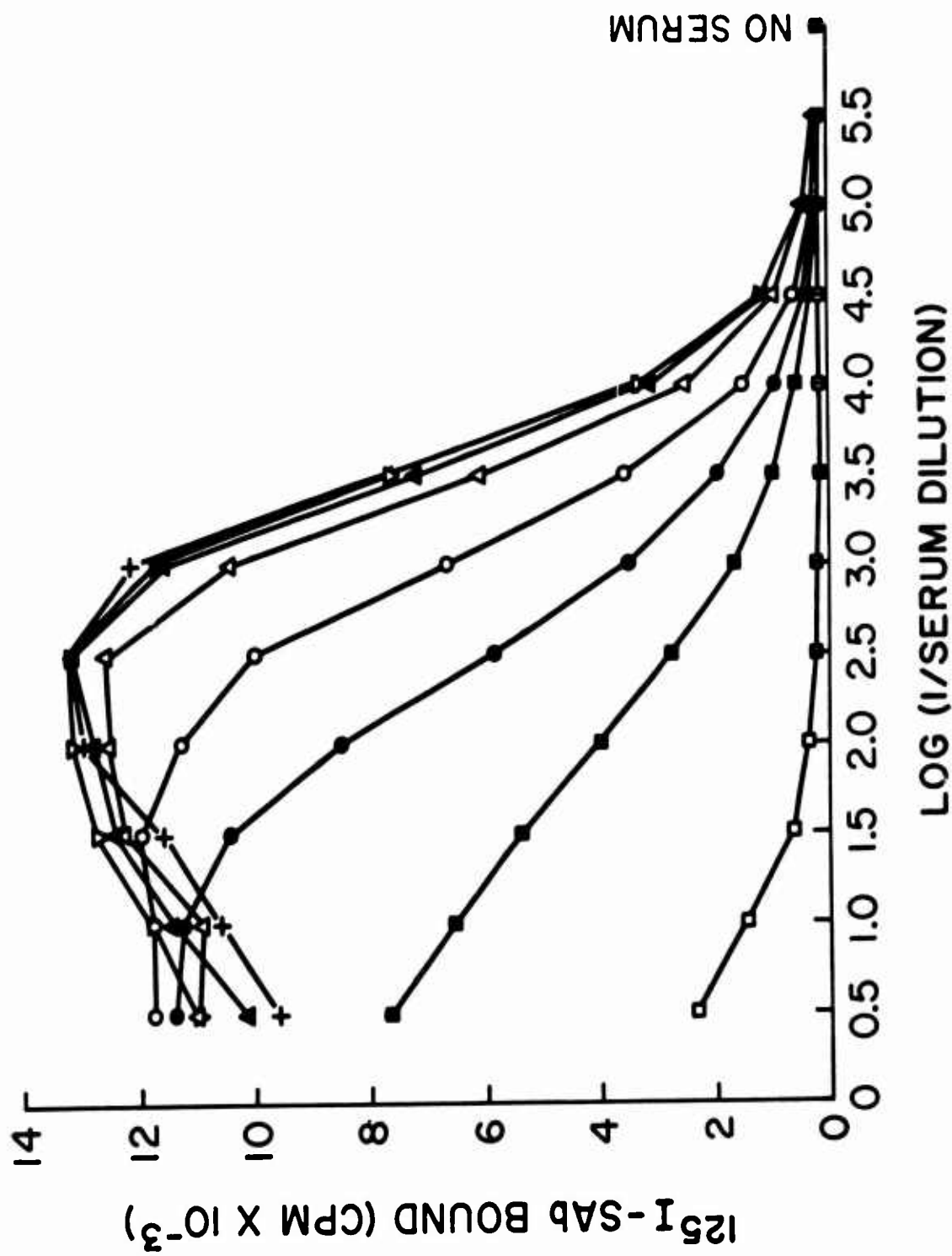
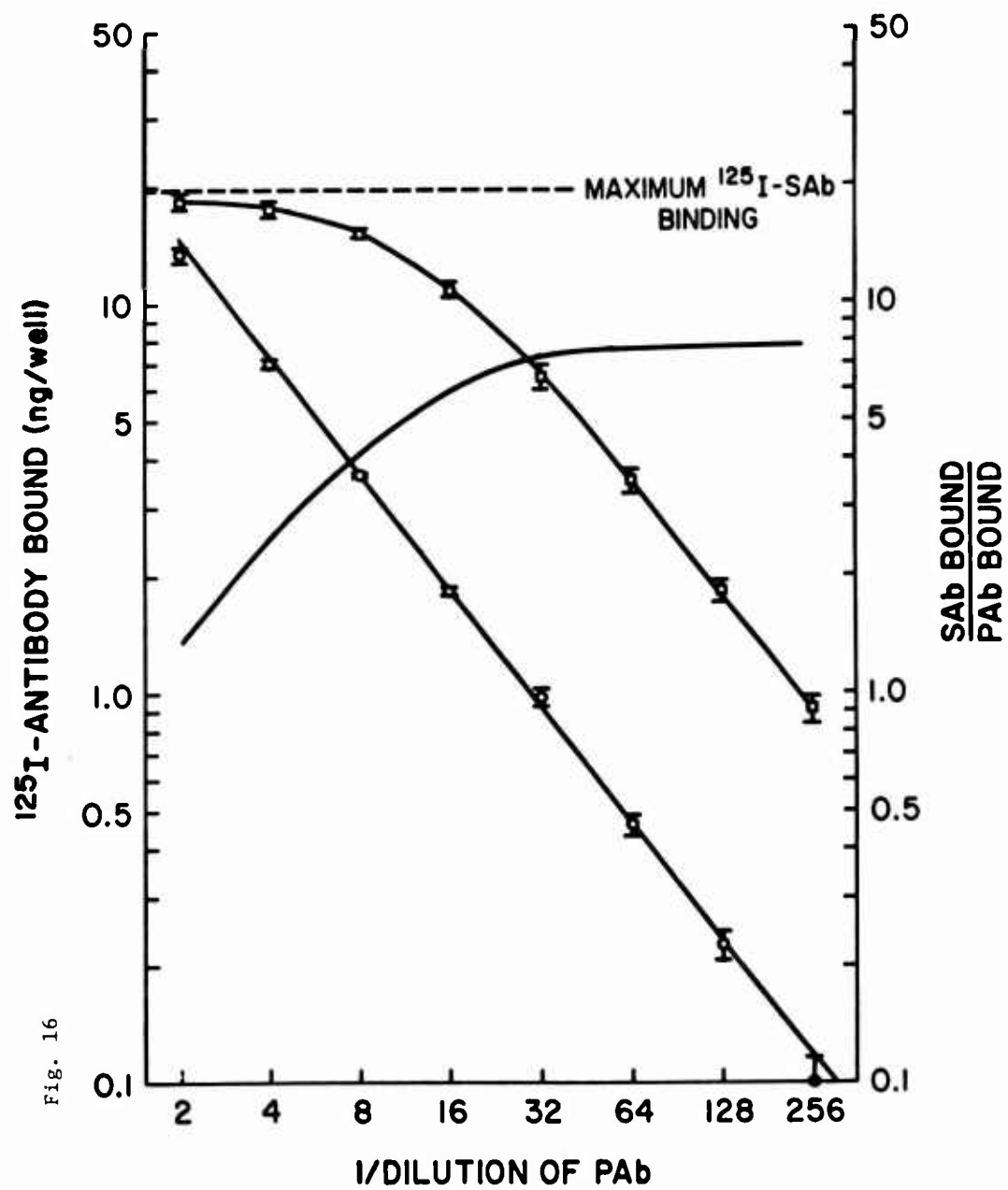


Fig. 16 Determination of the ratio of SAb bound to PAb bound at different points on the binding curve. The SPRIA was performed by the standard procedure using two-fold dilutions of  $^{125}\text{I}$ -PAb (rabbit anti-6275 OM protein antibodies, specific activity 2580 CPM/ng) and unlabelled or  $^{125}\text{I}$ -labelled SAb (goat anti-rabbit immunoglobulin, specific activity 1170 CPM/ng).  $^{125}\text{I}$ -PAb bound ( $\bullet$ ) was determined following binding of unlabelled SAb at 20 ng active antibody/25  $\mu\text{l}$ .  $^{125}\text{I}$ -SAb bound ( $\square$ ) was determined by subtracting the  $^{125}\text{I}$ -PAb bound from the total  $^{125}\text{I}$  bound to wells that received both  $^{125}\text{I}$ -PAb then  $^{125}\text{I}$ -SAb. Maximum binding of  $^{125}\text{I}$ -SAb was determined using excess unlabelled PAb. Each point is the mean of 4 (PAb) or 6 (SAb) wells.



are summarized in the following expression:

$$\mu\text{g Ab protein ml} = \frac{40 \cdot \text{CPM}_{\text{ep}} \cdot D_{\text{ep}}}{\text{SA} \cdot R_{\text{ep}} \cdot F_{\text{ep}}}$$

Here the factor 40 adjusts the volume from 25  $\mu\text{l}$  to 1 ml.

We examined two approaches to determine the value of these parameters: The first was direct experimental determination of the three separate parameters and the second was to combine the three into a single coefficient  $K(t)$ , which due to radioactive decay is time dependent, and to determine the value of this coefficient by performing the solid phase assay on several sera calibrated by the quantitative precipitin test. Thus,  $K(t) = \text{SA} \cdot R_{\text{ep}} \cdot F_{\text{ep}}$  and  $K(t) = (40 \cdot \text{CPM}_{\text{ep}} \cdot D_{\text{ep}}) / (\mu\text{g Ab protein/ml})$ . From the latter expression it is clear that since the Ab content is known and the quantities in the numerator are obtained from the S.P. assay, the coefficient  $K(t)$  can be determined.

Values for the three separate parameters obtained experimentally in the rabbit system are shown in Table 11. The ratio of SAB bound to PAb bound was found to be about 7.4. The fraction of PAB bound, at the completion of the assay, was found to be 0.6 and the specific activity of the SAB was 500-1200 CPM ng for different preparations.

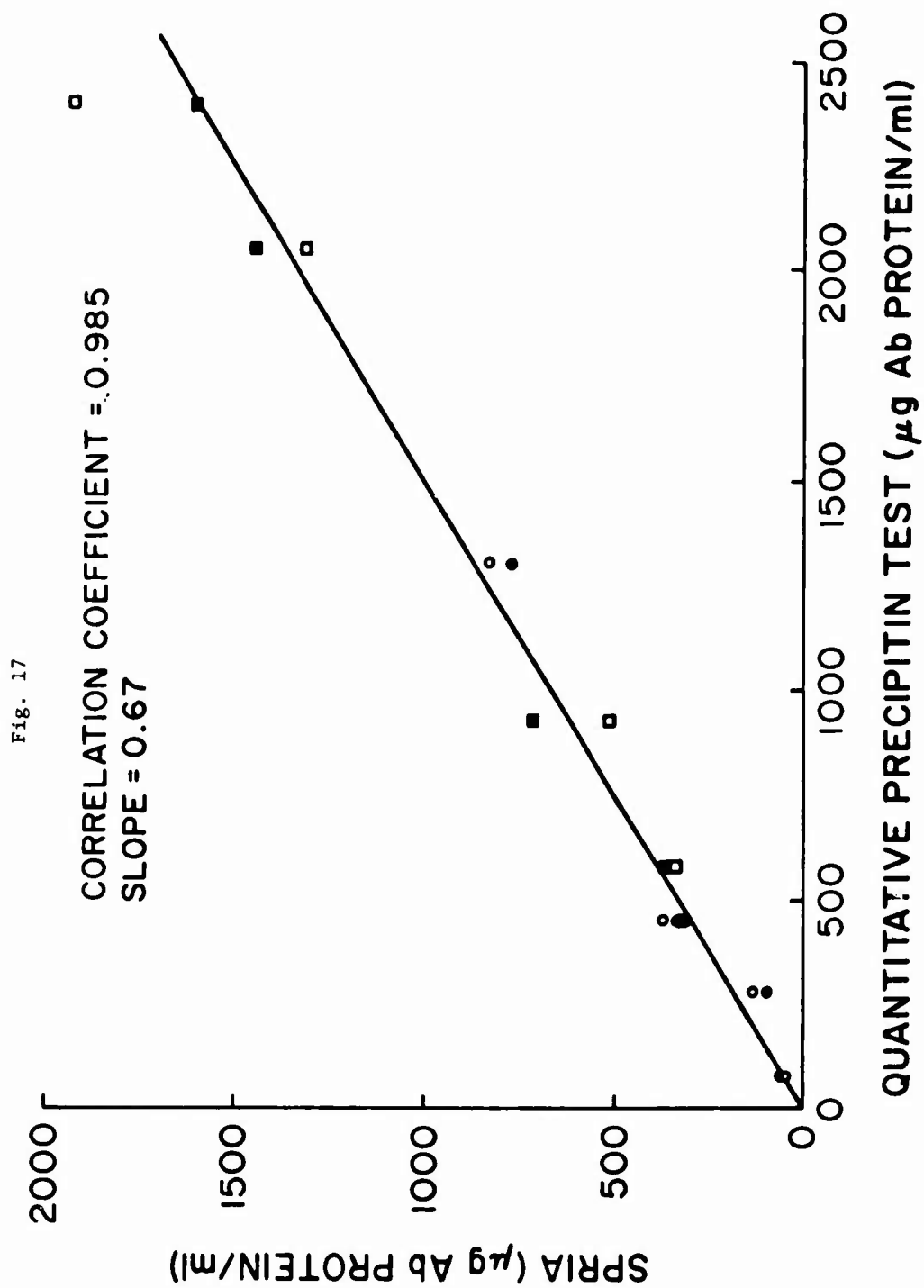
Table 11. Experimental values for SPRIA calibration coefficient parameters.

Parameter	PAb	SAb	Value
$R_{\text{ep}}$	$^{125}\text{I}$ -rabbit anti-OM protein	$^{125}\text{I}$ -GARG or GARG	7.2, 7.6
$F_{\text{ep}}$	$^{125}\text{I}$ -rabbit anti-OM protein	GARG	0.60, 0.59
SA	-	$^{125}\text{I}$ -GARG	500-1200 (CPM/ng protein)

Using these values the antibody content of nine rabbit anti-meningococcal antisera was determined and compared to quantitative precipitin values obtained with the same sera and antigens (Fig. 17).

The points fit a straight line regression through the origin and the correlation coefficient of 0.985 indicates a high degree of

Fig. 17. Correlation between the quantitative SPRIA and the quantitative precipitin test. The results of the quantitative SPRIA were calculated using experimentally determined values for SA,  $R_{ep}$  and  $F_{ep}$ . Two SPRIA results representing the mean of duplicate determinations with two different  $^{125}\text{I}$ -SAb preparations are given for each serum. Nine sera from rabbits immunized with whole viable meningococci of different strains were tested against the same homologous antigen in both assays. The symbols  $\circ$ ,  $\bullet$  represent sera tested against homologous LPS antigens and the symbols  $\square$ ,  $\blacksquare$  denote sera tested against the homologous OMC.



correlation. The value of the slope indicates that the SPRIA values were on the average about 33% lower than the quantitative precipitin values.

The results suggest that a reasonably good estimate of serum antibody can be obtained without reference to independently calibrated antisera.

More accurate results are obtained, however, using reference sera prepared by quantitative precipitin analysis to directly calibrate the solid phase assay. Four human reference sera were prepared in this way using three different antigens. These sera were each used to determine the calibration coefficient for a preparation of  $^{125}\text{I}$ -antihuman gamma-globulin (Table 12).

Two pools of immune human sera were tested against the C-capsular polysaccharide, one serum against meningococcal OMC and commercial tetanus immune gammaglobulin was tested against tetanus toxoid. The resulting values for  $K(t)$  given in CPM/ $\mu\text{g}$  protein are very similar. This suggests that the calibration coefficient may be relatively independent of the particular antigen and antiserum used.

The assay is very versatile and has been successfully applied to the quantitation of human and rabbit antibodies to a variety of different bacterial antigens.

#### IV. Antibody-dependent cellular cytotoxicity (ADCC) of meningococci.

It has been well demonstrated that lymphocytes can kill a variety of target cells either directly or in cooperation with specific antibody (21,22). Previous studies have shown that lymphocyte killing of *N. meningitidis* (Mgc) not only occurs but is antibody dependent (ADCC). Studies were, therefore, undertaken to more precisely define the killer-cell population and to characterize the mediating antibody.

##### A. Cellular basis of ADCC.

Human peripheral white blood cells were fractionated into three populations by separation over Ficoll-hypaque, followed by overnight incubation in plastic flasks and passage over nylon wool columns. Those mononuclear cells, which were non-adherent to the flasks after overnight incubation, were harvested and found to be 95% lymphocytes with 5% contaminating monocytes and will be referred to as non-adherent mononuclear cells (MNC; Table 13). After passage over nylon wool, the mononuclear cell population was 99.5% pure lymphocytes with only 0.1-0.9% contaminating monocytes. This cell population is designated as purified lymphocytes (Ly-P; Table 13). Those mononuclear cells which adhered to the plastic flasks were harvested and found to be approximately 90% pure monocytes (Mn; Table 13).

Table 12. SPRIA calibration coefficients obtained with calibrated human sera and three different antigens.

Sera <sup>a</sup>	Antigen	μg Ab protein precipitated per ml	CPM <sub>ep</sub> <sup>b</sup>	D <sub>ep</sub>	K(t) x 10 <sup>-6</sup>
Pool A	meningococcal group C polysaccharide	100	1785	3,000	2.14
Pool B	meningococcal group C polysaccharide	32	2284	1,000	2.86
H.S.	meningococcal 99M(B) outer membrane complex	28	2284	1,000	3.26
Tetanus immune human gamma globulin	Tetanus toxoid	2380	1575	100,000	2.65



Table 13. Functional, enzymatic and morphologic characteristics of cell populations<sup>1</sup>.

Cell Type	Percent Lymphocytes <sup>2</sup>	Percent Monocytes			
		Percent Myeloperoxidase Positive <sup>3</sup>	Percent Latex Ingesting <sup>4</sup>	Percent Morphologically Positive <sup>5</sup>	Percent Granulocytes <sup>6</sup>
Initial Mononuclear Cells <sup>7</sup>	79 (69-98)	17 (9-26)	11 (6-18)	23 (15-28)	1 (0-2)
Nonadherent Mononuclear Cells <sup>8</sup>	95 (90-97)	5 (2-8)	4 (2-10)	6 (4-11)	0
Purified Lymphocytes <sup>9</sup>	99.5 (99.1-99.9)	0.5 (0-0.9)	0	0.6 (0-1.2)	0
Monocytes <sup>10</sup>	11 (3-21)	86 (76-99)	77 (68-86)	91 (82-95)	0

<sup>1</sup> All percentages expressed as the mean with the range in parenthesis.

<sup>2</sup> Calculated by subtraction of percent of monocytes and granulocytes as determined by morphologic and enzymatic examination.

<sup>3</sup> Cells staining with Kaplow's myeloperoxidase stain.

<sup>4</sup> Cells ingesting 2 or more 0.81  $\mu$  latex particles.

<sup>5</sup> Cells morphologically resembling monocytes under methylene blue wet mount examination.

<sup>6</sup> Cells morphologically resembling granulocytes by Wright's stain.

<sup>7</sup> Cells obtained directly from Ficoll-hypaque interface.

<sup>8</sup> Ficoll-hypaque separated cells that were nonadherent to plastic flasks following overnight incubation.

<sup>9</sup> Nonadherent cells that were gently eluted from nylon wool columns.

<sup>10</sup> Adherent cells that were removed from plastic flasks following overnight incubation.

In the cytotoxicity assay group C meningococci were harvested during mid-log growth, aliquoted into tubes and incubated with group C polysaccharide (Csss) post-immunization heat-inactivated human antisera (Ab) and/or cell populations of either MNC, Ly-P, or Mn at 37°C for one hr. Viability of Mgc prior to and following the test incubation was measured by colony counting methods. Data is expressed as both colony forming units at zero and at one hr and as percent specific kill according to the formula:

$$\% \text{ specific kill} = 100 \frac{\text{Number of colonies in experimental tubes (cells plus media or cells plus antibody)}}{\text{Number of colonies in control tubes (media alone or antibody alone)}} \times 100$$

Both non-adherent mononuclear cells and purified lymphocytes were capable of killing meningococci only in the presence of antibody (Figs. 18 and 19). Cytotoxicity was most efficient at a killer to target cell ratio of 800:1 and was greater for MNC than Ly-P (Fig. 20). Monocytes were considerably more efficient at killing meningococci, approaching 100% kill at ratios of 400:1 (Fig. 21). Since MNC contain 5% monocytes, at a MNC killer:target ratio of 800:1, there would be a monocyte:target ratio of 40:1. From Fig. 21 it can be calculated that a MN:target ratio of 40:1 would contribute 30% to the total cytotoxicity of around 95% seen at the MNC:target ratio of 800:1 (Fig. 20). Thus, the 30% difference in cytotoxicity between Ly-P and MNC at a killer target ratio of 800:1 can be precisely ascribed to the differences in monocyte contamination between the two populations of lymphocytes, and it can be seen that while both cell populations participate in ADCC, lymphocytes are indeed functionally active alone.

Prior immunization with Csss did not affect lymphocyte participation in ADCC. In the presence of Ab there was no difference between the killing ability of cells purified from immunized individuals as compared to that of cells from non-immune individuals at any killer target ratio (Table 14). Nor were cells from immune individuals able to kill in the absence of antibody (Table 15). Pre-incubation removal of platelets and post-incubation removal of leukocytes likewise had no effect on ADCC (Table 16).

Thus, it can be concluded that both lymphocytes and monocytes participate in the killing of meningococci in the presence of specific antibody.

#### B. Inhibition of lymphocyte-mediate ADCC of group C meningococci by group C capsular polysaccharide (Csss).

In the previous section it was demonstrated that lymphocytes, in cooperation with heat-inactivated antisera (Ab) from subjects immunized with group Csss, can kill group C meningococci in vitro. In

Fig. 18. Non-adherent mononuclear cell-mediated ADCC of Mgc.

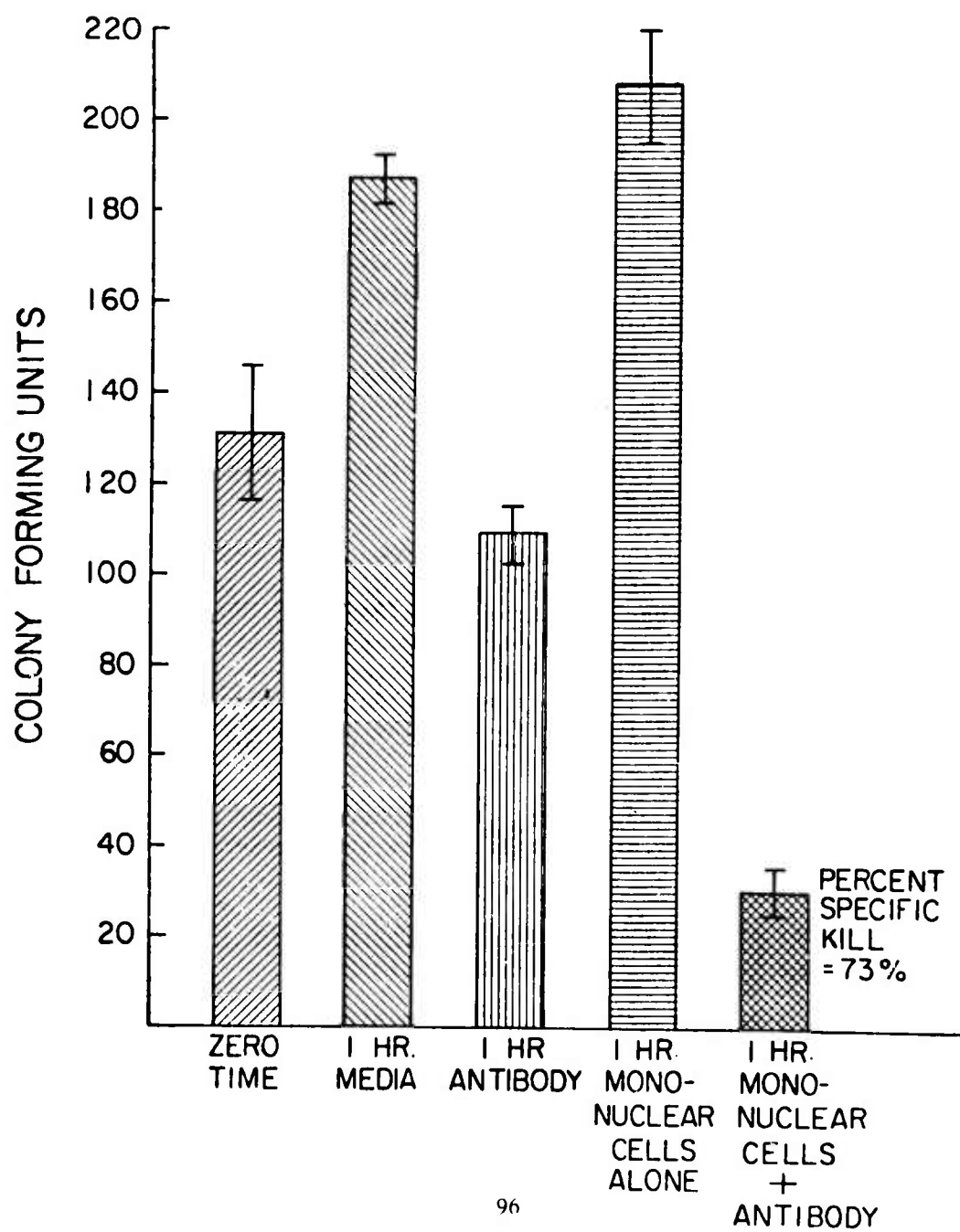


Fig. 19. Purified lymphocyte-mediated ADCC of Mgc.

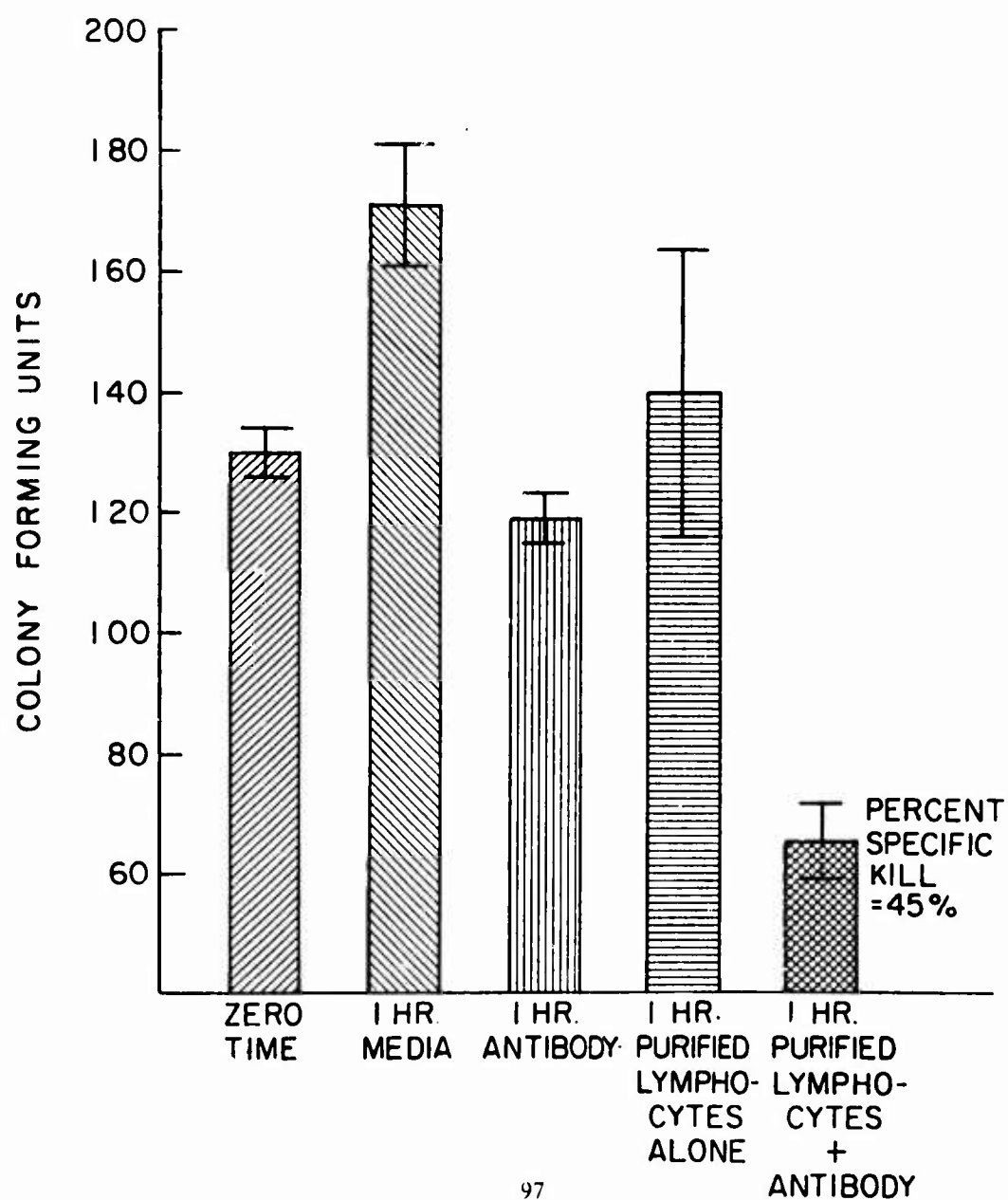


Fig. 20. Effect of killer/target cell ratio on non-adherent mononuclear cell-mediated and purified-mediated ADCC of Mgc.

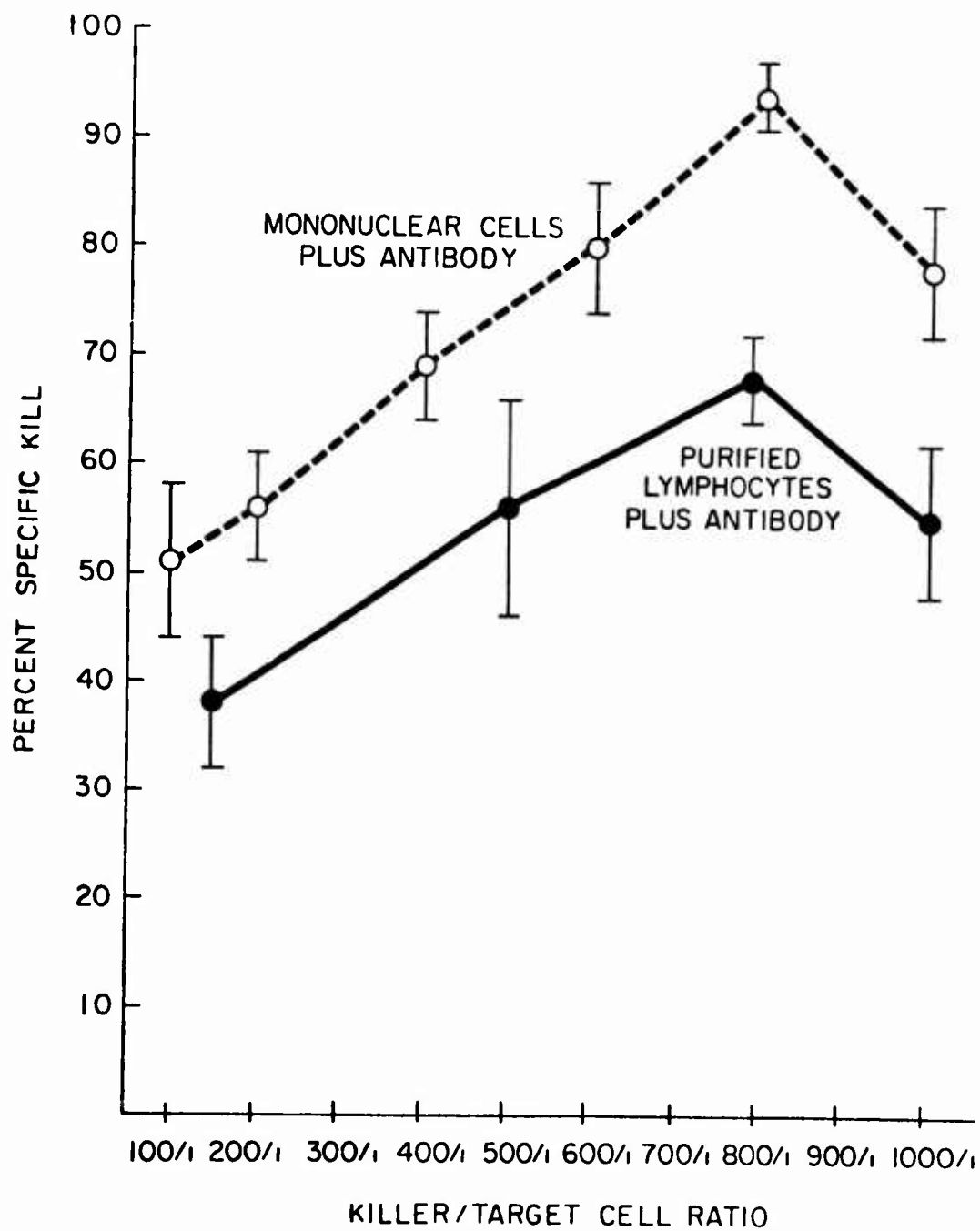


Fig. 21. Effect of killer/target cell ratio on monocyte-mediated ADCC of Mgc.

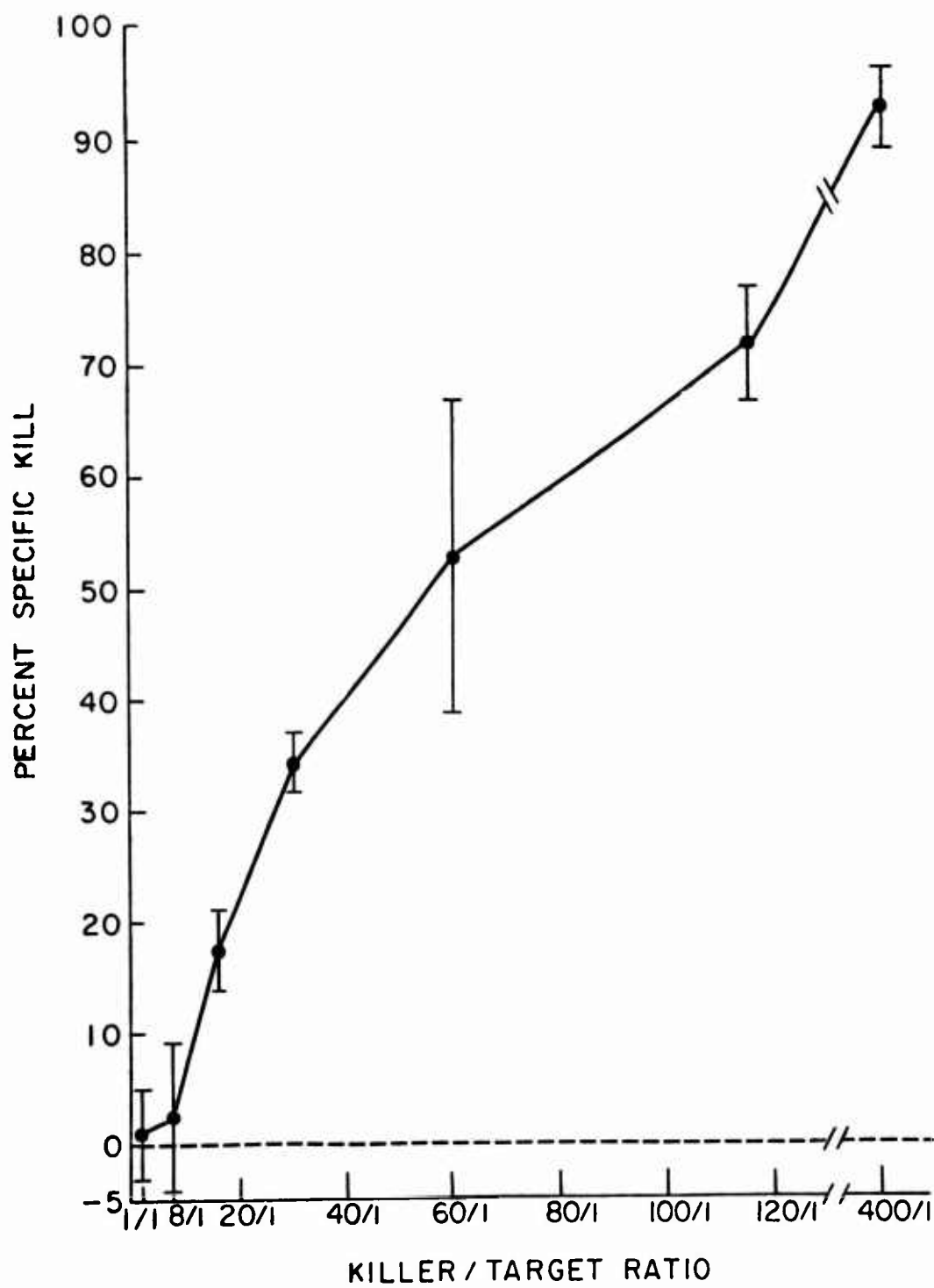


Table 14. Similarity of ADCC of meningococci by cells from immunized and non-immunized individuals<sup>1</sup> using A) mononuclear cells<sup>2</sup> and B) purified lymphocytes<sup>3</sup>.

Cell Type	Killer/Target Cell Ratio	Percent Specific Kill <sup>4</sup>	
		Non-immunized	Immunized
A) Non-adherent Mononuclear Cells <sup>2</sup>	100/1	51 ± 7	51 ± 2
	200/1	43 ± 6	60 ± 6
	500/1	72 ± 4	76 ± 10
	800/1	89 ± 3	96 ± 2
B) Purified Lymphocytes <sup>3</sup>	150/1	44 ± 9	40 ± 12
	500/1	63 ± 18	53 ± 14
	900/1	59 ± 7	69 ± 4

<sup>1</sup> Immunized individuals were vaccinated with group C polysaccharide vaccine; non-immunized individuals were not.

<sup>2</sup> For characterization see Table 13, footnote "8".

<sup>3</sup> For characterization see Table 13, footnote "9".

<sup>4</sup> Each percentage represents the arithmetic mean of 2-11 experiments ± S.E.M. as determined by the formula

$$\% \text{ specific kill} = 100 - \left( \frac{\text{bacteria in exptl. tubes}}{\text{bacteria in control tubes}} \right) \times 100$$

Table 15. Inability of A) mononuclear cells<sup>1</sup> or B) purified lymphocytes<sup>2</sup> from either immunized or non-immunized individuals<sup>3</sup> to kill meningococci in the absence of antibody.

Cell Type	Killer/Target Cell Ratio	Percent Specific Kill <sup>4</sup>	
		Non-immunized	Immunized
A) Non-adherent Mononuclear Cells <sup>2</sup>	100/1	-20 ± 11	-6 ± 7
	200/1	-43 ± 32	-1 ± 11
	600/1	11 ± 22	-24 ± 30
B) Purified Lymphocytes <sup>2</sup>	100/1	3 ± 9	7 ± 2
	200/1	-28 ± 11	-2 ± 14
	500/1	1 ± 6	0 ± 14
	800/1	0 ± 8	-2 ± 7

<sup>1</sup> For characterization see Table 13, footnote "8".

<sup>2</sup> For characterization see Table 13, footnote "9".

<sup>3</sup> See Table 14, footnote "1".

<sup>4</sup> See Table 14, footnote "4". Negative values represent growth of bacteria in experimental tubes as compared to control tubes.



Table 16. Lack of effect of pre-incubation removal of platelets<sup>1</sup> and post-incubation removal of leukocytes<sup>2</sup> upon ADCC of meningo-cocci by A) mononuclear cells<sup>3</sup> and B) purified lymphocytes<sup>4</sup>.

Cell Type	Killer/Target Cell Ratio	Platelets <sup>1</sup>	Selective WBC Lysis Prior to Agar Plating <sup>2</sup>	Percent Specific Kill <sup>5</sup>
A) Non-adherent Mononuclear Cells <sup>3</sup>	200/1	Present	No	57 ± 9
		Removed	Yes	61 ± 10
	400/1	Present	No	76 ± 3
		Removed	Yes	61 ± 1
	600/1	Present	No	71 ± 5
		Removed	Yes	85 ± 9
	1000/1	Present	No	74 ± 12
		Present	Yes	76 ± 3
		Removed	Yes	87 ± 4
B) Purified Lymphocytes <sup>4</sup>	150/1	Present	No	38 ± 10
		Present	Yes	43 ± 6
	500/1	Present	Yes	45 ± 3
		Removed	Yes	58 ± 12
	800/1	Present	No	69 ± 9
		Removed	Yes	68 ± 6
	1000/1	Present	No	46 ± 7
		Present	Yes	41 ± 7
		Removed	Yes	66 ± 2

<sup>1</sup> Platelets were removed by using defibrinated instead of heparinized blood.

<sup>2</sup> Following the test incubation but before agar plating, leukocytes were selectively lysed by freeze-thaw treatment.

<sup>3</sup> For characterization see Table 13, footnote "8".

<sup>4</sup> For characterization see Table 13, footnote "9".

<sup>5</sup> See Table 14, footnote "4".

order to determine the antigenic specificity of the antibody mediating this lethal activity outer membrane complex antigens, previously shown to be immunologically active in a variety of serological assays, were used as inhibitors.

Ficoll-hypaque separated mononuclear cells were depleted of granulocytes by overnight incubation in plastic flasks. The non-adherent cells consisting of  $95 \pm 4\%$  lymphocytes and  $5 \pm 4\%$  monocytes were used either directly or following passage over nylon wool columns. The column purified lymphocytes had  $0.5 \pm 0.4\%$  monocyte contamination. Group C meningococci were incubated with either 95% or 99.5% pure lymphocytes and/or Ab for one hr at  $37^{\circ}\text{C}$  without complement. Killing of meningococci was measured by enumeration of colony forming units following plating of aliquots of experimental and control tubes prior to and following test incubation. Bactericidal activity ranged from 53% to 84% and was found only in the presence of both lymphocytes and Ab.

Pre-incubation of antisera at  $37^{\circ}\text{C}$  for one hr with increasing quantities of Csss produced total inhibition at a ratio of 25 mcgm Ag:0.1 ml of antisera diluted 1:64. Increasing quantities of heterologous group Asss were ineffective (Fig. 22). Similarly, homologous type II protein isolated from both the test organism and group B type II meningococci and lipopolysaccharide isolated from the test organism were ineffective as inhibitors (Table 17).

These studies indicate that the antibody in post-Csss immunization sera that participates in lymphocyte-mediated antibody-dependent killing of meningococci is directed against the group specific Csss with which the individual was immunized.

#### Summary and Conclusions:

The presence of bactericidal activity in acute sera from recruits with systemic meningococcal disease has been demonstrated by selective removal of serum IgA which blocks the lytic activity of IgG and IgM. This suggests that host susceptibility may depend upon the serum level of specific IgA blocking antibody. A solid phase radioimmunoassay (SPRIA) was developed which permits quantitation of human antibody to meningococcal serotype protein and lipopolysaccharide (LPS) antigens. Antigen-specific protein and LPS serotyping of 90 strains of meningococci was performed using a new SPRIA inhibition procedure. A significant correlation was found between the occurrence of type II protein and type 3,7 LPS. A significant antibody response to the LPS determinants of strain 135B (LPS type 1,3,4,7) was demonstrated in recruits with systemic meningococcal disease due to types II and II,IV epidemic strains. Human antibodies specific for the group C polysaccharide were shown to cooperate with lymphocytes and monocytes in complement independent killing of group C meningococci.

Fig. 22. Csss inhibition of purified lymphocyte-mediated ADCC of group C Mgc.

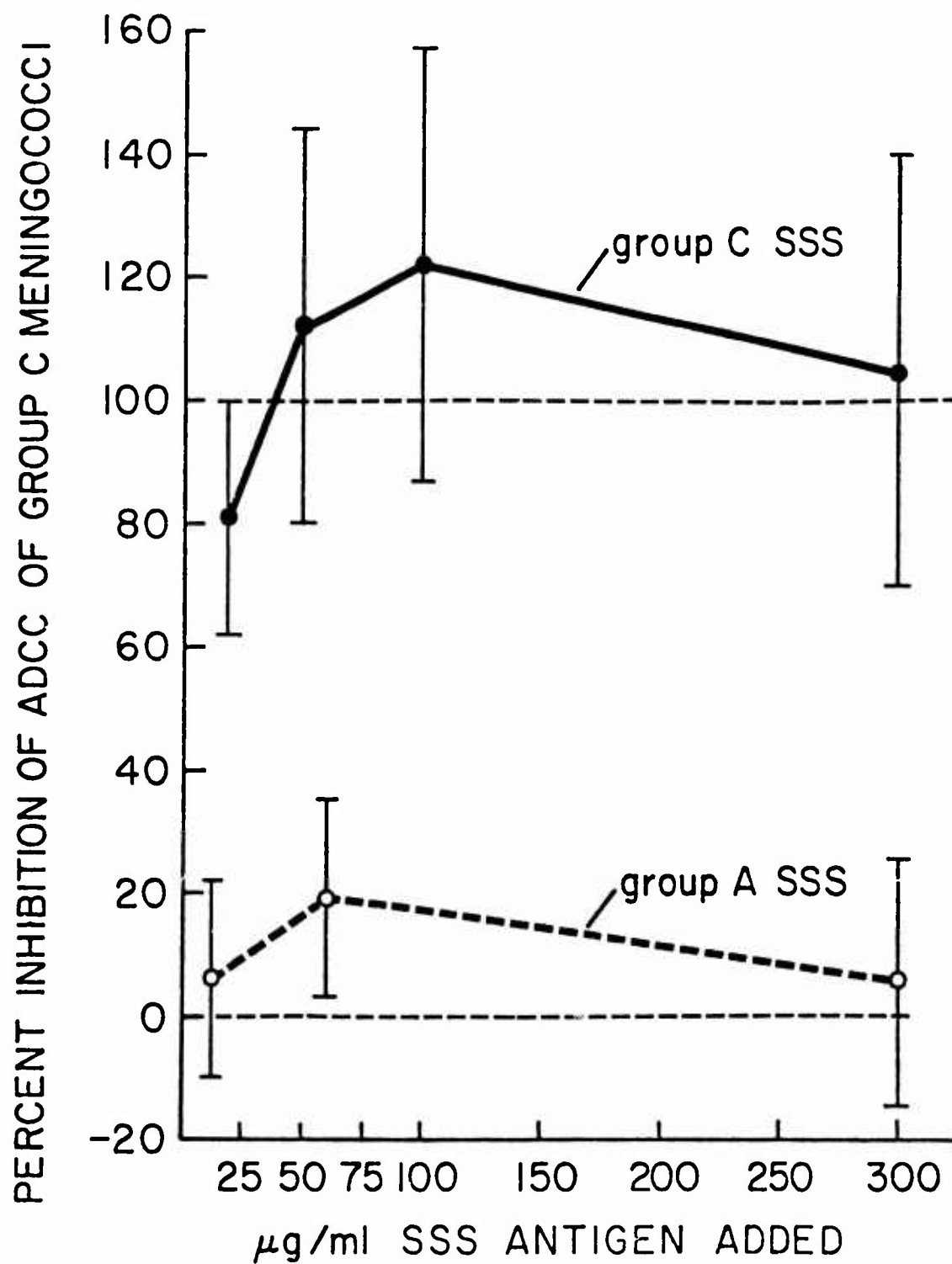


Table 17. Lack of inhibition of purified lymphocyte<sup>1</sup>-mediated ADCC of group C meningococci by A) isologous LPS, B) homologous protein and C) heterologous protein.

Antigen Added			Percent Specific Kill <sup>4</sup>		
Type <sup>2</sup>	Amount ( $\mu$ g/ml) <sup>3</sup>		Antigen Absent (Control)	Antigen Present (Exptl.)	Percent Inhibition of Specific Kill <sup>5</sup>
A) Isologous LPS (str 138-I)	50		70 $\pm$ 28	74 $\pm$ 24	-31 $\pm$ 32
	100		70 $\pm$ 28	70 $\pm$ 26	0 $\pm$ 1
	200		70 $\pm$ 28	73 $\pm$ 26	-17 $\pm$ 16
	400		70 $\pm$ 28	71 $\pm$ 29	-1 $\pm$ 1
B) Homologous Protein (Type II, str 986)	50		79 $\pm$ 11	75 $\pm$ 2	7 $\pm$ 14
	200		79 $\pm$ 11	71 $\pm$ 5	8 $\pm$ 18
	500		79 $\pm$ 6	71 $\pm$ 10	8 $\pm$ 18
C) Heterologous Protein (Type III, str 990)	125		79 $\pm$ 6	63 $\pm$ 12	7 $\pm$ 26
	500		79 $\pm$ 6	65 $\pm$ 15	12 $\pm$ 25

<sup>1</sup> See footnote "1", Table 16.

<sup>2</sup> See footnotes "2-5", respectively, Table 14.

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

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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 6517	76 06 30	DD-DR&T(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>	8. DWSN INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACC <sup>a</sup> YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>	10. LEVEL OF S <sup>a</sup> A. WORK UNIT
75 07 01	R. Corp.	U	U	NA	NL		
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	61101A	3A161101A91C		00	185		
b. CONTRIBUTING							
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Speciation of Biomolecules by Mass Spectrometry							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002300 Biochemistry		008300 Inorganic Chemistry					
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		76 06		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				b. RECEIVING		c. FUNDS (in thousands)	
d. NUMBER <sup>a</sup>				FISCAL YEAR		75	
e. TYPE:				CURRENT		4	
f. CUM. AMT.				76		3	
20. RESPONSIBLE DOD ORGANIZATION				21. 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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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
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				NAME: Kazyak, L., BS			
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23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Mass Spectrometry; (U) Drug Metabolism; (U) Gas Chromatography; (U) Analytical Chem							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Pursue 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) This work unit is terminated with this reporting period.</p> <p>25. (U) 75 07 - 76 06 Using urine specimens from a Department of Defense supported research project a correlation has been found between amount of marijuana smoking and the urinary concentration of 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid. The study showed a much higher concentration of the 9-acid metabolite in urine of heavy smokers than in the urine of light smokers. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.</p>							

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

DD FORM 1498  
1 MAR 66

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.



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 Gale E. Demaree, MSC

Associate: Billy G. Bass, M.S.; CPT James A. Cella, MSC;  
Laurence R. Hilpert, B.S.; SP5 Piyush K. Gandhi, B.S.;  
Leo Kazyak, M.A.; CPT James A. Kelley, MSC; Robert C.  
Permisohn, M.S.

The technical 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. Efforts during the past year have been focused on the following:

Correlation of cannabis (marihuana) metabolite concentrations in human urine with the concomittant physiological and pharmacological effects.

Relative to studies being concluded on cannabis metabolism, work in our laboratory on the detection of 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid in urine of marihuana smokers indicates a very significant difference between casual smokers and heavy smokers in the excretion of this metabolite of cannabis.

Urines from a study that involved six volunteer marihuana users have been analyzed for the "9-acid" (11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid). According to the designation of casual and heavy smoker, ascribed to the individual on the basis of previous use of marihuana, the latter group exhibited concentrations of the 9-acid metabolite in a range of 200-750 ng./ml. of pooled urine on the 24th day of the study (period of peak excretion of the metabolite). This is quite different from the 9-acid metabolite excretion of casual smokers who never produced concentrations above 20 ng./ml. The values depend on the amount of marihuana previously ingested and the 24 hr. urinary volume. Analysis of urines from heavy marihuana users obtained four days after cessation of smoking also show persistence of the 9-acid metabolite.

Casual smokers are regarded as those individuals with a history of smoking marihuana on an average of 17 cigarettes or reefers per month and no more than 20 for the same period. Heavy smokers are designated as those who smoked no less than 24 reefers per month and as a group

averaged about 12 reefers per month.

In the interest of further corroborating our findings and to expand the basis of our study to include data on the metabolic rate in relation to cannabis ingestion and the formation of the 9-acid metabolite, additional specimens were obtained from other volunteers of the study. Specimens from two of these individuals have been analyzed for the entire 30 day period, and the results have verified our initial findings.

The attempt to produce a deuterated analogue of the metabolite has been discontinued because of the limited time to complete the work on this entire project. The tri-deuterated analogue of 11-nor- $\Delta^8$ -THC-9-carboxylic acid has been ordered from NIDA to enable us to incorporate isotope dilution techniques into our procedure. Presumably, the isotope dilution technique will improve the precision of the method to the extent that lower values will have greater significance, and the quantification limits can be determined more precisely.

As soon as the isotope dilution technique has been checked on some of the specimens for which complete data have been obtained, the project will then be terminated.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 185 Speciation of biomolecules by mass spectrometry

Literature Cited.

Publications:

1. Kelley, J.A. and Arnold, K.P.: Detection of urinary cannabis metabolites: A preliminary investigation. J. of Forensic Sciences. 21: No. 7. pp 252 ff, April 1976.

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)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>	8. DRG/H INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM <sup>a</sup>
75 07 01	H. Term	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	61101A	3A161101A91C	00	192			
B. CONTRIBUTING							
C. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Antiarrhythmic Effects of Aliphatic Amines							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
012600 Pharmacology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		76 06		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>a</sup>				FISCAL YEAR		75	
C. TYPE				CURRENT		1.0	
D. KIND OF AWARD				76		1.0	
E. CUM. AMT.						39	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME <sup>a</sup> Heiffer, Dr. M.H.			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Korte, CPT D.			
				NAME: Schuster, MAJ B.			
22. 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) 75 07 - 76 06 The aliphatic amine, WR 2823, has been shown to protect against experimental shock. WR 2823 was tested to determine whether a possible antiarrhythmic action could contribute to its other protective properties. No protection in anesthetized cats against either ventricular tachycardia or fibrillation induced by ouabain was seen; however WR 2823 did offer statistically significant protection against digoxin-induced arrhythmias. These studies will be continued and reported under work unit number 076, project 3A161102B71P. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.</p>							

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

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1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65  
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 192 Antiarrhythmic effects of aliphatic amines

Investigators.

Principal: Melvin H. Heiffer, Ph.D.

Associate: CPT D. Korte, MAJ B. Schuster, COL L. Miner, P.  
Tilton, SP4 M. Neidig, Jr.

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 the continued pharmacological investigation of the important new drug, the aliphatic amine designated WR 2823.

2. Antiarrhythmogenic potential of WR 2823.

a. Background:

WR 2823, an aliphatic sulfur-containing compound, has both immediate and prolonged cardiovascular activity. This activity consists of an immediate hypotension and bradycardia followed by a long-lasting alpha adrenergic blockade (Heiffer *et al.*, 1969). WR 2823 has been reported to decrease mortality from hemorrhage in animals (Vick *et al.*, 1973). Hemorrhage has been reported to produce subendocardial lesions which, by disrupting conduction, may produce arrhythmias that result in a decrease in cardiac output which would augment the positive feedback cycle leading to irreversible shock (Hackel *et al.*, 1974). Thus, prevention of arrhythmias may be one aspect of the protective property of WR 2823 in shock states. To determine whether a possible antiarrhythmic action could contribute to its protective properties in shock states, WR 2823 was evaluated for its antiarrhythmic potential against both ouabain- and digoxin-induced toxicity.

b. Methods:

Adult cats of either sex (2.2 - 3.8 kg) were anesthetized with sodium pentobarbital (40 mg/kg, ip). The trachea was cannulated with an endotracheal tube for monitoring respiration. The left femoral artery was cannulated for recording blood pressure. Lead II of the electrocardiogram was recorded for measuring heart rate and detecting changes in rhythm. The right femoral vein was cannulated for glycoside infusions and the left vein was cannulated for other drug injections. Lead II of the electrocardiogram, blood pressure, heart rate and respiration were recorded on a Hewlett-Packard Model 7868A recorder. Lead II of the electrocardiogram was also displayed on the visoscope component of the recorder for continuous monitoring of rhythm changes. Rectal temperature was measured with a Yellow Springs telethermometer and maintained at 37°C by a heating pad.

The cats were divided into two groups: those animals given ouabain (Calbiochem) at an infusion rate of 2 µg/kg/min and those animals given digoxin (Lanoxin<sup>R</sup>) also at an infusion rate of 2 µg/kg/min. The animals in these two groups were divided into three subgroups. The first subgroup was the control and consisted of those animals infused with the glycoside without drug pretreatment. The second subgroup consisted of those animals given WR 2823 (50 mg/kg, iv) slowly over a 5-minute period followed one hour after the start of the WR 2823 injection by the glycoside infusion. The third subgroup consisted of those animals given propranolol HCl (Ayerest Company), (2 mg/kg, iv) slowly over a 5-minute period followed 15 minutes from the start of the propranolol injection by the glycoside infusion. An additional dose of WR 2823 (35 mg/kg, iv) was administered to a separate group of 5 cats that received the digoxin infusion. The ouabain was dissolved in and the digoxin was diluted with 0.9% saline so that the appropriate dose was administered at a rate of 0.181 ml/min.

The toxic dose and lethal dose of the two glycosides were calculated from the infusion time. The toxic dose was considered to be the dose of glycoside that produced three consecutive ventricular beats. The lethal dose was considered to be the dose of glycoside that produced ventricular fibrillation. The doses necessary to produce the toxic and lethal endpoints were compared by Newman-Kuels Test of Significance (Snedecor and Cochran, 1967) with a p value of 0.05 or less considered significant.

c. Results:

The results of WR 2823 or propranolol pretreatment on ouabain toxicity are given in Table 1. Ouabain-induced ventricular tachycardia was observed after infusion of  $68.6 \pm 3.6$  (mean  $\pm$  SEM) µg/kg

in control animals. Pretreatment with WR 2823 (50 mg/kg, iv) did not alter the dose of ouabain infused before ventricular tachycardia was observed. Pretreatment with propranolol (2 mg/kg, iv) increased by 15.1  $\mu$ g/kg over control values the average dose of ouabain infused before ventricular tachycardia was observed. However, this increase was not significant at the 5% level of significance. The fibrillatory or lethal dose of ouabain in control animals was  $97.2 \pm 6.0$   $\mu$ g/kg. The dose of ouabain necessary to produce fibrillation after pretreatment with WR 2823 (50 mg/kg, iv) was  $89.8 \pm 4.3$   $\mu$ g/kg. This decrease in lethal dose of ouabain was not significant. Propranolol (2 mg/kg, iv) produced a significant increase of 26.1  $\mu$ g/kg in the lethal dose of ouabain.

Table 2 contains the data from the studies of digoxin toxicity after pretreatment with WR 2823 or propranolol. Digoxin was less toxic than ouabain; the dose of digoxin producing ventricular tachycardia was  $126.6 \pm 7.9$   $\mu$ g/kg in control cats. Both WR 2823 and propranolol pretreatment produced significant increases in the dose of digoxin necessary to produce ventricular tachycardia. WR 2823 (50 mg/kg, iv) and propranolol (2 mg/kg, iv) increased the digoxin dosage that produced ventricular tachycardia by 36.4% and 58.0%, respectively. The fibrillatory dose of digoxin in control cats ( $158.8 \pm 6.3$   $\mu$ g/kg) was also greater than the lethal dose of ouabain. Again, pretreatment with WR 2823 and propranolol significantly increased the dose of digoxin necessary to produce fibrillation in cats. WR 2823 and propranolol increased the fibrillatory dose of digoxin by 30.5% and 50.2%, respectively.

The comparative effects of pretreatment with a lower dose of WR 2823 (35 mg/kg, iv) on digoxin toxicity are given in Table 3. This dose produced an increase of 27.0% in the dose of digoxin necessary to produce ventricular tachycardia and 25.7% in the lethal dose of digoxin. Thus, the antiarrhythmogenic action of WR 2823 appears to be related to the dose administered.

#### d. Discussion:

The action of WR 2823 against digitalis-induced arrhythmias was investigated in order to determine whether an antiarrhythmic effect may, in part, be responsible for the effectiveness of WR 2823 in treatment of shock states. Phentolamine and phenoxybenzamine, alpha-adrenoreceptor blocking agents, have been shown to be effective against ouabain-induced arrhythmias (Ettinger et al., 1969) and digoxin-induced arrhythmias (Rothaus and Powell, 1975), respectively. Since WR 2823 also has alpha-adrenoreceptor blocking activity, a similar antiarrhythmic spectrum was anticipated. However, analysis of the data indicated that WR 2823 was selectively effective against digoxin-induced arrhythmias while it was ineffective against

ouabain-induced toxicity. These findings were therefore inconclusive in establishing an antiarrhythmic effect as a mechanism for the protective action of WR 2823 in shock states.

The results of these studies are interesting since only recently has differentiation of the toxicity produced by cardiac glycosides been reported. Kelliher and Roberts (1974) suggested that beta-adrenoreceptor blocking agents protected against digitalis toxicity by different mechanisms depending on the glycoside used. Digoxin-induced arrhythmias could be prevented by beta-blocking doses of these agents, doses that were ineffective against ouabain-induced arrhythmias. Only doses of the beta-blocking agents which produce neural depression were effective against ouabain-induced arrhythmias. The present study demonstrates that the alpha-blocking agent, WR 2823, is effective against digoxin-induced toxicity but not ouabain-induced toxicity. Thus, the varying efficacy of WR 2823 in preventing digitalis toxicity may be a function of inherent differences in the toxicity of the cardiac glycosides.

Digitalis glycosides have both a direct toxic action on the heart and an indirect effect mediated via an interaction with the sympathetic nervous system. Recent studies with transplanted and denervated hearts indicate that the indirect neural aspects of digitalis glycosides may be a more important component of their toxicity than the direct effects (Gillis *et al.*, 1975). The indirect neural effect associated most often with digitalis toxicity has been sympathetic stimulation (Gillis *et al.*, 1975). However, the site(s) of digitalis interaction with the sympathetic nervous system has not been defined. Recent evidence indicates that the primary site of digoxin interaction is peripheral rather than central (Weaver *et al.*, 1976) while ouabain has little effect on peripheral reflexes (McRitchie and Vatner, 1976) but has profound effects on the posterior hypothalamus to produce sympathetic stimulation (Saxena and Bhargava, 1975). In addition, Dutta and Marks (1966) indicated that digoxin was concentrated to a greater extent in the adrenal gland than is ouabain. Furthermore, 6-hydroxydopamine, an agent that depletes 95% of the catecholamines in the heart but only 35% of the catecholamines in the adrenal gland protects against ouabain toxicity but not digoxin toxicity (Roberts *et al.*, 1976). Thus, it appears that ouabain may have more of an effect centrally to produce an increase in sympathetic tone while digoxin may act through the adrenal gland directly, as well as other peripheral components, to produce an increase in sympathetic tone. Consequently, both beta-blockade and alpha-blockade may be effective against digoxin toxicity by interfering with peripheral components of the sympathetic nervous system, including release of catecholamines from the adrenal gland. However, since ouabain



apparently produces toxicity by central mechanisms, only those alpha- and beta-blocking agents that produce neural depression are effective in preventing ouabain toxicity. Thus, WR 2823 may not be effective against ouabain toxicity because it does not cross the blood-brain barrier in quantities sufficient to produce neural depression or because it lacks appreciable neural depressant activity.

Another explanation for the protective action of alpha-blocking agents against digitalis-induced arrhythmias has recently been proposed. Rothaus and Powell (1975) suggested that phenoxybenzamine was effective against digoxin-induced arrhythmias because it antagonized the alpha-mediated sympathetic constriction of coronary arteries produced by digitalis. Another alpha-blocking agent, phentolamine, had also been reported to be beneficial against digitalis-induced (ouabain) arrhythmias (Ettinger *et al.*, 1969). However, since WR 2823, an agent with alpha-blocking properties, was not successful against ouabain-induced arrhythmias, it appears that the mechanism for its protective action is not related to an effect on alpha-mediated coronary artery constriction.

An important consideration in digitalis toxicity is the experimental model. Different anesthetics produce a change in the toxicity of ouabain (Stickney, 1974). Thus, the choice of anesthetic may influence the relative toxicity of the various glycosides. Secondly, ouabain, when infused at equal rates, produces toxic effects at lower doses than digoxin. Consequently, the ouabain model may be too toxic to show any protective action by WR 2823 and could account for the observation that propranolol only increased by 19.1% and 25.8% the dose of ouabain necessary to produce ventricular tachycardia and fibrillation while it increased the dose of digoxin necessary to produce ventricular tachycardia and fibrillation by 58.0% and 50.2%, respectively. Additional studies are being designed using lower infusion rates of ouabain to approximate the toxicity of digoxin in order to determine whether the ouabain model was too toxic to show the protective effects of WR 2823.

Table 1  
Effect of WR 2823 and Propranolol on Ouabain Toxicity

Pretreatment	Ouabain Toxicity			
	VT <sup>a</sup>		VF <sup>b</sup>	
(n = 6)	Dose $\mu\text{g/kg}$	% of Control	Dose $\mu\text{g/kg}$	% of Control
Control	68.6 $\pm$ 3.6 <sup>c</sup>	100.0	97.2 $\pm$ 6.0	100.0
WR 2823 (50 mg/kg)	69.7 $\pm$ 4.0	101.6	89.8 $\pm$ 4.3	92.4
Propranolol (2 mg/kg)	83.7 $\pm$ 4.9	119.1	123.3 $\pm$ 8.4 <sup>d</sup>	126.9

<sup>a</sup> VT - dose infused when ventricular tachycardia occurred.

<sup>b</sup> VF - dose infused when ventricular fibrillation occurred.

<sup>c</sup> Values are mean  $\pm$  SEM.

<sup>d</sup> Significant difference ( $p < 0.05$ ) from control Newman-Kuel's Test of Significance.

Table 2  
Effect of WR 2823 and Propranolol on Digoxin Toxicity

Pretreatment	Digoxin Toxicity			
	VT <sup>a</sup>		VF <sup>b</sup>	
(n = 6)	Dose ( $\mu$ g/kg)	% of Control	Dose ( $\mu$ g/kg)	% of Control
Control	126.6 $\pm$ 7.9 <sup>c</sup>	100.0	158.8 $\pm$ 6.3	100.0
WR 2823 (50 mg/kg)	172.7 $\pm$ 18.4 <sup>d</sup>	136.4	207.3 $\pm$ 15.6	130.5
Propranolol (2 mg/kg)	200.0 $\pm$ 8.6 <sup>d</sup>	158.0	238.5 $\pm$ 9.1 <sup>d</sup>	150.2

a VT - dose infused when ventricular tachycardia occurred.

b VF - dose infused when ventricular fibrillation occurred.

c Values are mean  $\pm$  SEM.

d Significant difference ( $p < 0.05$ ) from control Newman-Kuel's Test of Significance.

Table 3  
Dose-Response Relationship of Antiarrhythmic Action of WR 2823 vs Digoxin Toxicity

WR 2823 (mg/kg)	Increase in Arrhythmic Dose of Digoxin			
	Ventricular Tachycardia		Ventricular Fibrillation	
	N	$\frac{\mu\text{g/kg}}{\%}$	$\frac{\mu\text{g/kg}}{\%}$	$\frac{\mu\text{g/kg}}{\%}$
35	5	34.2	40.8	25.7
50	6	46.1	48.5	30.5

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 192 Antiarrhythmic effects of aliphatic amines

Literature Cited.

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1. Korte, D.W. Jr. and Nash, C.B.: Ventricular electrophysiology of quinidine-propranolol combinations in the dog heart. *J. Pharm. Exper. Therap.* 197:452-457, 1976.

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)636	
3 DATE PREV 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>	8A DISTR INSTR <sup>a</sup>	8B SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	9 LEVEL OF SUM <sup>a</sup>
75 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	61101A	3A161101A91C	00	194			
B. CONTRIBUTING							
C. CONTRIBUTING							
11 TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Development of an Organ Culture Method from Intestinal Biopsies							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</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:				PRECEDENCE		B. FUNDS (in thousands)	
C. TYPE:				FISCAL YEAR		76	
D. KIND OF AWARD:				CURRENCY		1.5	
E. AMOUNT:				77		45	
F. CUM. AMT.							
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
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21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Ewing, E., Richardson, W.			
				NAME:			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Organ culture; (U) Intestine; (U) Phase Microscopy; (U) Electron Microscopy							
23 TECHNICAL OBJECTIVE <sup>a</sup> 24 APPROACH 25 PROGRESS (Furnish 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) 75 07-76 06 Utilizing a modified Rose's tissue culture chamber and conventional Falcon culture bottles, the viability and growth of the guts of the guinea pigs and mice <u>in vitro</u> have been continuously studied. Fetal small and large intestine epithelial cells (absorptive, goblet, Paneth and Argentaffine cells), endothelial cells, mesenchymal cells (lymphocytes, macrophages, mast cells, fibroblasts, smooth muscle and nerve cells) could be grown and remain viable up to one week while adult counterparts do not survive more than 2 days. Current efforts have been directed toward synthesis of immunoglobulin and distribution of immuno-competent cells in guts in this organ culture system. Studies of the effects of virulent shigella derived toxins on cultured guts have been also initiated.</p> <p>For technical reports, see Walter Reed Army Institute of Research Annual Reports, 1 Jul 75 - 30 Jun 76.</p> <p>Support in the amount of \$19,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</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: Gertrude Issac

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 (Takeuchi et al. 1974) (See 1974-75 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 established. Sequential ultrastructural observations have been initiated on the growth of intestinal epithelial cells of cultured guts.

4. Cytochemical Reaction of Absorbed Horseradish Peroxidase (HRP): Studies of absorption of macromolecular substances by absorptive cells of cultured small intestine has been continuously investigated. 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 hour or 3 hours 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 hour 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. 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. At 3 hours, HRP was totally absent in the lumen and at the surface of the microvilli. Moderate HRP activity was still present between epithelial cells. On the other hand, the intercellular spaces of the lamina propria revealed strong HRP activity. These observations indicate that the absorption of HRP in cultured guts is identical to that reported in vivo (Cornell et al. 1971).

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 progressed. Likewise monitoring of the immunofluorescent localization of immunoglobulin as another parameter of the functional activity of the cultured gut has also been started.

6. Infection of cultured small bowel by enteroviruses: In collaboration with the Department of Veterinary Microbiology, WRAIR, studies of mouse hepatitis virus infection in cultured mouse guts has been initiated in order to obtain information on the site of viral replication and its cytopathic effects in the guts.

7. Shortage of Technical Personnel: A well trained technician who assisted our organ culture of the guts for the last two years has recently resigned due to RIF in WRAIR which affected progress of this research mission. This shortcoming will be hopefully overcome by training a new personnel assigned in other projects at our department.

#### 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 microscopy 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 continuously investigated. 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 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 DISPN INSTR <sup>a</sup>	8B SPECIFIC DATA- CONTRACTOR ACCESS	9 LEVEL OF SUM
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10 NO CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61101A	3A161101A91C	00	196			
B. CONTRIBUTING							
C. CONTRIBUTING							
11 TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Biochemical Characterization of Arbovirus Antigens							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002300 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 GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE NA EXPIRATION				PRECEDING		B. FUNDS (in thousands)	
D. NUMBER <sup>a</sup>				FISCAL YEAR		1.0	
C. TYPE				CURRENT		1.0	
E. KIND OF AWARD				77		1.0	
F. CUM. AMT.							
19 RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursuant to 38 USC 552 (a)(7)(D))			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: RUSSELL, COL Philip K.			
				NAME: BRANDT, Dr. Walter E.			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Arbovirus; (U) Antigen; (U) Immunology; (U) Immunopathology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pursuant to 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 nonstructural proteins of arboviruses of military importance and to describe the role of such antigens in the immune response, protection and immunopathology of arbovirus infections.</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. Animal model systems are investigated for evaluating protection and modification of disease.</p> <p>25 (U) 75 07 - 76 06 Virulent and avirulent strains of Semliki Forest Virus (SFV) were examined in inbred mice as a model for evaluating subunit vaccine efficacy. Comparative in vivo replication and pathology studies yielded pronounced differences between the strains. Infection with avirulent SFV resulted in protection against virulent SFV challenge. Protection was biphasic; early protection (2-3 days) was specific and could be transferred by serum whereas late protection (after 7 days) could be transferred by either serum or cells. Preliminary characterization of the virion subunits is in progress to test their efficacy in the inbred mouse protection system. Non-ionic detergent disruption of SFV strains, followed by isoelectric focusing of the components, has resolved nucleocapsid components. Complete separation of the envelope glycoproteins awaits further experimentation. This alphavirus system is concurrently applied to the more difficult flaviviruses (which includes dengue fever) as the methodology is developed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1975 - 30 June 1976. Support in the amount of \$24,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>							

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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;  
L. D. Agniel; SP5 S. A. Harrison; SP4 C. D. Page;  
PFC S. M. Shaver; K. M. Langenbach; G. P. Onley;  
R. J. Jackson

Description

To define the antigenicity of arbovirus structural and nonstructural components as potential immunogens of prophylactic value. To isolate 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 and the development of appropriate model systems for evaluating protection and the immune response.

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 offers many advantages over flaviviruses in preliminary studies concerning methodology because of more rapid replication to high titers, greater efficiency of radiolabeling, and more complete characterization. More recently a virulent and avirulent pair of alphaviruses (Semliki Forest viruses) have been investigated as a model system for a detailed investigation of the immune status of animals immunized with either live avirulent viruses or sub-virus components.

I. Introduction

Previous studies using the alphavirus (Sindbis) have been primarily concerned with the isolation and characterization of virion component antigens and antigenic analysis of these antigens as they react with humoral antibodies from immunized animals. In support of this goal, we



have separated the three structural polypeptides of Sindbis and prepared animal antisera to each of them. Antisera to the nucleocapsid protein are group reactive with most other alphaviruses, but exhibit no neutralizing activity. The E<sub>1</sub> envelope glycoprotein appears to represent the virus hemagglutinin and the corresponding antibody cross reacts with members of closely related virus complexes. In contrast, E<sub>2</sub>, or the other envelope glycoprotein is the virus specific antigen and antibody to this protein specifically neutralizes intact virus.

To pursue this avenue of research with Sindbis virus would involve the further investigation of the humoral antibody response to various immunogenic preparations of E<sub>2</sub>. This approach assumes that humoral antibody is the major, if not the only, mechanism responsible for an immunized animal's protection from infection. The Sindbis system is not readily adaptable to the measurement of complete protection because older animals do not routinely die following infection and the detection of infection via viremias, etc., is difficult and variable and entails a variety of assumptions that may prove invalid (e.g., no viremia equals protection). We have, therefore, investigated a variety of animal virus models that were applicable to our antigen isolation and characterization expertise, as well as providing an animal model system with a lethal endpoint and avirulent strains.

The Semliki Forest virus (SFV) system meets most of these requirements in that paired virulent and avirulent strains are available; these are alphaviruses very similar to SIN virus with an E<sub>1</sub> and E<sub>2</sub> envelope glycoprotein (although a smaller E<sub>3</sub> protein is reported to exist as well) and a nucleocapsid protein with very similar characteristics. Inbred mouse strains appear very susceptible to infection; there is a measurable subclinical infection with the avirulent strain and with the virulent strain infection is obvious, with death as an endpoint, allowing cell and serum transfer experiments to assess levels of protection and the role of cell-mediated immunity. Much of our recent work has, therefore, been directed toward describing the SFV model system.

## II. Interaction of virulent and avirulent strains of Semliki Forest virus in inbred mice

### A. History of strains

Semliki Forest Virus (SFV) strains A774C2L1/A/S and L10H6C1/A were supplied by Dr. C. J. Bradish (Microbiological Research Establishment, Porton Down, England). Both strains are closely defined clonal populations of differing virulence for mice (Bradish, et al., 1971). Viruses were received in the lyophilized state and were passaged once in suckling mice (Dr. P. B. Jahrling, USAMRIID); the resultant 20% (w/v) mouse brain suspension was stored at -70°C for use in these studies.

## B. Definition of the problem

In recent years much effort has been directed toward an understanding of the interactions between viruses and the natural defenses of host animals. Resistance of animals to reinfection with viruses is believed to be due to the presence of circulating neutralizing antibody (Worthington et al., 1972; Worthington, 1973, 1976; Zisman et al., 1971); however, some authors suggest that neutralizing antibody plays only a pivotal role in recovery from viral infections (Nathanson and Cole, 1971; Weiner, et al., 1970; Camenga et al., 1974). It has, however, been well established that those individuals who have defects in thymus-dependent immunity (but normal humoral immunity) suffer greater morbidity from viral infection (Cooper et al., 1968). In addition, most recent findings point to a critical role for cell-mediated immune (CMI) mechanisms in thwarting viral infections. Blanden (1970, 1971a, 1971b, 1974) has established the crucial nature of T-cell mediated responses in the murine host which facilitate recovery from ectromelia infection. The necessity for T-cell mediated defenses for recovery from Herpes simplex infections in humans has recently been suggested. (Jacobs et al., 1976; Steele et al., 1975; and Lodmell et al., 1973). In most arbovirus-induced encephalitides a prominent role for CMI has either been well established or suggested: i.e., Sindbis virus (McFarland, 1974; Griffin, 1976; Griffin and Johnson, 1973; Hackbarth et al., 1973; Johnson et al., 1972, McFarland et al., 1972; Bebaru Virus (Hapel, 1975), and Langat Virus (Vargin and Mayer, 1973). The work of others suggests the participation of both cellular and humoral aspects of the immune response in recovery from arboviral infections (Hirsch and Murphy, 1967; Rabinowitz and Adler, 1973; Adler and Rabinowitz, 1973; Shore et al., 1974).

The above-mentioned strains of Semliki Forest virus provide a unique opportunity to investigate the relative importance of cellular, humoral and nonspecific defense mechanisms which come into play during the interaction of the avirulent A774C2L1/A/S or lethal L10H6C1/A strains with an experimental murine host.

## C. Characterization of the strains

### 1. LD50 vs PFU

The virulence of defined strains of SFV has been documented for rabbits, guinea pigs, hamsters and Porton random bred mice (Bradish et al., 1971). In preliminary tests of virulence with "Kumba" and "standard" strains (WRAIR virus stock collection) of SFV it has been determined that among adult BALB/c, DBA/2, and A/J strain inbred mice that A/J mice were the most susceptible to death from SFV infection after intraperitoneal inoculation. The lethality of SFV strains L10H6C1 and A774C2L1/A/S (hereinafter referred to as L10 and A774, respectively) was determined for A/J mice inoculated i.p. Stock mouse brain suspensions (titers L10 =  $8.73 \times 10^8$  pfu/ml; A774 =  $8.4 \times 10^8$  pfu/ml) were



diluted and 0.1 ml of each dilution was injected i.p. into groups of 5 female A/J mice. Animals were observed for 14 days. No animal which developed clinical signs (ruffled fur, irritability, hind-leg paralysis) survived. Percent cumulative mortality and mean time to death are recorded below (Table 1). Calculations reveal that for L10 stock mouse brain seed there are 1.74 pfu/LD<sub>50</sub> and that for A774 there are  $> 8.40 \times 10^8$  pfu/LD<sub>50</sub>.

Table 1. LD<sub>50</sub> titrations of SFV strains A774 and L10

SFV strain	Dose (pfu)	% Cumulative mortality	Mean time to death (days)
A774	$8.4 \times 10^6$	0	-
	$8.4 \times 10^5$	0	-
	$8.4 \times 10^4$	0	-
	$8.4 \times 10^3$	0	-
	$8.4 \times 10^2$	0	-
	$8.4 \times 10^1$	0	-
	$8.4 \times 10^0$	0	-
	$8.4 \times 10^{-1}$	0	-
	$8.4 \times 10^{-2}$	0	-
L10	$8.7 \times 10^6$	100	3.4
	$8.7 \times 10^5$	100	4.2
	$8.7 \times 10^4$	100	4.4
	$8.7 \times 10^3$	100	4.8
	$8.7 \times 10^2$	100	4.4
	$8.7 \times 10^1$	100	5.4
	$8.7 \times 10^0$	80	7.0
	$8.7 \times 10^{-1}$	40	7.0
	$8.7 \times 10^{-2}$	0	-

## 2. Protection of survivors

Survivors of the above LD<sub>50</sub> titration were challenged on day 14 post infection with 100 pfu L-10 strain and observed for another 14 days. Results of this experiment are summarized in Table 2,

below:

Table 2. Protection of survivors of LD<sub>50</sub> titrations of SFV strains A774 and L10

SFV strains	Immunizing dose (pfu)	% Survivors at 14 days	% mortality following L10 challenge	Mean time to death
A774	$8.4 \times 10^6$	100	0	-
	$8.4 \times 10^5$	100	0	-
	$8.4 \times 10^4$	100	0	-
	$8.4 \times 10^3$	100	0	-
	$8.4 \times 10^2$	100	20*	2.0
	$8.4 \times 10^1$	100	10*	1.0
	$8.4 \times 10^0$	100	20*	1.0
	$8.4 \times 10^{-1}$	100	80	6.2
	$8.4 \times 10^{-2}$	100	80	6.2
L10	$8.7 \times 10^6$	0	-	-
	$8.7 \times 10^5$	0	-	-
	$8.7 \times 10^4$	0	-	-
	$8.7 \times 10^3$	0	-	-
	$8.7 \times 10^2$	0	-	-
	$8.7 \times 10^1$	0	-	-
	$8.7 \times 10^0$	20	0	-
	$8.7 \times 10^{-1}$	60	100	8.3
	$8.7 \times 10^{-2}$	100	100	6.2

\*Deaths (total of 3 animals) not typical of CNS symptomatology; no signs seen

From these data it appears that 100% protection to L10 challenge can be induced by 14 days post immunization with slightly less than  $8.7 \times 10^3$  pfu A774 (probably  $\sim 100$  pfu, as will be shown in subsequent results. It should be noted that of those animals receiving L10 strain, only 20% (1 mouse) of animals surviving primary immunization with  $8.7 \times 10^0$  pfu (calculated dose) were protected against subsequent lethal L10 challenge, i.e., sub-clinical infection with L10 leads to protection.

#### D. Pathology and course of infection in A/J mice

##### 1. Pathology

In preliminary studies of the pathology of SFV infection in A/J mice, pairs of females, 6-8 weeks old, were injected i.p. with low ( $10^2$  pfu) and high ( $10^5$  psu) doses of both "Kumba" and "standard" strains SFV. Six days later, when all mice were moribund, mice were autopsied and tissue samples were taken for histology. The following results were obtained for both doses and strains:

Table 3. Pathology of "Kumba" and "standard" SFV strains in A/J mice

Organ	Description
Lung	Focal interstitial pneumonitis
Liver	Focal round cell hepatitis
Kidney	Vague focal collections of lymphocytic cells
Small intestine	Cores of all villi inflamed, marked epithelial sloughing
Myocardium	Unremarkable
Thymus	Reactive hyperplasia
Spleen	Reactive hyperplasia, increased number of germinal centers, prominent giant cells in red pulp
Diaphragm	Unremarkable
Brain (cerebrum)	Periventricular inflammation; ventriculitis (choroid); meningitis; destructive foci, focal encephalitis
" (cerebellum)	Frank, destructive lesions; perivascular cuffing; ventriculitis; meningitis; focal encephalitis

Using strains A774 and L10 (100 pfu/mouse), pairs of mice were again inoculated i.p. and autopsied two and four days later. Results observed for the defined strains correlate well with virulence. On days 2 and 4 post inoculation with A774, brains showed only minimal inflammatory foci, whereas with L10 inflammation was moderate on day 2 and widespread by day 4, accompanied by widespread necrosis. In peripheral organs examined, liver, lung and kidney appeared to be possible sites of extraneural replication for both strains. The lymphoid organs examined (spleen, thymus and mesenteric nodes) exhibited reactive hyperplasia.

Spleens also exhibited multinucleated giant cells - implying infection.

## 2. Organ infectivities

In an attempt to further understand the course of SFV caused disease in mice, viral infectivities of the organs studied for pathology were determined. Pairs of animals were immunized in an experiment run in parallel with the A774 and L10-immunized mice used for pathology specimens. Mice were sacrificed on days 2 and 4 p.i. by CO<sub>2</sub> narcosis, and were exsanguinated by severing the right axillary artery. Blood and target organs were collected aseptically, weighed and frozen at -70°C until assayed for infectivity. For assay, organs were homogenized in modified Tenbrook tissue grinders in cell culture medium (10% FCS). Homogenates were made as 10% (w/v) suspensions, decimally diluted and dilutions were assayed by plaque titration in BHK 21/15 cells. Target organ infectivities are summarized below (Figures 1, 2).

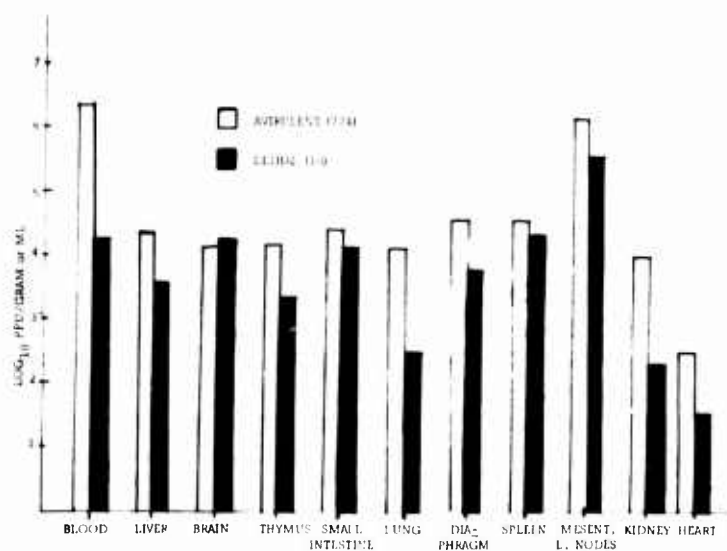


Figure 1. Organ infectivities 2 days following intraperitoneal inoculation of Semliki Forest virus strains A774 (avirulent) and L10 (lethal)

It is immediately evident that, while the brain titers of both strains are nearly equal on day 2, by day 4 the lethal strain has replicated exuberantly and the avirulent strain is being cleared from the brain or its replication is being severely limited. It is also significant that no viremia can be detected with either strain on day 4 despite the fact that spleen and mesenteric lymph node titers remain high (A774 shows higher titers in these organs than L10 on day 4).

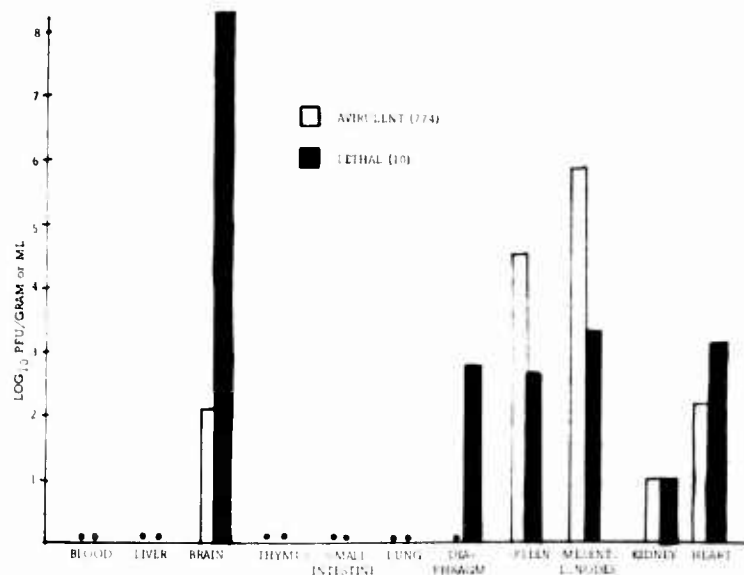


Figure 2. Organ infectivities 4 days following intraperitoneal inoculation of Semliki Forest virus strains A774 (avirulent) or L10 (lethal).

#### E. Protective capacity of strain A774

Since animals immunized with strain A774 were solidly immune to challenge with 100 pfu of strain L10 at 14 days, it became of immediate interest to examine the kinetics of the appearance of this protection. To this end, multiple groups of 5 mice were inoculated with 100 pfu of strain A774 and challenged with 1000 pfu L10 (>500 LD<sub>50</sub>) simultaneously and periodically from +6 hours to +14 days. Results of this experiment are recorded in Figure 3 on the following page. One hundred percent survival is obtained by 48 hrs post immunization (80% at 24 hrs p.i.), is maintained for a day, and then begins to wane; by day 6 no animal survives L10 challenge and protection returns abruptly thereafter. This experiment is being repeated with larger animal groups and more early time points to verify the unusual nature of the curve obtained.

### III. Replication characteristics and temperature sensitivity of Semliki Forest virus strains

The inverse correlation of temperature sensitivity and virulence has been either implied or demonstrated with many viruses. As a general rule, mutants selected on the basis of their temperature sensitivity are less virulent than the parent virus. Since the SFV strains L-10 and A-774 were selected on the basis of their mouse neurovirulence,

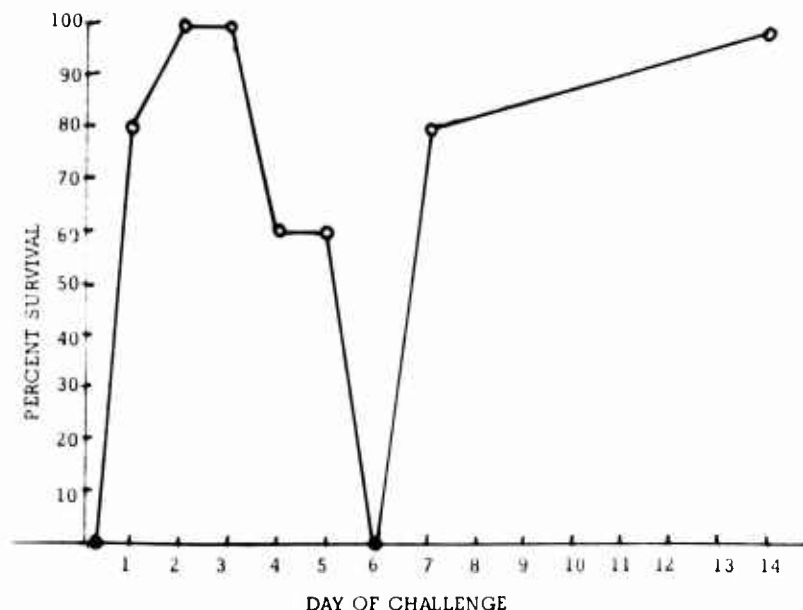


Figure 3. Survival of A/J mice from challenge with 1000 pfu of lethal Semliki Forest virus strain L10 following infection with 100 pfu of the avirulent strain A774.

we compared their replication characteristics at two temperatures, 36 - 36.5°C - or normal incubator temperatures, and 40°C, a temperature normally nonpermissive for most temperature sensitive viruses. A variety of cell lines were initially examined for their ability to support the replication of the SFV strains and of these, the BHK21 clone 15 cells and primary chick embryo cell (CEC) cultures appeared superior and were, therefore, compared in the growth curve experiments at the two temperatures. The growth curves of the SFV strains at two temperatures in each of the cell lines are shown in Figures 4 and 5 on the following page.

The SFV lethal strain (L-10) exhibited little temperature sensitivity to the log phase (5-11 hr) of its replication. In fact, replication at 40°C was actually more rapid in the BHK-21/15 cells. Although time to maximum titer appeared the same for each cell type, the CEC cultures yielded slightly higher titered virus than the BHK-21 cells. Also, the lethal strain decreased in titer after 14 hours in the CEC cultures; heat stability might be one of the factors, and these experiments have yet to be done. In contrast, the avirulent SFV strain (A-774) exhibited marked temperature sensitivity as a slower replication in BHK 21/15 cells and a greatly diminished yield from CEC cultures (less than 1% of the 22 hour virus yield at 22 hrs). The reduced virus yield observed in the CEC cultures at 40°C and the delayed release of infectious virus in BHK 21/15 cells may be important

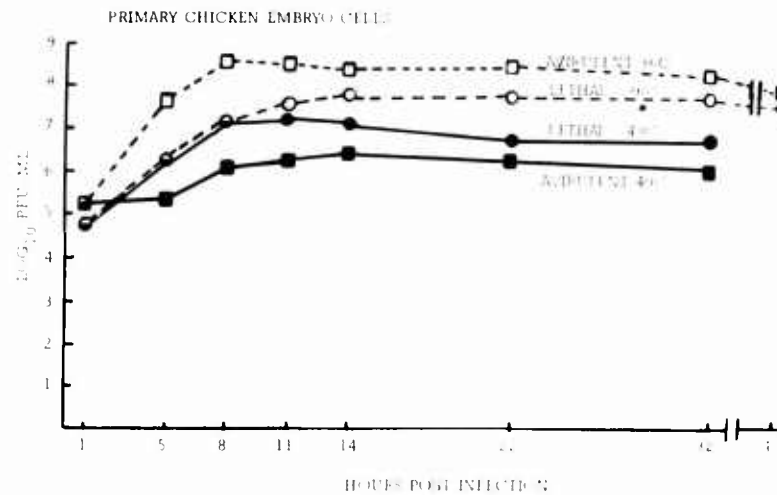


Figure 4. Replication of avirulent (A774) and lethal (L10) strains of Semliki Forest virus in primary chicken embryo cells incubated at two temperatures.

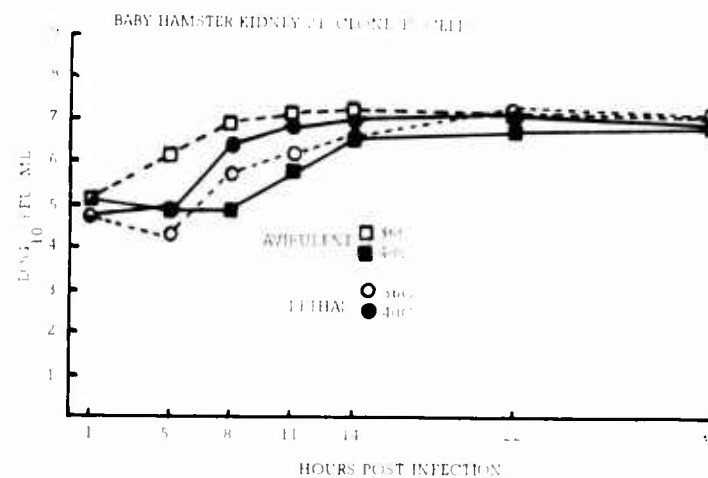


Figure 5. Replication of avirulent (A774) and lethal (L10) strains of Semliki Forest virus in baby hamster kidney cells incubated at two temperatures.

characteristics of the A774 virus which contribute to the decreased virulence of this strain.

#### IV. Radiolabeling of SIN and SFV strains

Virion antigen isolation and characterization experiments require radiolabeling of virion proteins to a high specific activity. Experiments were conducted to determine if SFV strains could be intrinsically labeled to the same activity as routinely obtained with SIN virus. Most of our alphavirus radioisotope experience has been with  $^3\text{H}$ -amino acids, a low energy radioisotope requiring the slicing and spectrophotometric counting in expensive scintillation cocktails. Labeling with the  $^{35}\text{S}$ -methionine amino acid offers particular advantages for autoradiography, etc., and consequently was incorporated into these experiments. A comparison of the  $^{35}\text{S}$ -methionine and  $^3\text{H}$ -amino acids labeling efficiency for each of the viruses in two cell lines is shown in Table 4. Cells were infected and, following virus adsorption, washed - and amino-acid-free medium supplemented with 2% dialysed fetal bovine serum - was replaced. Following a 4-5 hour amino acid starvation, the indicated concentrations of radioactive amino acids were added and the supernatant fluids harvested 20 hrs post infection. The virus containing medium was clarified, precipitated by ammonium sulfate and centrifuged on rate zonal sucrose gradients. The specific activities expressed in the Table represent the pooled samples from the virion rich peak fractions of the gradient. Control flasks containing the normal amino acid concentration exhibited very little radioactivity in the virion region.

Table 4. Radiolabeling of SIN and SFV strains

Virus	Cell type	I s o t o p e (conc)		
		$^3\text{H}$ -Amino acids	$^{35}\text{S}$ -Methionine	
		(10 $\mu\text{Ci/ml}$ )	(10 $\mu\text{Ci/ml}$ )	(1 $\mu\text{Ci/ml}$ )
Sindbis:	BHK-21	12.3*	38.7	35.8
	1 $^0$ CEC	43.5	174.3	137.5
SFV L-10:	BHK-21	2.8	10.2	7.3
	1 $^0$ CEC	44.9	236.2	218.4
SFV A-774:	BHK-21	2.1	5.8	6.4
	1 $^0$ CEC	36.4	183.9	154.6

\* cpm  $\times 10^3$  per 50  $\mu\text{l}$



As can be seen from Table 4, the CEC cultures yielded consistently higher radioactivity in virion fractions. Since no virus quantification was performed, this could represent the reduced virus replication exhibited by the BHK cells in the growth curve experiments. The  $^{35}\text{S}$ -methionine labeled preparations contained much more radioactivity than did the  $^3\text{H}$ -amino acid preparation, even at one-tenth the isotope concentration. Although the low dose  $^{35}\text{S}$ -methionine labeled preparations ( $1\ \mu\text{Ci/ml}$ ) were lower, the differences were not great or linear and it appears that radiolabeling with  $^{35}\text{S}$ -methionine in infected CEC cultures will yield higher specific activities than routine  $^3\text{H}$ -amino acid labels, even with a tenfold decrease in added isotope.

#### V. Adoptive transfer experiments

Preliminary adoptive immunizations have been performed using donor mice 1, 2, 3 and 6 days post immunization (i.p., with 100 pfu strain A774). Briefly, 5 donor mice (each day) were exsanguinated by axillary artery cut and blood was pooled for serum collection. Spleens from the same animals were aseptically removed and processed in Leibowitz medium (L-15) to form single-cell suspensions; cells were washed twice in L-15. Serum and cells obtained in this way were both divided into 5 equal doses, arbitrarily referred to as mouse-equivalents of serum or spleen cells. Serum or cells were injected i.v.; i.p. virus challenge followed in 4 hours. Table 5 is a summary of the results of serum and spleen cell adoptive immunizations. Full protection against lethal L-10 challenge is affected by 1 and 2-day serum; most of this effect is lost with day-3 serum but returns by day 6. Protection by spleen cell transfer first appears on day 6; normal cells and serum are without effect. More extensive experiments are underway to examine the nature of the successful adoptive transfers, the possible role of contaminating virus or other factors in serum transfers.

The specificity of the early protection demonstrated *in vivo* is being examined at USAMRIID by Dr. P. B. Jahrling by challenging animals inoculated with A774 with a virulent strain of Venezuelan equine encephalitis virus.

#### VI. Separation of virion components by isoelectric focusing

As a preliminary experiment in the degradation and separation of virion components,  $^{35}\text{S}$ -methionine labeled preparations of SIN and SFV, strains L-10 and A-774, were disrupted with 1% Triton X-100 and electrofocused on pH 3-10 gradients. Figure 6 depicts the radioactive profile observed with the  $^{35}\text{S}$  labeled SIN virus with three principal peaks of radioactivity representing the three virion structural proteins. The smallest of the three peaks near the acid region of the gradient has been shown to contain the nucleocapsid protein, probably in some intimate association with the nucleic acid. The two major peaks represent the two envelope glycoproteins  $\text{E}_1$  and  $\text{E}_2$ . The  $\text{E}_1$

Table 5. Survival of mice of L-10 challenge following adoptive immunization with serum or spleen cells from donors inoculated with A774

Adoptive immunization Donor material	% Survivors
1 day spleen cells . . . . .	0
1 day serum . . . . .	100
2 day spleen cells . . . . .	0
2 day serum . . . . .	100
3 day spleen cells . . . . .	0
3 day serum . . . . .	20
6 day spleen cells . . . . .	100
6 day serum . . . . .	100
Normal spleen cells . . . . .	0
Normal serum . . . . .	0
Untreated control . . . . .	0

protein at pH 6 represents the virus hemagglutinin and the E-2 protein at the higher pH is the virus specific antigen.

Electrofocusing of SFV strain L-10 yielded a slightly different profile (Figure 7). Although radioactivity was still observed in a peak near the acid region of the gradient, two widely separated peaks such as were observed with SIN virus were not evident. A single major peak existed near pH 7.5, with some indication that this profile represented two peaks very close together. The distribution of radioactivity from SFV strain A-774 was quite similar to its virulent member of the pair L-10 (Figure 8). The major peak near the neutral pH range exhibits considerable evidence of a split peak although the distribution of radioactivity is considerably more disproportionate.

It becomes considerably more important to these studies to determine if the envelope proteins of the SFV strains can be separated by the techniques of isoelectric focusing. Since isoelectric focusing can be performed over a gradient range of virtually any two or more pH units between pH 3 and 10, the split peaks of the SFV strains and the high pH single peak of SIN Virus were collected and refocused over the

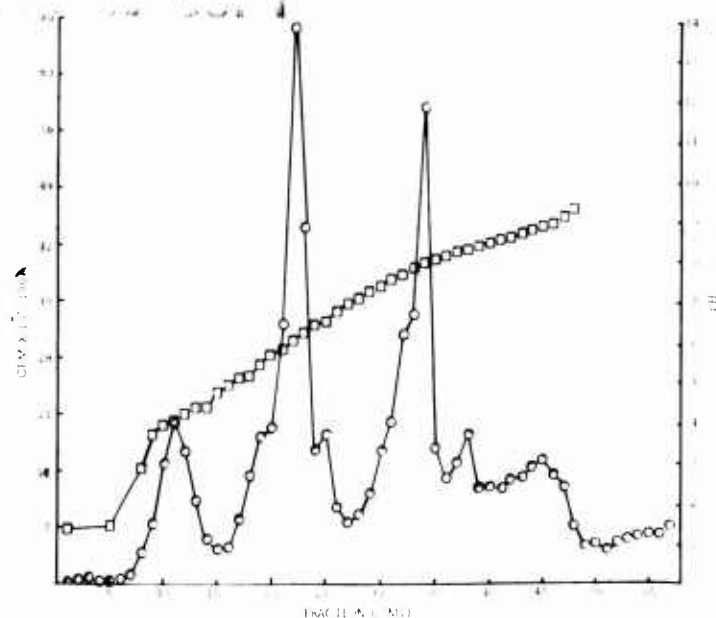


Figure 6. Distribution of <sup>35</sup>S-methionine labeled Sindbis virus on a Triton X-100 containing pH 3-10 gradient. Fractions 32 to 45 were selected for refocusing.

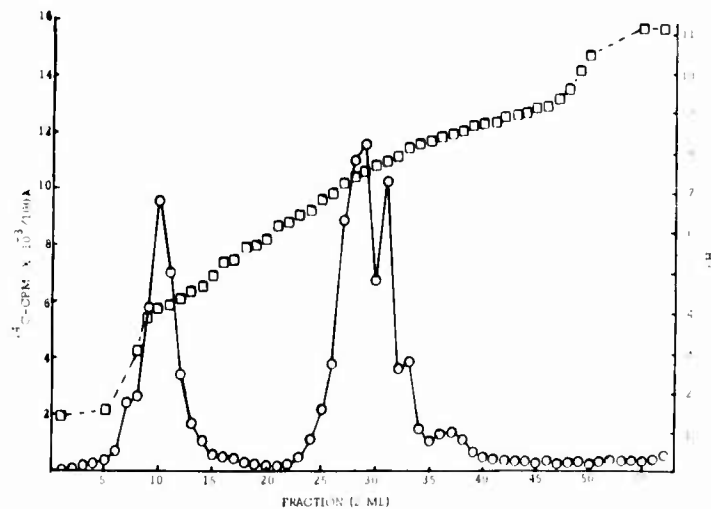


Figure 7. Electrofocusing profile of Triton X-100 treated Simliki Forest virus strain L-10 on a pH 3-10 column. Fractions 25 to 40 were selected for refocusing.

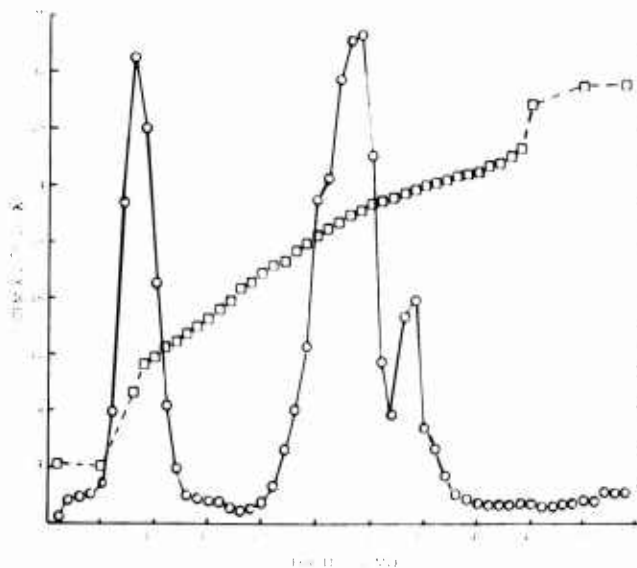


Figure 8. Distribution of  $^{35}\text{S}$ -methionine labeled Semliki Forest virus strain A774 components on a pH 3-10 electrofocusing gradient following disruption with 1% Triton X-100. Fractions 23 to 37 were pooled for refocusing.

pH range 7-10. These electrofocusing profiles are shown in Figures 9, 10, and 11. The high pH peak of SIN virus focused to a nice single peak; however, the radioactivity distribution of the SFV strains closely resembled their distribution on the pH 3-10 gradients. The profile of strain A774 appears to represent a family of peaks, a situation which may represent incomplete dissociation of the envelope proteins with various combinations representing each of the peaks.

These studies are continuing, employing a variety of virion component disruption procedures and numerous separation procedures followed by animal immunization with the isolated components. This separation system is concurrently applied to a representative flavivirus (Japanese encephalitis) as the methodology is developed (see Work Unit 166). Although the SFV strains may not follow the same pattern of SIN virus, they may be even more analagous to the flaviviruses and the model for testing protection and immunity becomes even more important.

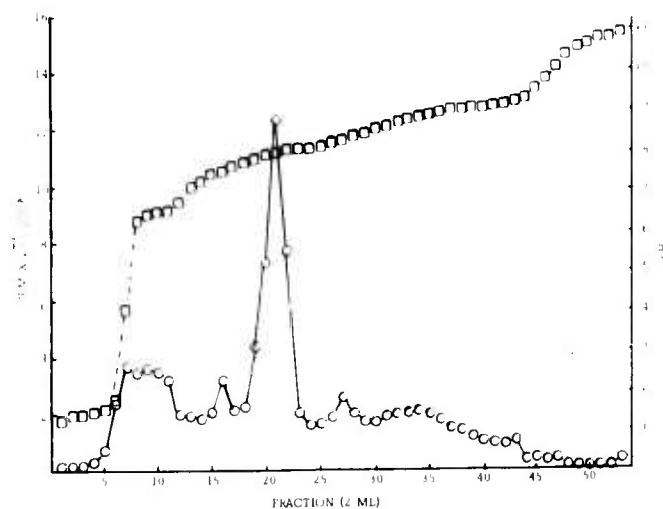


Figure 9. Refocus of the E<sub>2</sub> envelope glycoprotein of Sindbis virus on a pH 7-10 gradient.

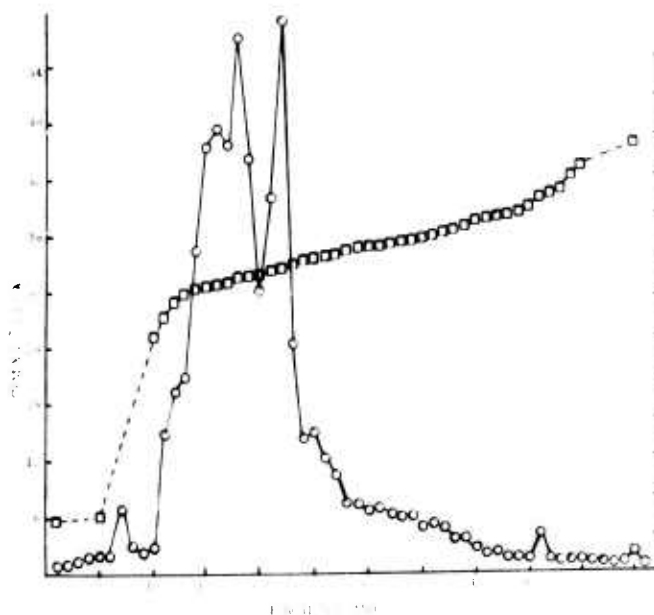


Figure 10. Refocus of pH 7 to 8 fractions from Semliki Forest virus strain L-10 on a pH 7-10 gradient.

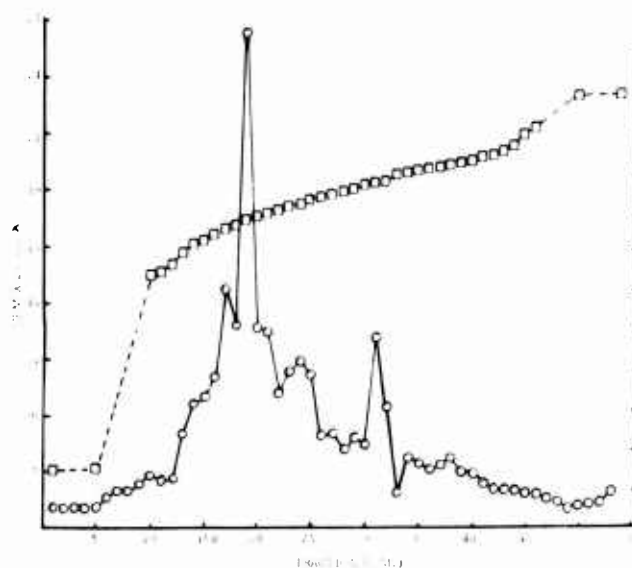


Figure 11. Distribution of radioactivity on a pH 7-10 narrow range electrofocus gradient of Semliki Forest virus strain A-774 fractions from previous pH 3-10 electrofocus experiment (pool of pH 7 to 8.5).

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 196, Biochemical Characterization of Arbovirus Antigens

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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

Publications.

Dalrymple, J. M., Schlesinger, S., and Russell, P. K. Antigenic characterization of two Sindbis envelope glycoproteins separated by isoelectric focusing. *Virology* 69: 93, 1976

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Relationships: (U) Vaccines							
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 genetic basis for susceptibility to disease in laboratory animals. Studies required to improve rickettsial vaccines to prevent disease in troops operating in 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 genetically homogeneous inbred mice to determine basis of rickettsial virulence.							
25. (U) 75 07 -76 06. 1. 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, nitrosoguanidine and gamma radiation have been determined. 2. Biochemical studies of rickettsial macromolecules have focused on identification of peripheral proteins and glycoproteins of whole rickettsiae. Selective extrinsic radioiodination provides a sensitive method for identification of external proteins and intrinsic radiolabeling with galactose or glucosamine aids in identification of glycoproteins. 3. The use of highly inbred strains of mice has indicated that differences in virulence of scrub typhus organisms are related to inheritable traits of the mice. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							
Support in the amount of \$61,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 197 Rickettsial Genetics

Investigators.

Principal: Joseph V. Osterman, Ph.D.; MAJ Michael Groves, VC  
Associate: Christine S. Eisemann; CPT Stanley C. Oaks, Jr., MSC;  
SP5 John Dewitt; SP4 Denise Caron

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. Biochemical and immunological studies of rickettsial macromolecules.

A. Identification of peripheral proteins by intrinsic and extrinsic radiolabeling.

SDS-polyacrylamide gel electrophoresis of whole organisms has demonstrated 24 rickettsial proteins in representatives of typhus and spotted fever groups (1). The structural location of each of these proteins is a major determinant of its role in the interaction between the host and the organism during the infectious process. Those proteins located on the external surface of the rickettsiae are most important in this interaction with the immune system of the animal host, and thus, are of greatest interest as potential subunit vaccines.

The identification of peripheral rickettsial proteins was attempted with typhus group (R. prowazekii) and spotted fever group (R. conorii) rickettsiae as model systems for the future analysis of scrub typhus rickettsiae (R. tsutsugamushi). The approach to this investigation included the identification of glycoproteins by intrinsic labeling with radioactive sugars, localization of external proteins by specific labeling with <sup>125</sup>I, and subsequent extraction of labeled cell wall components to assess their immunogenicity and protective capacity against subsequent challenge of immunized animals.

1. Rickettsiae grown in spinner cultures of gamma-irradiated, cycloheximide (1 µg/ml)-inhibited L-929 cells were labeled with radioactive galactose or glucosamine and purified by differential and sucrose velocity centrifugation. The organisms were free from any detectable contamination by L-929 cell protein as determined by either L-929 cell CF activity or radioisotope contamination. Fig. 1 shows the results of combining  $^{14}\text{C}$  amino acid labeled R. prowazekii infected L-929 cells with  $^3\text{H}$  galactose labeled uninfected L-929 cells and subjecting the mixture to the purification scheme. No radioactive contamination of the rickettsial band was observed, even though the uninfected L-929 cells in this experiment were uninhibited by cycloheximide. The concentration of cycloheximide present in rickettsial spinner cultures has been shown to inhibit L-929 cell uptake of radioactive sugars by 85% (Table 1).

Analysis of rickettsial glycoproteins was performed on 12 cm polyacrylamide gels, which were determined to be more sensitive than the 7 cm gels previously employed. Fig. 2 shows the results of co-electrophoresis of  $^{14}\text{C}$  amino acid labeled R. prowazekii and  $^3\text{H}$  galactose labeled R. prowazekii. There are 2-3 major glycoproteins which correspond to the previously identified proteins #1, #3, and #4 (1). Analysis of glucosamine labeled organisms yielded similar results. Fig. 3 is a co-electropherogram of  $^{14}\text{C}$  amino acid labeled R. prowazekii and  $^3\text{H}$  glucosamine labeled R. prowazekii. The aminosugar label again identifies proteins #1 and #3 as glycoproteins. The molecular weights of these R. prowazekii proteins are in the order of 87,000 and 30,000 daltons, as determined by reference to the mobilities of rickettsial proteins. The relative mobilities of these glycoproteins are, of course, affected by the sugar moiety and preclude precise determination of molecular weights.

Similar analysis of R. conorii glycoproteins is shown in Fig. 4. The labeled sugar co-migrates with protein #1, which suggest a molecular weight of 85,000 daltons.

2. Further identification of external rickettsial proteins was performed by extrinsic labeling with  $^{125}\text{I}$ . The use of the enzyme lactoperoxidase allows radiolabeling of only exposed, surface proteins, due to the inability of the enzyme to penetrate the cell membrane (2). An alternate method of iodination, utilizing the compound chloramine-T, is generally used for non-specific labeling of total proteins (3). Recent studies have indicated that the permeability of biological membranes is greatly altered by the concentration of iodide present in the test system (4). The concentration effect proved to have a significant impact on the permeability of rickettsial membranes, especially in the spotted fever group.

Radioiodination of R. prowazekii was carried out with both high (50 µM) and low (0.5 µM) concentrations of iodide. In all experiments,

Table 1. Radioactive sugar incorporation into TCA precipitable components of irradiated uninfected cells

Concentration of cycloheximide	Pretreatment time with cycloheximide (hrs)	Isotope incorporation 24 hrs after addition		Isotope incorporation 72 hrs after addition	
		galactose	glucosamine	galactose	glucosamine
0 $\mu\text{g/ml}$	--	3280 <sup>a</sup>	1707	5651	4602
1 $\mu\text{g/ml}$	24	516	553	1038	605
	48	627	332	1110	509
2 $\mu\text{g/ml}$	24	705	386	928	596
	48	586	357	944	506

<sup>a</sup>  
Counts per minute/ $4.0 \times 10^5$  cells

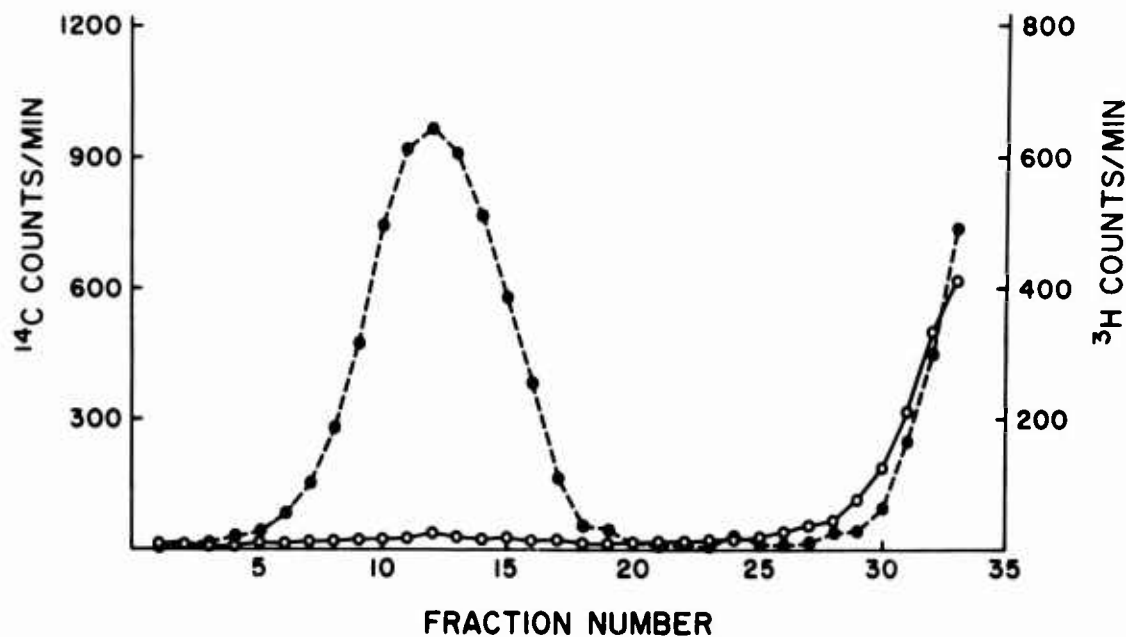


FIG. 1 Purification of  $^{14}\text{C}$  amino acid labeled *R. prowazekii* (●----●) from  $^3\text{H}$  galactose labeled uninfected L-929 (o—o) by rate zonal centrifugation in a linear 5-30% sucrose gradient.

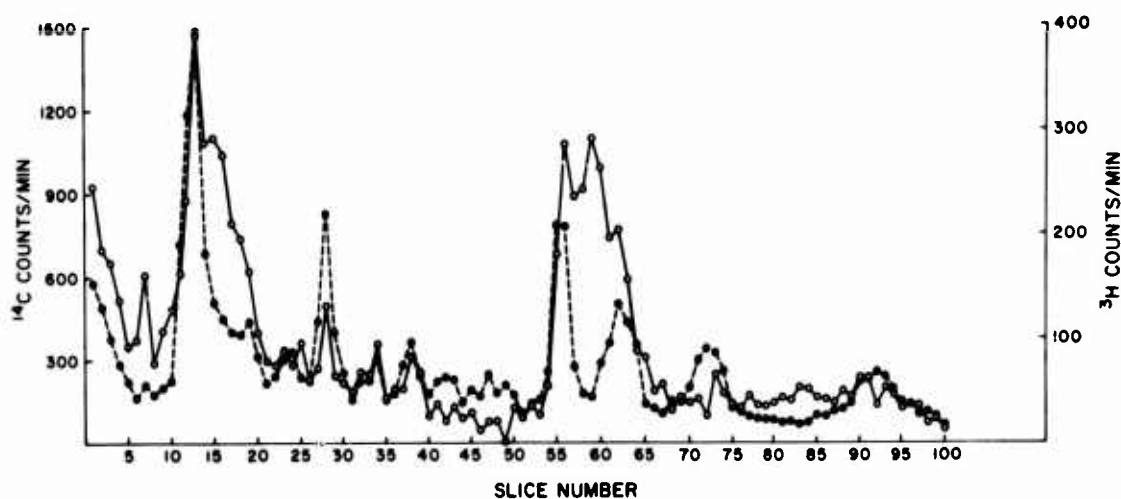


FIG. 2 Co-electrophoresis of  $^{14}\text{C}$  amino acid labeled *R. prowazekii* (●----●) and  $^3\text{H}$  galactose labeled *R. prowazekii* (o—o).

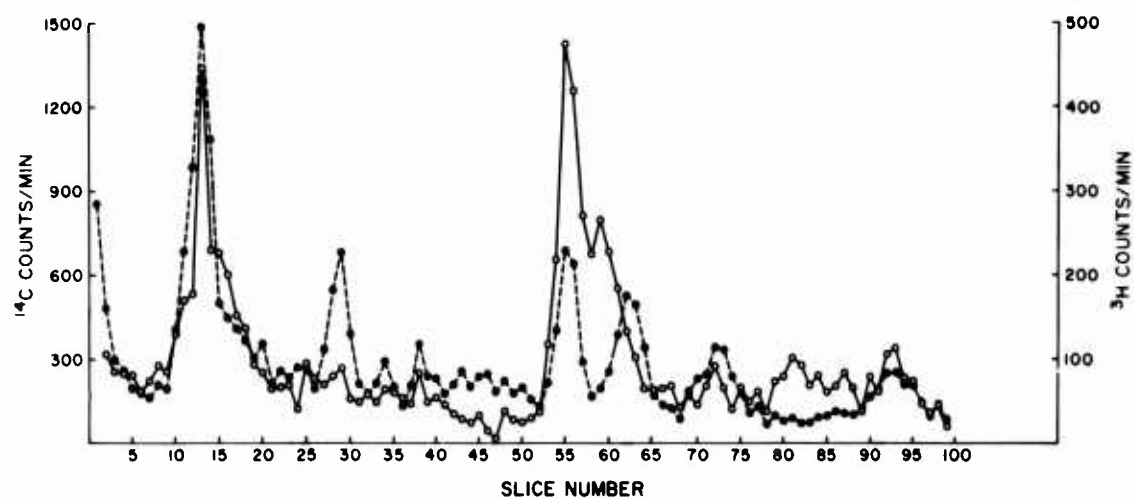


FIG. 3 Co-electrophoresis of  $^{14}\text{C}$  amino acid labeled *R. prowazekii* (●----●) and  $^3\text{H}$  glucosamine labeled *R. prowazekii* (○—○).

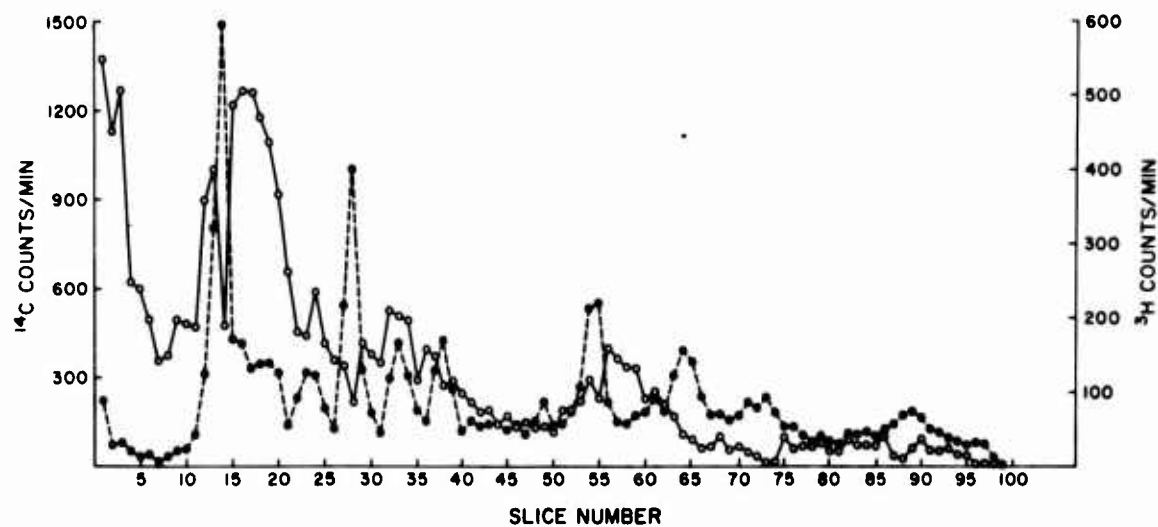


FIG. 4 Co-electrophoresis of  $^{14}\text{C}$  amino acid labeled *R. conorii* (●----●) and  $^3\text{H}$  galactose labeled *R. conorii* (○—○).



the concentration of  $^{125}\text{I}$  was constant at 0.5  $\mu\text{M}$ , and was increased in appropriate samples by the addition of cold potassium iodide. Identical results were obtained whether the samples were iodinated by lactoperoxidase or chloramine-T.

Fig. 5 shows superimposed electropherograms of  $^{125}\text{I}$  labeled (50  $\mu\text{M}$ ) and intrinsically labeled  $^{14}\text{C}$  amino acid labeled R. prowazekii. The profiles were identical, indicating general radioiodination of the proteins independent of their positions in the rickettsial cell.

Decreasing the iodide concentration to 0.5  $\mu\text{M}$  resulted in the specific labeling shown in Fig. 6. This electropherogram identifies protein #4 as a major external component. This data is compatible with the results obtained with intrinsic radioactive sugar labeling of R. prowazekii. The 27,000 dalton molecular weight region was again shown to contain an external structural component.

Similar radioiodination of R. conorii yielded strikingly different results. High concentrations of iodide again achieved general protein labeling, comparable to the intrinsic labeling with  $^{14}\text{C}$  amino acids. Fig. 7 shows superimposed electropherograms of  $^{125}\text{I}$  labeled and  $^{14}\text{C}$  amino acid labeled R. conorii.

Decreasing the iodide concentration, however, did not result in the specific labeling of external proteins as in the experiments with R. prowazekii. Fig. 8 shows an electropherogram of R. conorii organisms radioiodinated with the lower concentration of iodide (0.5  $\mu\text{M}$ ). The profile generally resembled the results formerly achieved with high iodide concentration, although reduced in radio-activity. The R. conorii cell membrane is possibly more sensitive than that of R. prowazekii to alterations in permeability induced by even minute quantities of iodide. Further experiments are planned to investigate the variations in permeabilities of these two groups of organisms.

3. Fractions of yolk-sac grown rickettsiae prepared by 1) ether extraction (soluble and corpuscular antigens) (5) and 2) ether extraction followed by boiling in 0.2N sodium hydroxide (ESS) (6) have been shown to be antigenically active. Extracts of radioiodinated R. prowazekii and R. conorii were analyzed by density gradient centrifugation and by electrophoresis on SDS-polyacrylamide gels. Fig. 9 shows superimposed potassium tartrate-glycerol density gradients of R. prowazekii ether extract and R. prowazekii ESS. Ether-extracted material exhibited a uniformly-banding component which sedimented at the same density as a major component of the ESS. Both gradients exhibited additional low density material, banding diversely in the ether extract gradient and appearing as soluble material in the ESS gradient. The major radioactive peaks of these gradients, although exhibiting the same



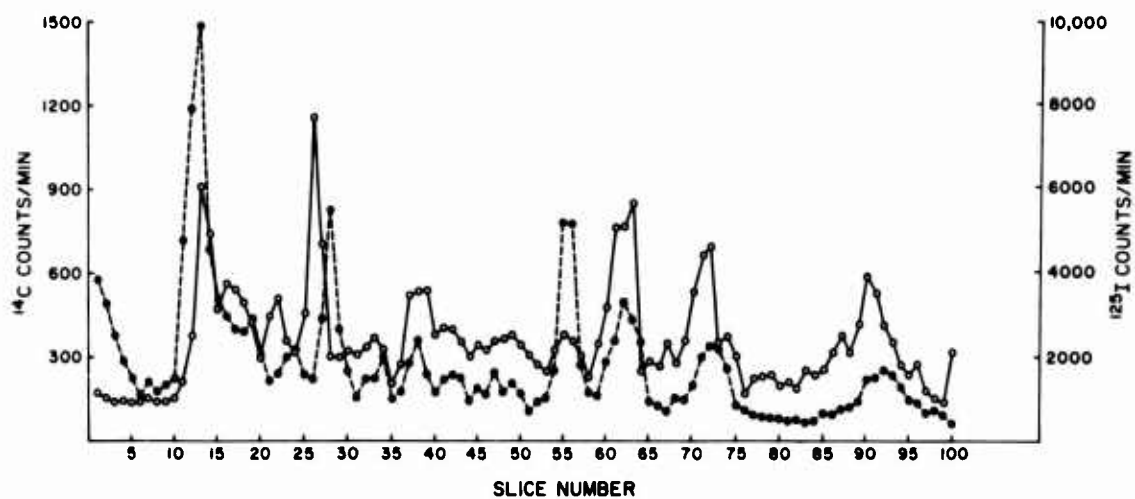


FIG. 5 Superimposed electropherograms of <sup>14</sup>C amino acid labeled *R. prowazekii* (●----●) and <sup>125</sup>I labeled (50 μM) *R. prowazekii* (o—o).

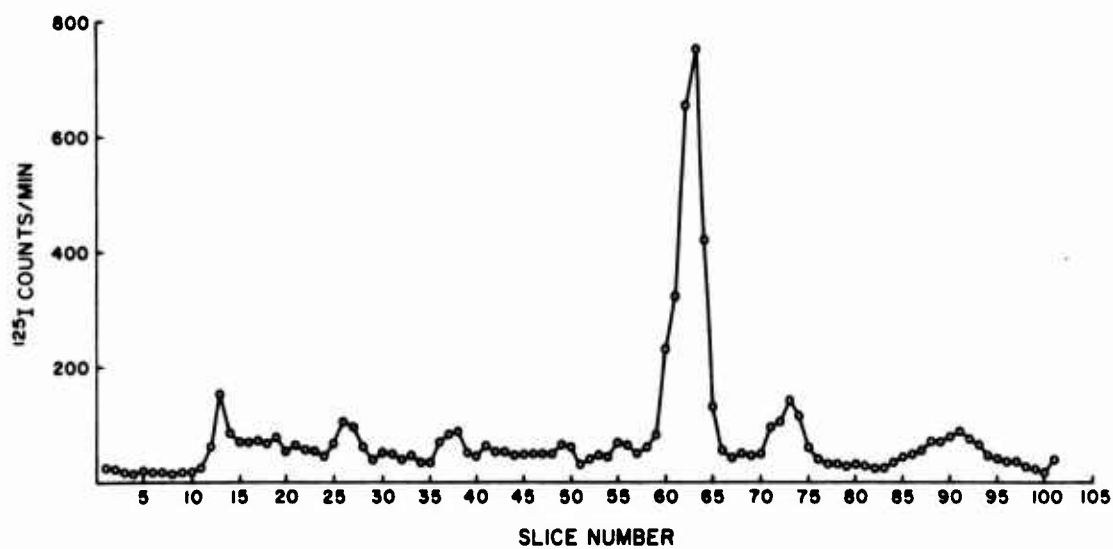


FIG. 6 Electrophoresis of peripherally labeled <sup>125</sup>I (0.5 μM) *R. prowazekii*.

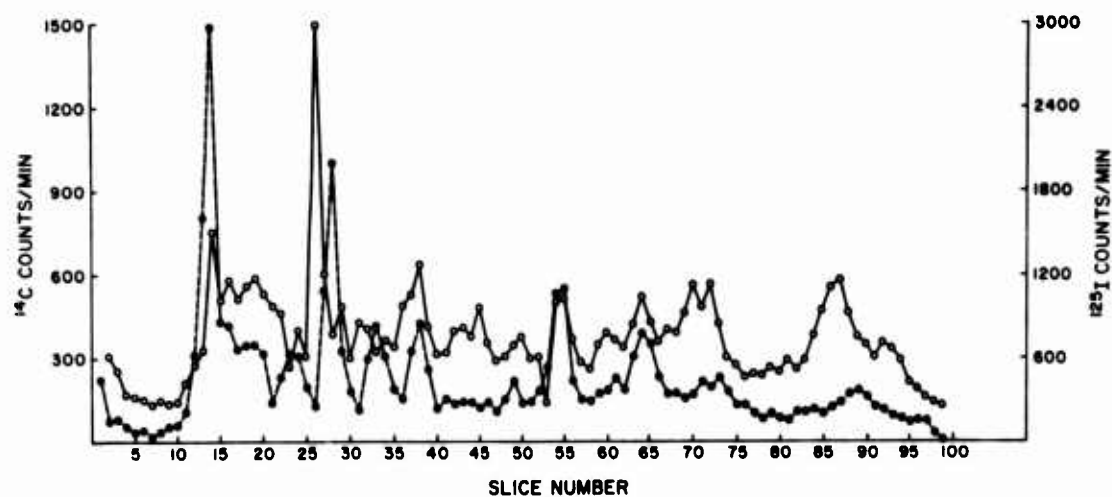


FIG. 7 Superimposed electropherograms of <sup>14</sup>C amino acid labeled *R. conorii* (●----●) and <sup>125</sup>I labeled (50 μM) *R. conorii* (○—○).

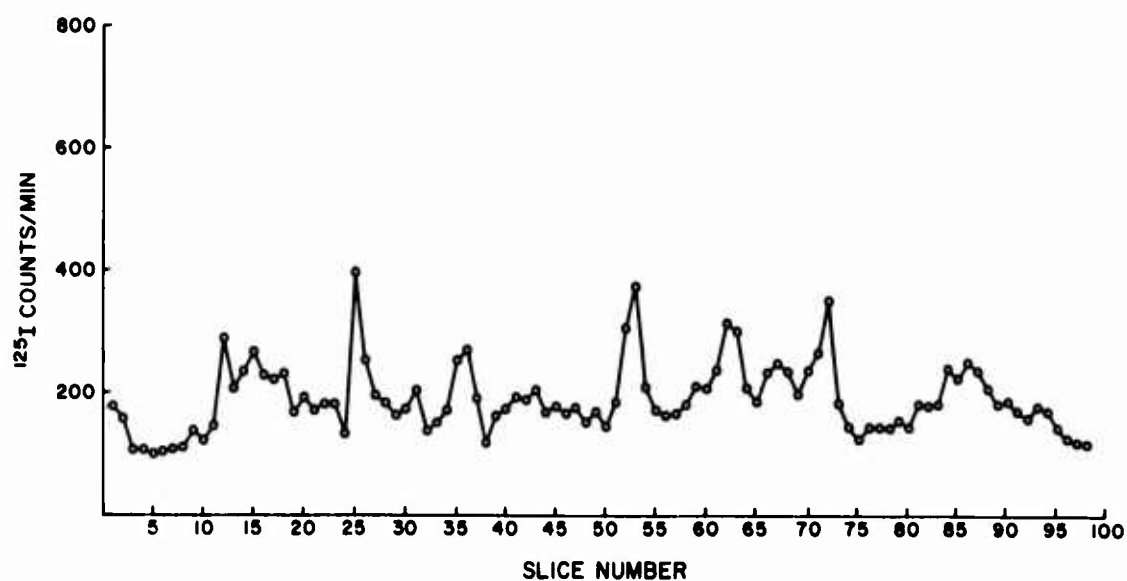


FIG. 8 Electrophoresis of <sup>125</sup>I (0.5 μM) *R. conorii*, demonstrating the lack of specific labeling at low iodide concentration.

buoyant densities, do not represent the same fractions of the rickettsial cell. Ether-extracted organisms retain the intact structure of whole cells, can be pelleted from the supporting menstruum, and electrophoretically resemble unextracted whole cells (Fig. 10). ESS, although exhibiting the same density as whole organisms, appears in gel electropherograms as a mixture of low molecular weight protein fractions (Fig. 11), devoid of any recognizable proteins of the whole cell. The radioactive ESS peak in density gradients may represent cell wall fragments of various sizes and molecular weights, but of the same density as the whole organism.

#### B. Studies on the purification of scrub typhus group organisms and application of model systems.

Scrub typhus group rickettsiae, grown in either yolk-sacs or cell culture, exhibit unusual adherence to host cell material and are not readily isolated by conventional methods. The various physical and chemical techniques utilized in this laboratory have included differential centrifugation; sonic disruption; treatment with EDTA, NaCl, Amphotericin B; the ion-exchange resins IRF and Cellite; the detergents Triton X-100 and Sarkosyl; the enzymes trypsin, pronase, phospholipase C, and neuraminidase; centrifugation on sucrose velocity gradients and on sucrose, renografin, cesium chloride and potassium tartrate-glycerol density gradients; and chromatography on Ecteola, Sepharose 4B and Con A-sepharose 4B columns. None of these techniques either alone or in combination yielded highly purified scrub typhus organisms, as indicated by L-929 cell activity in complement fixation or immunofluorescence tests.

Analysis of scrub typhus macromolecules is facilitated by, although not dependent upon, purification of these organisms from the host cell. Data obtained from previous studies with typhus and spotted fever group rickettsiae enabled us to intrinsically label scrub typhus group organisms while inhibiting the incorporation of isotope by the host cell.

Scrub typhus group organisms (strains Karp and Gilliam) were grown in spinner cultures of gamma-irradiated, cycloheximide-inhibited, L-929 cells, as previously described for typhus and spotted fever group rickettsiae (1). Cultures were maintained for 5-7 days (until the cells were maximally filled with organisms), then were homogenized, and subjected to differential centrifugation. The final high speed centrifugation was increased from 5,000 to 12,000 x g for 1 hr at 4C, because of the small size of R. tsutsugamushi.

The rickettsiae and L-929 cell material in the pellet were resuspended to 1 ml with TEN buffer, pH 7.5, mixed with an equal volume of 2M NaCl in distilled water, layered on a 4.3 x 5.5 cm Sepharose 4B column, and eluted with 2M NaCl in distilled water. Fractions (250  $\mu$ l) were collected

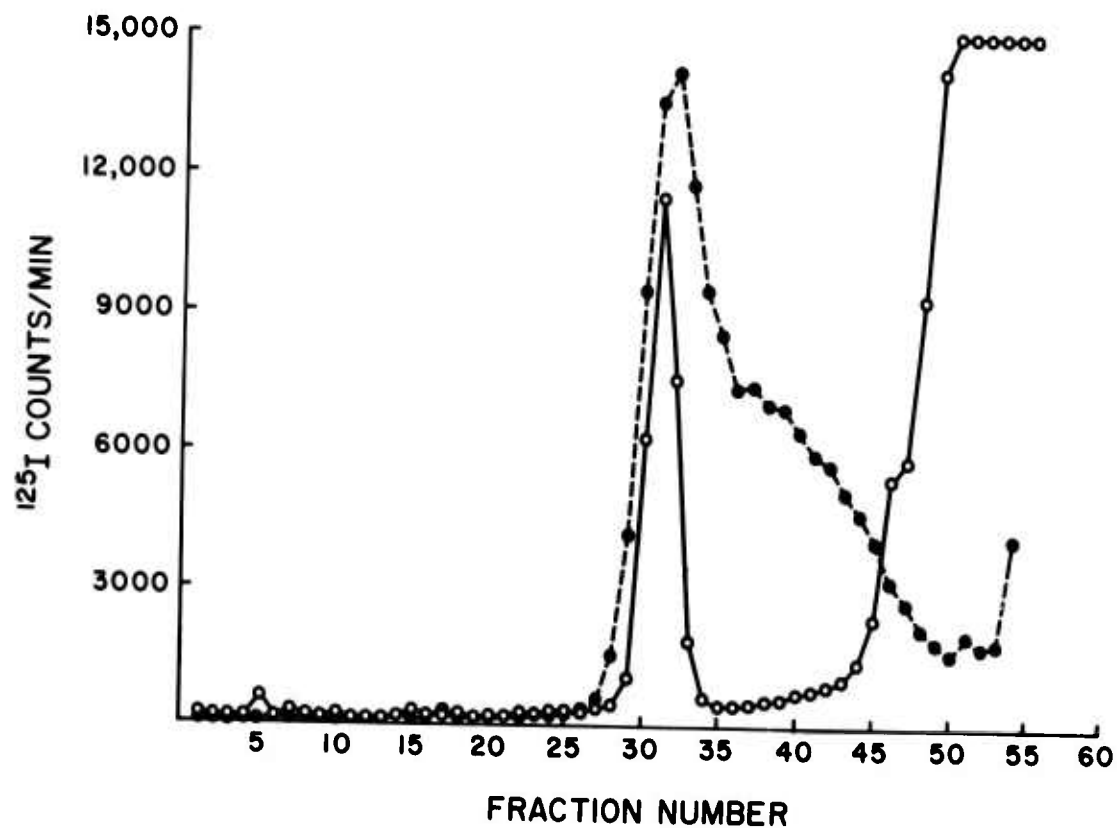


FIG. 9 Superimposed potassium tartrate-glycerol density gradients of  $^{125}\text{I}$  *R. prowazekii* ether extract (●----●) and  $^{125}\text{I}$  *R. prowazekii* ESS (o—o).

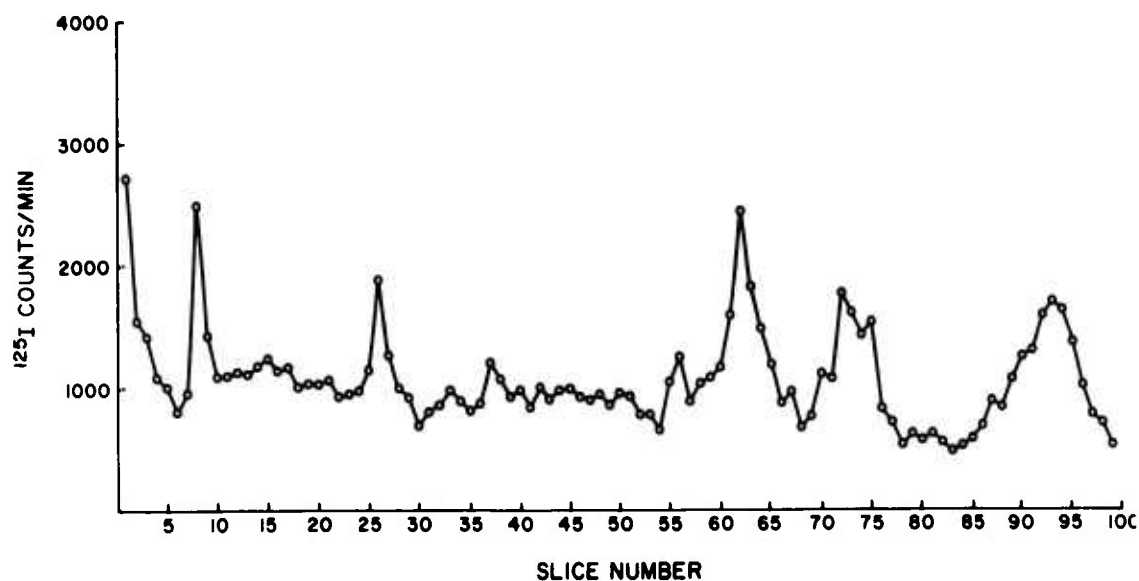


FIG. 10 Electrophoresis of ether extracted  $^{125}\text{I}$  *R. prowazekii*.

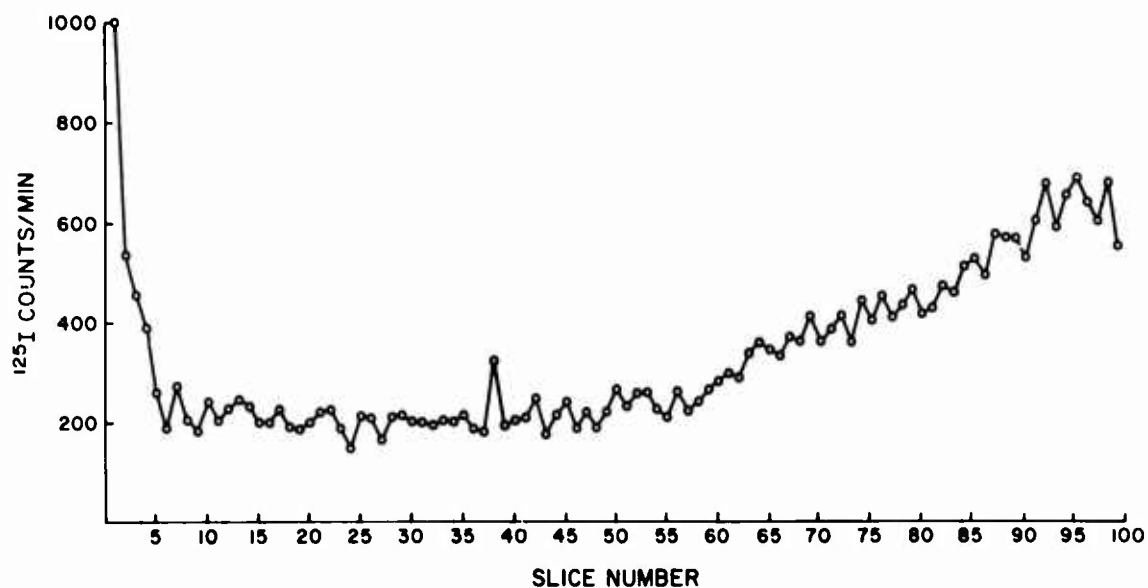


FIG. 11 Electrophoresis of  $^{125}\text{I}$  ESS extracted from *R. prowazekii*.

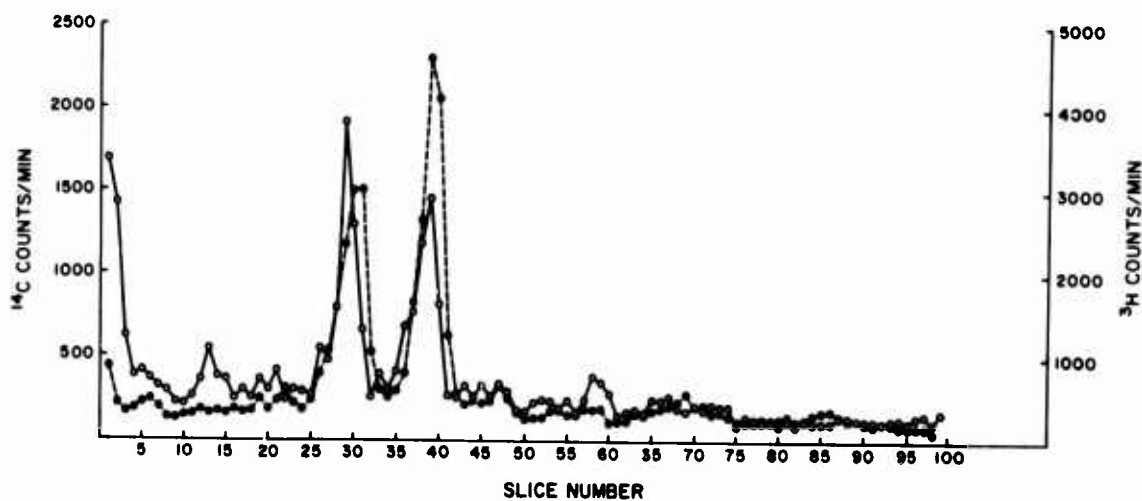


FIG. 12 Superimposed electropherograms of  $^{14}\text{C}$  amino acid labeled Karp strain (●---●) and  $^3\text{H}$  amino acid labeled Gilliam strain (o—o) of *R. tsutsugamushi*.

with an Isco model 328 fraction collector at 25C, and aliquots were counted after drying on glass fiber filters. Peak radioactive fractions were pooled and centrifuged at 57,700 x g for 1 hr at 4C. Pellets were resuspended in a small volume of TEN buffer, shell-frozen in dry ice-95% ethanol, and stored at -70C until use.

Suspensions of R. tsutsugamushi purified by these techniques were analyzed by complement fixation and immunofluorescence tests and were found to be still substantially contaminated by L-929 cell material. However, the intrinsically labeled, partially purified organisms were free of enough unlabeled L-929 cell protein that analysis on SDS-polyacrylamide gels was possible.

Fig. 12 shows superimposed electropherograms of <sup>14</sup>C amino acid labeled Karp strain and <sup>3</sup>H amino acid labeled Gilliam strain of R. tsutsugamushi. The profiles are strikingly different from the complex electrophoretic profiles of other rickettsial organisms. Two proteins of approximately 54,000 and 44,000 daltons are common to both strains and appear to be specific to the scrub typhus group of rickettsiae. Further investigation of these two isolated proteins may elucidate their antigenic and immunogenic potentials in the animal host.

II. The effect of the genetic background of mice on the virulence of scrub typhus strains.

A. Survey of inbred and random bred mouse strains for Gilliam susceptibility.

The laboratory mouse has been used more extensively than any other animal in the study of scrub typhus infections. When R. tsutsugamushi strains are inoculated intraperitoneally (IP) in mice, the observed death patterns can generally be categorized as either "susceptible", "resistant", or "selectively resistant". Susceptible patterns are typified by IP titrations with the Karp strain and are characterized by less than one log<sub>10</sub> difference between the lethal dose 50% (LD<sub>50</sub>) and the infectious dose 50% (ID<sub>50</sub>). Conversely, the resistant patterns show a 4 log<sub>10</sub> or greater difference between LD<sub>50</sub> and ID<sub>50</sub>. Titrations of the Gilliam strain in BALB/c inbred mice characterize this pattern. The third, and most intriguing, pattern is the selectively resistant type. The Gilliam titration in outbred ICR mice shown in Table 2 illustrates this pattern. Deaths and survivors occur in almost every dilution throughout the titration range. One explanation for this erratic pattern is that mouse susceptibility to various scrub typhus strains can be genetically determined. That is to say, mice which are genetically predetermined to be susceptible die when infected IP with only a few organisms while genetically resistant ones survive the IP inoculation of extremely large numbers of organisms. We describe here a survey of

several mouse strains for susceptibility to Gilliam infection and a more detailed study of some of the characteristics of one of these strains.

Currently, 10 inbred and 6 random bred strains have been studied for susceptibility to IP Gilliam infection. Karp susceptibility was also tested, but over a more limited dose range. All inoculums were 0.2 ml. Survivors of Gilliam and Karp titrations were back challenged with a lethal  $10^3$  LD<sub>50</sub> Karp challenge. If animals survived back challenge, they were considered to have been infected but survived the original challenge, and the results used to calculate the ID<sub>50</sub>.

The Gilliam titrations in the inbred mouse strains are summarized in Table 3. Five strains; the SWR, AKR, C57B1/6, and two BALB/c, exhibited a typical resistant pattern. The DBA, CBA, and 2C3H/He strains, on the other hand, were remarkably sensitive to Gilliam infection. These strains showed a uniform death pattern which was dose dependent and, with one exception, had an LD<sub>50</sub> which matched the ID<sub>50</sub>. This latter observation indicates that less than 10 organisms per mouse are lethal. The one unexpected finding was the selectively resistant response observed in the A mice. Considering the degree of genetic homogeneity of inbred strains, this erratic pattern seems unusual.

In contrast to the inbred mice, one-half of the random bred strains exhibited the selectively resistant death pattern to Gilliam challenge (Table 4). Two strains, CD<sub>1</sub> and CF<sub>1</sub>, were uniformly resistant to Gilliam. Only the Swiss Webster titration was characterized as a susceptible death pattern. Interestingly, the resistant CF<sub>1</sub> mice are, according to the breeder's literature, also Swiss Webster mice. This finding may not be unusual, however, since most commercial colonies were begun from a small number of breeders and are maintained as a closed colony. Depending on the degree of inbreeding, selection pressures, and genetic makeup of the original breeding stock, considerable homogeneity of certain characteristics can be expected in random bred mice.

Only the SWR strain evidenced any apparent resistance to the Karp challenge. Two of 15 mice survived the original challenge and subsequent back challenge. This strain also had a longer "average day of death" time than other mouse strains challenged in the same experiment (Table 5). Additional studies on the susceptibility of SWR mice to Karp are currently in progress.

#### B. Response of C3H/He mice to R. tsutsugamushi.

The inbred C3H/He strain was selected for extensive studies because of its sensitivity to Gilliam infection and its widespread use in biological research. The susceptibility of C3H mice to two strains of R. tsutsugamushi, Kostival and TA678,, which do not produce sensitive death patterns in ICR

mice was tested. A summary of the Kostival and TA678 titrations in C3H/He and BALB/c mice is shown in Table 6. C3H/He mice responded to a graded Kostival infection with a susceptible death pattern, but were resistant to TA678 infection. The BALB/c mice were also resistant to TA678. Interestingly, the BALB/c strain response to Kostival infection was a selectively resistant one similar to the pattern observed when Kostival is titrated in outbred ICR mice. Only one other inbred strain, A mice infected with Gilliam, has shown a selectively resistant pattern.

The ability of C3H/He mice to respond immunologically to Gilliam was evaluated. Mice were vaccinated with potentially lethal  $10^3$  and  $10^4$  LD<sub>50</sub> Gilliam challenges (LD<sub>50</sub> calculated in C3H/He mice inoculated IP) by one of two methods. In one vaccination procedure, mice were given chloramphenicol (2.5 mg/ml) ad lib in their drinking water from the third through the seventeenth day post Gilliam inoculation. Chloramphenicol is a bacteriostatic drug which apparently prevents the growth of scrub typhus organisms and allows the animal to establish a protective immunity. The alternate vaccination procedure employed subcutaneous inoculation of the Gilliam vaccine dose. This method uses to advantage the findings of Japanese investigators who demonstrated that mice can survive subcutaneous inoculation of several times the lethal IP dose. Both vaccine groups were challenged with either homologous Gilliam or heterologous Karp organisms on day 28 post vaccination with virulent Gilliam. Results of challenge indicate C3H/He mice are capable of responding immunologically to Gilliam infection (Table 7). Mice inoculated with the lowest vaccination dosage of  $10^3$  LD<sub>50</sub> were solidly protected against lethal IP Gilliam and Karp challenges of  $10^3$  LD<sub>50</sub>.

Table 2. IP Gilliam titration in ICR mice

Infected yolk sac dilution	Death/Total	Average Day of Death
$10^{-3}$	4/5	11.0
$10^{-4}$	3/5	11.6
$10^{-5}$	3/5	13.6
$10^{-6}$	5/5	12.4
$10^{-7}$	2/5	14.5
$10^{-8}$	4/5	14.7
$10^{-9}$	1/5	15.0



Table 3. IP Gilliam titration in inbred mice

Mouse strain	LD <sub>50</sub> dilution/gm yolk sac	ID <sub>50</sub> dilution/gm yolk sac	ID <sub>50</sub> - LD <sub>50</sub>	Death pattern
A <sup>a</sup>	10 <sup>-6.7</sup>	10 <sup>-8.5</sup>	10 <sup>-1.8</sup>	Selectively resistant
BALB/c <sup>a</sup>	< 10 <sup>-3.7</sup>	10 <sup>-7.7</sup>	> 10 <sup>-4.0</sup>	Resistant
BALB/c <sup>b</sup>	< 10 <sup>-3.0</sup>	10 <sup>-7.4</sup>	> 10 <sup>-4.4</sup>	Resistant
C3H/He <sup>a</sup>	10 <sup>-9.0</sup>	10 <sup>-9.0</sup>	10 <sup>0</sup>	Sensitive
C3H/He <sup>b</sup>	10 <sup>-8.8</sup>	10 <sup>-9.2</sup>	10 <sup>-0.4</sup>	Sensitive
SWR <sup>a</sup>	< 10 <sup>-3.7</sup>	10 <sup>-8.2</sup>	> 10 <sup>-4.5</sup>	Resistant
AKR <sup>a</sup>	10 <sup>-3.9</sup>	10 <sup>-8.0</sup>	10 <sup>-4.1</sup>	Resistant
DBA <sup>a</sup>	10 <sup>-8.4</sup>	10 <sup>-8.4</sup>	10 <sup>0</sup>	Sensitive
CBA <sup>a</sup>	10 <sup>-8.4</sup>	10 <sup>-8.4</sup>	10 <sup>0</sup>	Sensitive
C57B1/6 <sup>a</sup>	< 10 <sup>-3.0</sup>	10 <sup>-7.5</sup>	> 10 <sup>-4.5</sup>	Resistant

<sup>a</sup> Jackson Laboratories  
<sup>b</sup> Flow Laboratories

Table 4. IP Gilliam titration in random bred mice

Mouse strain	LD <sub>50</sub> dilution/gm yolk sac	ID <sub>50</sub> dilution/gm yolk sac	ID <sub>50</sub> - LD <sub>50</sub>	Death pattern
ICR <sup>a</sup>	10 <sup>-6.1</sup>	10 <sup>-8.1</sup>	10 <sup>-2.0</sup>	Selectively resistant
ICR <sup>b</sup>	10 <sup>-4.1</sup>	10 <sup>-6.2</sup>	10 <sup>-2.1</sup>	Selectively resistant
Swiss Webster <sup>c</sup>	10 <sup>-8.5</sup>	10 <sup>-8.9</sup>	10 <sup>-0.4</sup>	Sensitive
CD <sub>1</sub> <sup>d</sup>	< 10 <sup>-3.7</sup>	10 <sup>-9.5</sup>	> 10 <sup>-5.8</sup>	Resistant
CF <sub>1</sub> <sup>d</sup>	< 10 <sup>-3.7</sup>	10 <sup>-9.5</sup>	> 10 <sup>-5.8</sup>	Resistant
CF <sub>w</sub> <sup>d</sup>	10 <sup>-8.1</sup>	10 <sup>-9.7</sup>	10 <sup>-1.6</sup>	Selectively resistant

<sup>a</sup> WRAIR  
<sup>b</sup> Flow Laboratories  
<sup>c</sup> Microbiological Associates  
<sup>d</sup> Charles River

Table 5. Comparison of Karp challenge in SWR and 5 other mouse strains

Mouse strain	10 <sup>1</sup> LD <sub>50</sub> Karp		10 <sup>2</sup> LD <sub>50</sub> Karp		10 <sup>3</sup> LD <sub>50</sub> Karp	
	Survivors/Total	Average Day of Death	Survivors/Total	Average Day of Death	Survivors/Total	Average Day of Death
SWR	0/5	18.2	<sup>a</sup> 1/5	15.3	<sup>a</sup> 1/5	14.5
A	0/5	13.8	0/5	12.6	0/5	10.8
BALB/c	0/5	14.3	0/5	11.6	0/5	10.7
C3H/He	<sup>b</sup> 1/5	13.5	0/5	13.4	0/5	11.4
Swiss Webster	0/5	17.2	0/5	14.2	0/5	12.2
ICR	0/5	16.2	0/5	14.8	0/5	12.4

<sup>a</sup> Survived back challenge with 10<sup>3</sup> LD<sub>50</sub> Karp.

<sup>b</sup> Died on back challenge with 10<sup>3</sup> LD<sub>50</sub> Karp.

Table 6. Results of Kostival and TA678 titrations in C3H/He and BALB/c mice

<u>R. tsutsugamushi</u> strain	Mouse strain	LD 50 dilution/gm yolk sac	ID 50 dilution/gm yolk sac	ID - LD 50 50	Death pattern
Kostival	C3H/He	$10^{-8.7}$	$10^{-9.3}$	$10^{-0.6}$	Sensitive
	BALB/c	$10^{-4.7}$	$10^{-8.0}$	$10^{-3.7}$	Selectively resistant
TA678	C3H/He	$< 10^{-1.7}$	$10^{-7.5}$	$> 10^{-5.8}$	Resistant
	BALB/c	$< 10^{-1.7}$	$10^{-7.3}$	$> 10^{-5.6}$	Resistant

Table 7. Gilliam and Karp challenge of Gilliam vaccinated C3H/He mice

Vaccine Group	Gilliam challenge (Survivors/total)			Karp challenge (Survivors/total)		
	$10^1$ LD <sub>50</sub>	$10^2$ LD <sub>50</sub>	$10^3$ LD <sub>50</sub>	$10^1$ LD <sub>50</sub>	$10^2$ LD <sub>50</sub>	$10^3$ LD <sub>50</sub>
<u>Chloramphenicol:</u>						
$10^3$ LD <sub>50</sub> Gilliam	5/5	5/5	6/6	5/5	4/5	6/6
$10^4$ LD <sub>50</sub> Gilliam	5/5	6/6	6/6	5/5	6/6	6/6
<u>Subcutaneous:</u>						
$10^3$ LD <sub>50</sub> Gilliam	5/5	5/5	5/5	5/5	5/5	5/5
$10^4$ LD <sub>50</sub> Gilliam	4/4	4/4	5/5	4/4	4/4	5/5
<u>Unvaccinated:</u>						
Controls	0/5	0/5	0/5	0/5	0/5	0/5

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

#### A. Chemical mutagenesis of rickettsiae.

The previous annual report described in detail the purpose and scope of this project and presented some of the work done in the developmental stages. Since that report, over 1,200 plaques from nitrous acid-treated Rickettsia conorii suspensions have been screened in an effort to isolate temperature sensitive mutants. The procedure has not been successful.

There are several possible reasons for the inability to induce mutants with nitrous acid under the present experimental conditions:

1. The concentration of nitrous acid might be too low. Studies with bacteria such as Escherichia coli or Bacillus subtilis have used a wide range of molarities, most of which were higher than that used in our study.

2. The number of plaques screened may have been insufficient to detect mutants; at least 12 temperature sensitive mutants should have been detected at a 1% mutation rate. It is conceivable that the mutation rate is not as high as originally predicted.

3. The time of treatment with nitrous acid may not be sufficient to achieve mutagenesis. The amount of inactivation achieved in other studies appears to correlate more with mutagenicity than does time of treatment.

4. The rickettsiae may be forming aggregates, a problem detected by investigators working with viral mutagenesis. Aggregations of mutant and non-mutant rickettsiae would result in the formation of plaques at the non-permissive temperature, where normally the mutant alone would not plaque. The use of filtration through membrane filters has been used by others in viral studies with success.

The effect of the concentration of nitrous acid on the inactivation of R. conorii was investigated. Fig. 13 demonstrates the relationship of nitrous acid concentration to rickettsial inactivation, as measured by plaque assay in gamma-irradiated L-929 monolayers. These data were developed using yolk-sac propagated rickettsiae treated for 20 minutes at 4 C. It appeared from these results that a concentration of 0.2 to 0.25 M could be utilized. As will be noted later, however, this was not possible when the rickettsial suspension had been propagated in cell culture, because these preparations were rapidly inactivated (Fig. 14). Approximately 650 plaques were processed utilizing the higher concentration of nitrous acid (0.2 M) without success.

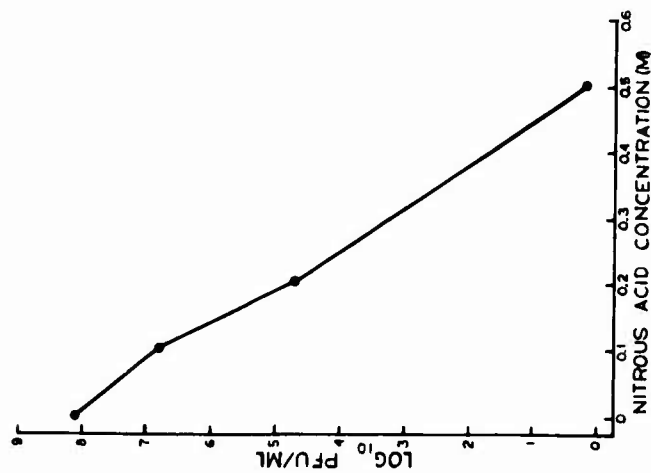


FIG. 13 Effect of nitrous acid concentration on the inactivation of yolk-sac(YS) propagated R. conorii treated for 20 minutes at 4C.

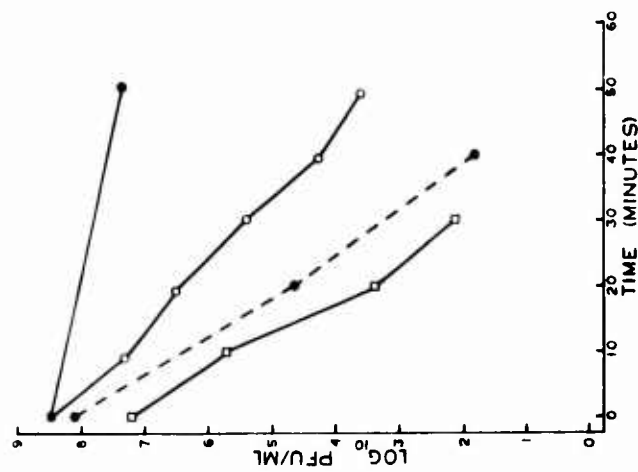


FIG. 14 Effect of time of treatment and nitrous acid concentration on the inactivation of cell culture(TC) and YS propagated R. conorii.  
 Untreated YS R. conorii held at 26C  
 0.1 M nitrous acid-treated YS R. conorii  
 0.2 M nitrous acid-treated YS R. conorii  
 0.1 M nitrous acid-treated TC R. conorii

The possibility that rickettsial aggregation is precluding identification of mutants is presently under investigation. Membrane filtration is employed to filter out aggregates.

The use of filtration created a need for cell culture propagated R. conorii, as the yolk-sac debris present in the standard suspensions clogged the membrane filters. Several lots of plaque-purified R. conorii were grown in L-929 cells at 37 C, centrifuged, resuspended in sucrose-phosphate-glutamate buffer and blended prior to quick-freezing in a 95% ethanol-dry ice bath. These suspensions were then plaque-titered prior to use.

Membrane filters of 0.45, 0.60, 0.80, and 1.2  $\mu$ m pore size were evaluated for their retention of rickettsiae. The 0.8  $\mu$ m pore size was selected since it allowed passage of R. conorii, yet did filter out aggregates as evidenced by a reduction in titer. A typical decrease in rickettsial titer after filtration through a 0.8  $\mu$ m membrane filter was from  $7.4 \times 10^7$  plaque forming units (PFU)/ml to  $3.5 \times 10^4$  PFU/ml; a loss of greater than 99.9% of the rickettsial PFUs.

As previously noted, the higher 0.2 M nitrous acid concentration could not be utilized with cell culture propagated rickettsiae as it resulted in rapid, total inactivation. The concentration was lowered to 0.1 M and the incubation period reduced to 15 minutes. This modified technique provided a suitable rate of rickettsial inactivation.

Investigations have also been initiated using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as a mutagenic agent for rickettsiae. Initial experiments performed using MNNG at a concentration of 200  $\mu$ g/ml and allowing a 15 minute contact with the rickettsial suspension at room temperature resulted in a decrease of greater than 4  $\log_{10}$  units ( $10^8$  to  $10^{3.3}$  PFU/ml). Control experiments showed that inactivation due to the lability of rickettsiae held at room temperature for a similar period is approximately 0.3  $\log_{10}$  units.

#### B. Mutagenesis by ionizing radiation.

The use of gamma-irradiation as a potential mutagen is also being evaluated. Fig. 15 illustrates the effect of gamma radiation on the titer of yolk-sac propagated R. conorii. Six hundred plaques from gamma-irradiated suspensions of yolk-sac propagated R. conorii have been screened with negative results. Mutagenesis experiments with cell culture grown organisms are currently in progress, using a gamma-irradiation dose of 150 Krads and incorporating the filtration modification.



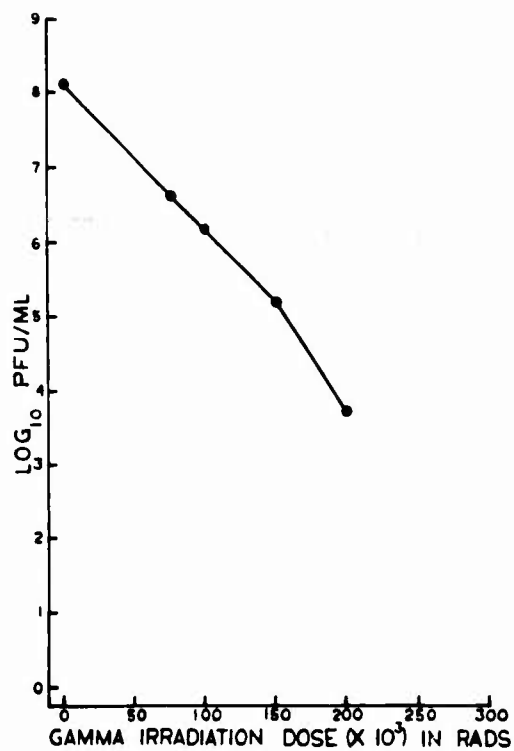


FIG. 15 Effect of gamma irradiation dose on the inactivation of YS propagated R. conorii.

#### IV. Growth of rickettsiae in mutant cell lines.

The basis for the obligate intracellular parasitism of rickettsiae is not known, but a detailed knowledge of the rickettsiae-host cell relationship is important because this information could conceivably lead to the acellular growth of rickettsiae.

The purpose of this investigation was to study the basis for rickettsial obligate intracellular parasitism by evaluating the growth characteristics of R. conorii in various mutant cell lines. In the past several years, a large number of natural and chemically-induced mutants of cell lines of human and animal origin have been isolated and often are well characterized as to the specific genetic defect. If a cell line can be found in which the rickettsiae do not proliferate or do so at a noticeably slower rate than controls, the specific defect in host cell metabolism might lead to an understanding of the basis for the obligate intracellular existence of rickettsiae.

At the present time, five well characterized mutant cell lines have been utilized for growth experiments with R. conorii. All five have been shown to support rickettsial growth at least as well as the control cell line, L-929. The five cell lines are:

a. XP-12BE (Jay Tim) - This cell line was developed from human skin fibroblast cells from a patient with xeroderma pigmentosum, a genetic disease in which the cells are defective in the repair of ultraviolet-induced cellular DNA damage.

b. Amdur II - These cells exhibit a deficiency in the conversion of methylmalonyl CoA to succinyl CoA, resulting in a condition known as methylmalonicacidemia.

c. CHP#3 - This cell line was from a patient with symptomatic galactosemia; a deficiency of UDP-galactose transferase.

d. LaBel - These cells are from a patient with a connective tissue disease, the Ehlers-Danlos syndrome, in which there appears to be a defective organization of collagen bundles into an intermeshing network. The molecular and genetic basis for this defect is not yet known.

e. On Ser - The defect in this cell line is in uric acid metabolism; there is a deficiency of hypoxanthine-guanine phosphoribosyl transferase.

f. Y1-13 - This cell line, derived by ethyl methane sulfonate mutagenesis of Chinese hamster ovary cells, is deficient in glucose-6-phosphate dehydrogenase.

Other mutant cell lines will be tested for their ability to support rickettsial growth.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 197 Rickettsial Genetics

Literature Cited.

References:

1. Eisemann, C.S., and Osterman, J.V. Proteins of typhus and spotted fever group rickettsiae. Infect. Immunity 00: 000-000, 1976 (accepted for publication).
2. Phillips, D.R., and Morrison, M. The arrangement of proteins in the human erythrocyte membrane. Biochem. Biophys. Res. Commun. 40: 284-289, 1970.
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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 6514	76 06 30	DD-DR&E(AR)636	
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B. CONTRIBUTING							
C. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Spin Labeling of Biomolecules							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002300 Biochemistry 008300 Inorganic Chemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		76 06		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		3	
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C. TYPE:				CURRENT		1	
D. KIND OF AWARD:				76		60	
E. 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				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME: Demaree, LTC G.E.			
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., Ph.D.			
				NAME: Copeland, E.S., Ph.D. 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, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede rest 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) 75 07 - 76 06 The following four projects were successfully completed. (1) Light-induced leakage of spin label marker from liposomes in the presence of phototoxic phenothiazines (2) phototoxic aspects of phenothiazine retinopathy. An electroretinographic evaluation of NP207 feline retinal damage in the absence and presence of UV light (3) an ESR study of secondary radical reactions in an irradiated sulfur containing glycoprotein and (4) electron spin resonance study of the synaptosome opiate receptor. Kinetics of stereo-specific binding of spin labeled morphine. For terminal technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

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

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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 198 Spin labeling of biomolecules

Investigators.

Principal: Gale E. Demaree, MSC

Associate: Edmund S. Copeland, Ph.D.; Carl R. Alving, M.D.;  
Marie M. Grenan, B.S.; Kenneth K. Kramer, M.D.;  
Larissa deBaare, M.D.; Bhupendra P. Doctor, Ph.D.

Four independent yet theoretically related projects have been conducted during the final year of this in-house laboratory independent research work unit. The first used spin label markers trapped within liposomes to study the mechanism of phototoxic phenothiazine mediated membrane damage. Concurrent studies in mice showed that those phenothiazines which had the greatest phototoxic activity in liposomal membranes also had the greatest phototoxic potency in the mammal. One finding of the study, that the phenothiazine, NP207, was markedly more phototoxic in both systems than structurally similar thioridazine, suggested phototoxicity might be involved in the severe retinal toxicity of the former compound.

Liposomes prepared from dipalmitoyl lecithin, cholesterol and dicetyl phosphate and containing a trapped spin label marker were exposed to longwave ultraviolet light in the presence of a series of phenothiazine tranquilizers. Electron spin resonance spectroscopy was used to detect spin label marker released from liposomes, taking advantage of the disappearance of line broadening from electron spin exchange which occurred on spin label release. The minimum effective phototoxic dose in mice of these phenothiazines was also determined. Kinetic studies of light-induced spin label release from phenothiazine-sensitized liposomes showed that membrane damage was rapidly induced and that the damaging species were short-lived. The damage process was oxygen dependent and could be temporarily prevented by cysteamine or  $\alpha$ -tocopherol added immediately before irradiation. Only those phenothiazines which mediated light-dependent liposomal membrane damage had phototoxic activity in mice and the degree of photosensitization was parallel in the two systems. In both photosensitization phenomena, the nature of the substituent at the phenothiazine 2-position was more important than the phenothiazine side chain. The data indicate that membrane lipids may be a part of the "biological target" in phenothiazine phototoxicity.

The second study was designed to determine whether there was a role of light in the development of NP207 toxic retinopathy.

Electroretinography was used to monitor retinal function during the course of NP207 administration to a cat one of whose eyes was exposed to UV light while the other was shielded from it. There was no difference in the development of retinopathic electroretinogram extinction between the two eyes. It was concluded that NP207 retinopathy is not light-dependent. These two studies with phenothiazines were conducted as prototypes for future studies with phototoxic antimalarial drugs (e.g., quinolinemethanols) for which the literature in this field is considerably less ample.

To test the hypothesis that phenothiazine retinopathy is in part a light-dependent phenomenon, we have evaluated by electroretinography the retinal function of both eyes of a cat for six months. The left eye was exposed to near ultraviolet light for two months at an irradiance of about  $1 \text{ mW/cm}^2$  while the right eye was shielded from light by an opaque contact lens. NP207 (piperidylchlorophenothiazine) was administered orally in daily doses which were gradually increased to 60 mg/kg. The B-wave of the electroretinogram (ERG) of each eye decreased to approximately one half of the pre-treatment mean value after 8 days of NP207 treatment and both ERG's were completely extinguished after 60 days of treatment. Both ERG's reappeared after cessation of NP207 administration but were less than half the pre-treatment mean value 48 days later. There was no demonstrable difference between the two eyes in the time of onset or in the extent of phenothiazine retinopathy. We conclude that NP207 retinopathy is not light-dependent.

The third study represents a continuation of earlier work which postulated a relation between the loss of enzymatic activity in irradiated ribonuclease (RNase) and the formation of secondary organosulfur radicals on its disulfide bonds. To learn whether the observed similarity in energy of activation for enzyme inactivation and for formation of secondary radicals was limited to RNase or was more generalized, similar studies were conducted with the glycoprotein enzyme, invertase. The results showed only that the free radical formation activation energy could be equal to that of enzyme inactivation but neither proved an equality nor identified the secondary radical formed in invertase. No organosulfur radicals were observed although the glycoprotein has five half-cystinyl residues per molecule.

Solid yeast invertase has been irradiated in vacuum at  $77^\circ \text{K}$  and subsequently heated. The kinetics of formation of a secondary radical have been studied by following its growth at different temperatures. Progressive power saturation studies have been applied to determine  $(T_1 T_2)^{1/2}$  for the primary and secondary radical populations.

It is shown that two different carbon radical populations constitute the primary and secondary species. Thus,  $(T_1 T_2)^{1/2}$  measured at  $77^\circ \text{K}$

increased from 1.0  $\mu$ sec to 1.7  $\mu$ sec as a result of secondary radical reactions. Both the primary and secondary radical populations have g-factors characteristic of carbon radicals. No evidence for organosulfur radicals was found for the cystine-containing glycoprotein, invertase. There was evidence for radical transformation in a period of little total radical decay. The principal secondary radical was formed with apparent activation energies of about 12 and 17 kcal/mole and are interpreted as upper limits of the true secondary radical energies of activation.

The fourth study has proven to be the terminal investigation of a series related to interaction of opiates with receptors on synaptosomes of the central nervous system. It is an extension of previous work using spin labeled morphine (SLM) to investigate opiate ligand binding with synaptosomal receptors. We have shown using displacement techniques that this binding is stereospecific for less than five per cent of the total ligand bound. We observed a kinetic binding curve characteristic of an allosteric interaction which varied with the concentration of added opiate agonist or antagonist and was temperature dependent.

Morphine, spin labeled on the 3- or 6- position has been used as the opiate ligand in a study of the time course of stereospecific opiate binding to intact synaptosomes isolated from non-cerebellar rat brain. The broadening of electron spin resonance lines induced by immobilization of the ligand on binding has been used to determine the concentration of bound opiate. The stereospecificity of the reaction was measured by comparing ligand binding in the presence of thousand fold molar excesses of dextrorphan or levorphanol. Using both static and flow techniques, the binding process has been continuously monitored at times greater than 4.8 seconds after mixing spin labeled morphine with synaptosomes. It is shown that for this ligand and receptor preparation, binding takes place primarily during a delayed, abrupt process whose rate and time of onset are temperature dependent and reflect the presence of added opiate agonist or antagonist.



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. Copeland, E.S., Alving, C.R., and Grenan, M.M.: Light-induced leakage of spin label marker from liposomes in the presence of photo-toxic phenothiazines. Photochem. Photobiol. 24: 41-48, 1976.



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)636	
3. DATE PREV SUMMARY <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTR <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM <sup>a</sup>
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11. PRIMARY						200	
12. CONTRIBUTING							
13. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Cardiac Performance in By-pass Surgery							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>a</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				B. PREVIOUS		C. FUNDS (in thousands)	
B. NUMBER <sup>a</sup>				FISCAL YEAR		2	
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E. 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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				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursuit SSAN if U.S. Academic Institution)			
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TELEPHONE: 202 576-3551				TELEPHONE: 202 576-3795			
				SOCIAL SECURITY ACCOUNT NUMBER			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Pfuetze, LTC K. D.			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>a</sup>							
(U) Myocardial Metabolism; (U) Cardiac Dynamics;							
(U) Extracorporeal Circulation; (U) Tissue pH; (U) Frozen Autologous Blood							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pursuit individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23 (U) 1. To evaluate the effects of alterations in systemic pH on the function of the heart; 2. to evaluate muscle surface pH measurements as an index of tissue perfusion; and 3. to establish a systematic approach for the procurement, freezing and use of autologous blood. The results of these studies may: (a) establish priorities for treatment of systemic acid-base derangements; (b) provide military surgeons with an atraumatic method for monitoring the adequacy of resuscitation; and (c) provide a method for the conservation of blood for emergency military use.</p> <p>24 (U) 1. Systemic acid-base derangements were created utilizing an extra-corporeal circulatory system; 2. the sensing element of a pH probe was placed in contact with the muscle surface of experimental animals; and 3. all patients who were scheduled for elective cardiac surgery at WRAMC were potential donors of blood which could be frozen and made available for their operative procedure.</p> <p>25 (U) 75 07 - 76 07. 1. Despite adequate perfusion pressures and blood oxygenation, cardiac function deteriorated rapidly when the pH of blood was dropped to 7.0 plus-minus 0.1. 2. Muscle surface pH measurements paralleled the systemic pH under experimental conditions and served as an index of acid-base status and tissue perfusion. 3. Over 200 patients have participated in the frozen autologous blood transfusion program. Up to 10 units of blood have been obtained safely from patients with severe cardiovascular disease. Support in the amount of \$15,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p> <p>For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75-30 Jun 76.</p>							

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 200 Cardiac performance in by-pass surgery

Investigators.

Principal: LTC Arthur W. Fleming, MC

Associate: LTC Karl D. Pfuetze, MC

I. Systemic Acidosis and Alkalosis.

A. Statement of the Problem. To determine the degree of alterations in coronary blood flow, myocardial metabolism and cardiac function that occurs secondary to metabolic and respiratory acidosis and alkalosis.

B. Background. Massive tissue trauma and hypotension which might be found in battle casualties are often associated with acidosis due to the accumulation of fixed anions in the blood. Acute ventilatory impairment may also occur in severe trauma cases and lead to a respiratory acidosis. Efforts at resuscitation may further exaggerate existing acid-base changes. Massive blood transfusions may initially lead to a systemic acidosis, followed by a systemic alkalosis due to citrate metabolism. The over-use of ventilatory support may lead to a respiratory alkalosis. The effects of severe acid-base changes on the heart are not completely understood.

C. Experimental Approach. An extracorporeal circulatory system was devised to accomplish the following: The entire circulatory volume in an intact dog could be controlled on a minute to minute basis; the sympathoadrenal system could be maintained intact or separated from the coronary circulation; coronary blood flow and oxygen consumption could be easily measured without manipulation of the coronary arteries, and the heart rate and work could be controlled so that experimental animals were comparable to each other.

D. Results and Discussion. The development of a system whereby the above parameters could be controlled and manipulated has been the most significant accomplishment. Severe acidosis ( $\text{pH } 7.0 \pm 0.1$ ) had a profound effect on cardiac rhythm, coronary blood flow, and oxygen consumption. Rhythm changes consisted usually of bradycardia, nodal rhythms and premature ventricular contractions. Coronary blood flow increased significantly while oxygen consumption decreased by approximately 50%. Cardiac rhythm, coronary blood flow and oxygen consumption were more stable at an alkaline pH.

II. Physiologic and Biochemical Responses to Multiple Phlebotomies in Dogs

A. Statement of the Problem. The physiologic and biochemical responses of normal dogs to frequent donations of large volumes of blood for subsequent autologous transfusions during experimental cardiopulmonary by-pass were studied.

B. Experimental approach. Adult mongrel dogs were bleed 12.5% of their blood volume (BV) 3 times per week for 3 to 7 weeks. Blood samples were obtained prior to each phlebotomy for hematocrit (HCT), S. proteins (SP), and S. electrolytes (SE). Dogs were randomly divided into four groups. Group A received only oral iron, Group B received multiple iron injections intravenously (IV). Group C received a single bolus dose of iron IV. Group D received multiple iron injections intramuscularly.

C. Results and discussion. Following 8 phlebotomies of 12.5% of the BV, dogs receiving only oral iron had a decrease in HCT from  $44.3 \pm 1.3\%$  to  $36.5 \pm 1.3\%$  and a decrease in the HGB from  $16.1 \pm 0.6 \text{ gm } \%$  to  $12.8 \pm 0.7 \text{ gm } \%$ . Erythropoiesis was not further enhanced by the addition of parenteral iron. SI, TIBC and SE were not significantly altered. SP decreased from  $7.7 \pm 0.4$  to  $6.3 \pm 0.1 \text{ gm } \%$ . Due to the rapid rate of erythropoiesis in dogs, 100% of the BV could be collected without demonstrable detrimental physiologic or biochemical alterations. Removal of 120 to 166% of the BV was also well tolerated. However, the removal of greater than 200% of the BV led to weight loss, fatigue and lethargy. Cardiopulmonary by-pass experiments performed using autologous blood exclusively eliminates the risk of hemodynamic changes secondary to the homologous blood syndrome. (An abstract of this work has been submitted for publication.)

### III. Clinical Autologous Blood Transfusions

A. Statement of the problem. To develop a systematic approach for obtaining a sufficient volume of autologous blood for use during and after all elective thoracic and open heart surgical procedures, thus eliminating the need for homologous blood transfusions.

B. Background. The risk associated with the use of homologous blood transfusions are well documented and include significant acute problems, as well as long-term morbidity and/or mortality (viral hepatitis and other infectious diseases, allergic reactions, febrile reactions, Rh sensitization and non-red cell antigen sensitization). The exclusive use of autologous blood for elective operations would eliminate most of the disadvantages of homologous blood transfusions.

C. Experimental approach. All patients who were scheduled for elective surgery on the Thoracic Surgery Service, WRAMC, were potential candidates. Each unit of blood was collected in CPD preservative by AABB Standards. After separation of each unit into packed cells and plasma, each component was then frozen separately and stored until the time of surgery. Phlebotomies were performed at four days to two week intervals.

D. Results and Discussion. Up to 10 units of blood have been obtained safely using a combination of preoperative and intraoperative phlebotomies. Many of the open heart surgery patients donated 50 to 90% of the total blood used during their hospitalization. None of the

patients who have been asked to donate blood for their operation have refused. No serious complications have occurred in patients who have predeposited blood for their own operation. Further progress has been made in the design and development of forms so that information can be disseminated to the anesthesiologist, operating room technicians, recovery room nurse, ward nurses and doctors in an expeditious manner. As a result of our efforts in demonstrating that patients with severe cardiac disease could donate blood to be used for their own operations, all of the other clinical surgical services now use autologous blood transfusions to some degree. In addition, the forms that we have developed are being used at other military medical centers in the United States.

#### IV. Muscle Surface pH

A. Statement of the Problem. To evaluate muscle surface pH as an index of cardiac output, peripheral tissue perfusion and acid-base status.

B. Background. A technique for measuring surface hydrogen ion activity (expressed as pH) is currently available. The assertion, however, that muscle surface pH is an index of tissue perfusion and systemic acid-base status has not been supported by actual measurements of blood flow to the involved muscle.

C. Experimental Approach. Femoral arterial blood flow was measured electromagnetically and the muscle surface pH of the same extremity was continuously monitored in a group of dogs which were placed on total cardiopulmonary by-pass and which underwent manipulation of their systemic acid-base status.

D. Results and Discussion. Data generated thus far has demonstrated that  $pH_m$  reflects the systemic acid-base status when systemic blood flow is maintained constant as was possible using total cardiopulmonary by-pass. Results were reproducible only when very careful attention was given to the maintenance of hemostasis and electrode placement. A second group of dogs are currently under evaluation to determine specifically the reliability and reproducibility of pH measurements as well as to determine the delay in changes in central arterial pH and pressure measurements in comparison to changes in  $pH_m$  under conditions of controlled blood loss.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 200 Cardiac performance in by-pass surgery

Literature Cited.

References:

Maher, F. T., Watkins, L. C., Jr., Broadbent, J. C. and Bollman, J. L.: Significance of homologous donor blood to the toxic reaction in dogs. *Circ. Res.* 6: 47-54, 1958.

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Publication

Kessler, K. M., Foianini, J. E., Anderson, W. T., and Pfuetze, K.: Tricuspid insufficiency due to nonpenetrating trauma. *Am. J. Cardiol.* 37: 442-444, 1976.

Exhibit

Fleming, A.W., Green, D.C., St. James, D., Radcliffe, J.H., and Olson, P.: An Expanded Use of Autologous Blood Transfusions in Elective Thoracic and Open Heart Surgery. Exhibited at the American Association of Blood Banks Twenty-Eighth Annual Meeting, 9-14 November 1975, Chicago.



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)636	
3. DATE PREV. SUMMARY <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REQUIRING <sup>a</sup>	8a. DISSEM INSTR <sup>a</sup>	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
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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/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER:				FISCAL		76	
c. TYPE:				YEAR		1	
d. KIND OF AWARD:				CURRENT		42	
e. AMOUNT:				77		1	
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				SOCIAL SECURITY ACCOUNT NUMBER			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME:			
				NAME:			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Fibrinolysis; (U) Wound Healing; (U) Tendon Repair; (U) Peritoneal Adhesions							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23 (U) To evaluate the significance of fibrin deposition and fibrinolytic (fibrin removing) mechanisms in the healing of wounded tissues. To understand mechanisms of the early healing response, with the ultimate aim of gaining pharmacologic control over the deposition of scar tissue, particularly within the peritoneum and in the vicinity of wounded and repaired long tendons. Such studies may produce insights into the general mechanisms of wound healing, over which there is currently no control. Understanding and control of the wound healing process would dramatically reduce morbidity and disability of wounded soldiers.</p> <p>24 (U) Fibrin deposition and organization to form scar are studied by special histochemical techniques. Fibrinolysis is assessed in vitro by a fibrin plate technique and in vivo by histochemical methods. Effects of systemic defibrinogenation are studied by use of venom extract anrod. Gliding function of tendons is measured on an Instron tensiometer.</p> <p>25 (U) 75 07 - 76 06. Prolonged local failure of peritoneal fibrinolysis has been shown to be a unifying pathogenetic mechanism in peritoneal adhesion formation. Evidence for a similar mechanism has been found in adhesions to healing long tendons; short term defibrinogenation has been shown to improve the gliding function of wounded and repaired long tendons. Some effects of surgical technique on tendon adhesion formation have also been discovered.</p> <p>Support in the amount of \$10,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p> <p>For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75-30 Jun 76.</p>							

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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 Robert F. Buckman, Jr, MC

## I. 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. Prolonged local failure of endogenous peritoneal fibrinolytic mechanisms has been shown in vitro and in vivo to be a unifying pathogenetic mechanism in peritoneal adhesion formation caused by operative trauma. Moreover, the physiologic basis for the adhesion-free healing of deperitonealized surfaces and for the ischemic stimulus to peritoneal adhesion has been shown in vivo and in vitro to involve local variations in peritoneal fibrinolytic activity. It has been demonstrated also that ischemic tissues release a factor capable of inhibiting normal fibrinolysis. To our knowledge, this

represents the initial demonstration of fibrinolytic inhibitors in association with the wound-healing process. These data may be of importance in determining the basic surgical biology of peritoneal healing and post-traumatic peritoneal adhesion formation.

## II. Peritonitis

A. Background and Statement of the Problem. It has been shown by Das and associates that the intraperitoneal administration of platelet-rich plasma protects experimental animals from the lethal effects of intraperitoneal endotoxin. Because of the possible significance of this observation in the treatment of patients who have suffered severe bowel injuries, resulting in massive fecal contamination of the peritoneum, a study was undertaken in rats to determine whether peritoneal thrombocytosis was a naturally occurring defense in peritonitis.

B. Experimental Approach. Peritoneal fluid platelet counts, by the phase contrast method of Brecher were obtained at intervals from one to 24 hours following the intraperitoneal administration of endotoxin or a 20% filtered fecal suspension.

C. Results and Discussion. It was found that peritoneal fluid thrombocytosis does not occur as part of the natural defense of the rat in peritonitis. If this observation is confirmed in higher animals including man, it may indicate that platelets must be administered intraperitoneally, from an exogenous source, to confer protection against intraperitoneal endotoxin.



Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 201 Fibrinolysis in peripheral blood vessels

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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>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pursue 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) 75 07-76 06 In vitro studies of the interaction of antibody with Trypanosoma rhodesiense indicate alternative complement pathway mediation. A sensitive antigen assay based on absorption of in vitro active antibody has been developed. Immunity to T. rhodesiense can be induced in athymic mice. Transferability of immunity to T. rhodesiense in mice, with cells is transient, but splenocytes retain memory for long periods as assayed in recipients given sub immunogenic doses of antigen. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1975 - 30 June 76.							
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<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 202 Antigenic composition of trypanosomes

Investigators: COL Carter L. Diggs, MC, M.D., CPT Gary H. Campbell, MSC, Ph.D.; John Barbaro, Ph.D.; D.T.O. Wong, Ph.D.  
Assistants : Andre J. Toussaint, M.S.; Barbara J. Flemmings, B.S.; James Dillon, B.S.; Klaus Esser, B.S.; John Bussey, Chris Goodhue, B.A.; Ruta Hajkowski, B.S.

I. Studies of the antigens of Trypanosoma rhodesiense

A. Bioassay of Trypanosoma rhodesiense Protective Antigens.

Objective. This study was designed to develop a highly sensitive assay for the detection of the surface antigens of African trypanosomes in the fluid phase.

Description. The principle used in the development of this bioassay is the absorption of antibody which is cytotoxic for intact trypanosomes as determined in an in vitro assay of cytotoxicity based on inhibition of incorporation of leucine into the organisms by antibody. The feasibility of this approach was presented in last years annual progress report. Difficulties have been encountered in using the antigen assay in a practical way for purification studies, since relatively large amounts of antigen were required and anti-complementary effects encountered. This report is concerned with data indicating that these problems can be in large part circumvented by a simple maneuver which allows the use of fewer organisms and less antibody, and therefore requires less antigen for inhibition of cytotoxicity. This assay was achieved through the removal of low molecular weight materials (presumably predominantly free leucine) which inhibit incorporation <sup>3</sup>H-leucine by the organisms.

Progress. A comparison of the incorporation of leucine into trypanosomes in the presence of dialysed versus non-dialysed serum is presented in Table I. The use of high concentrations of serum has previously been necessary because of the requirement for large amounts of immune serum and, in particular, for large amounts of fresh normal serum as a source of complement. Using G 25 chromatography to remove leucine from the complement, it was possible to lower the numbers of organisms by tenfold, the amount of antibody tenfold and to still achieve a comparable degree of inhibition of the reaction. This result is shown in Table II. Using this lowered concentration of organisms, and antiserum the antigen dose response relationship presented in Table III was obtained. The antigen preparation used for this experiment is an incubate of intact organisms, and is presumed to be simpler than the whole homogenates which have been previously used. This preparation had no detectable anti-complementary activity when used undiluted.

TABLE I

Effect of dialysis on inhibition of incorporation of 3-H leucine by normal rat serum

Serum dil	CPM $\times 10^{-4} \pm 95\%$ Confidence limits	
	Non-dialysed	Dialysed
1/1	2.3 $\pm$ 0.5	48 $\pm$ 3
1/3	6.0 $\pm$ 0.9	85 $\pm$ 11
1/9	20 $\pm$ 3	114 $\pm$ 9
1/27	50 $\pm$ 4	140 $\pm$ 7
0.15 MNaCl	149 $\pm$ 13	

TABLE II

Effect of number of trypanosomes on antiserum mediated inhibition of 3H-incorporation

Microliters of Immune Serum/ Reaction mixture	CPM $\times 10^{-3} \pm 95\%$ Confidence limits	
	$5 \times 10^6$ cells	$5 \times 10^5$ cells
0	37 $\pm$ 3	2.7 $\pm$ 0.6
0.05	41 $\pm$ 3	3.0 $\pm$ 0.7
0.1	42 $\pm$ 6	1.6 $\pm$ 0.8
0.2	43 $\pm$ 2	0.2 $\pm$ 0.1
0.4	41 $\pm$ 3	0.2 $\pm$ 0.2
0.8	34 $\pm$ 2	0.3 $\pm$ 0.3
1.6	21 $\pm$ 4	0.2 $\pm$ 0.4
3.1	11 $\pm$ 1	0.3 $\pm$ 0.3
6.3	7 $\pm$ 2	0.3 $\pm$ 0.1
12.5	5 $\pm$ 2	0.3 $\pm$ 0.2

TABLE III

Absorbtion of anti-T. rhodesiense cytotoxic activity by soluble antigen

Antigen/	Microliters of Reaction mixture	% absorbtion ± 95% Confidence limits
	0.1	1 ± 0.3
	0.2	3 ± 2
	0.4	13 ± 4
	0.8	36 ± 6
	1.6	57 ± 9
	3.1	79 ± 4
	6.3	81 ± 11

Discussion. The more sensitive methodology for the detection of antigen is expected to allow accelerated progress in the characterization of antigen preparations.

B. Isolation of Trypanosoma rhodesiense Antigens by Potassium chloride extraction.

Objective. The aim of this study is the isolation and purification of protective antigens from T. rhodesiense.

Description. Last years WRAIR Annual Report established that the in vitro Trypanosome cytotoxicity assay could be used to monitor new antigen preparations and that some antigen extracts could inhibit the cytotoxicity assay. In an attempt to obtain purer and more specific antigen preparations a potassium chloride (KCl) extraction of T. rhodesiense was used. This procedure was developed by Mr. Gore, Department of Parasitic Diseases, WRAIR and Dr. White, Department of Viral Diseases. Their data showed considerable promise.

Progress. The protein content of the KCl extract of trypanosomes was determined by micro-Kjeldhal and by Lowry's method of protein determination. This particular lot of KCl antigen extract contained between 8.6 and 9.5 mg protein per ml. Analyses of the antigen extract was done by immunoelectrophoresis (IEP) and by polyacrylamide gradient gel electrophoresis (PAGE). Immunoelectrophoresis of the KCl extract in agarose gel and reaction with rat anti- T. rhodesiense sera demonstrated a single band in the beta region that was split at the ends

indicative of more than one component. This compares well with data obtained with soluble extracts obtained by ultrasonic oscillation indicating a similar precipitin pattern. When approximately 65 micrograms of KCl antigen extract was analyzed by PAGE and stained for protein, over 10 bands were visible ranging in molecular weights from 54,000 to 1,350,000 the most prominent in the 400,000 to 800,000 molecular weight.

The antigen extract was also tested for its ability to inhibit the in vitro trypanosomal cytotoxicity assay and was active.

Discussion: The KCl antigen extraction procedure of White and Gore is a distinct improvement over the sonicated Trypanosome antigen preparation. There are fewer contaminating components and less anticomplementary activity. Further purification of this antigen extract by sephadex G200 filtration, preparative PAGE and isoelectric focusing is contemplated.

## II Mechanisms of Immunity in African Trypanosomiasis

Objective. These studies attempt to define mechanisms of immunity involved during resistance to Trypanosoma rhodesiense infection.

Description. Immunity to T. rhodesiense can be induced in experimental animals by injection of irradiated organisms. Elucidation of 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 production of the immunity. As reported in the 1975 progress report an in vitro correlate of cell mediated immunity was first studied. Spleen cells from animals immunized against a variant population of T. rhodesiense organisms will undergo blast transformation when exposed in vitro to soluble T. rhodesiense organisms. This response is not, however, variant specific. Variant organisms that will kill the immune animal will also induce the blastogenic response. The cells undergoing blastogenesis are T cells. This implication that T cells might be involved in an immune response to T. rhodesiense was further studied in adoptive transfer experiments. The Wellcome strain of T. rhodesiense was used. All studies were performed with C57Bl/6 mice.

### Progress.

#### A. Studies on the Development of Resistance to T. rhodesiense

##### 1. Studies on the Natural Course of Disease

The optimal conditions for establishing reproducible consistent infections were first determined. Animals were injected with  $10^0$  -  $10^6$  viable organisms. As shown in Figure 1, a single organism was capable of establishing an infection which led to death in 7 days in 4 of 7 animals.  $10^3$  organisms produced a highly predictable, uniform death pattern, which occurred 5 days after initial injection and was chosen as the challenge dose in all further experiments. Parasites could be observed on a wet mount preparation of tail blood two days before death of the mice. When

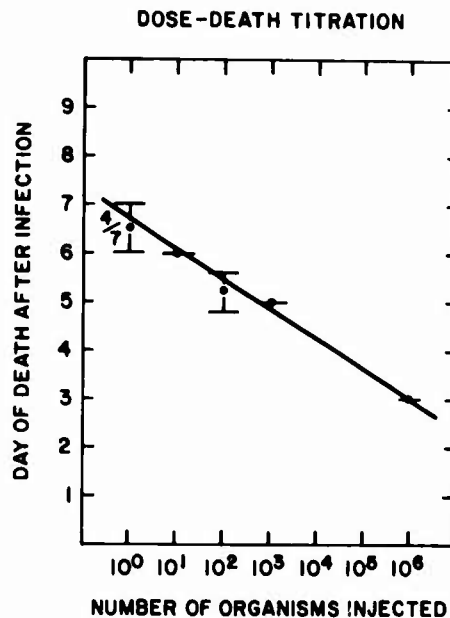


Figure 1. Relationship of Time of Death to Exposure Inoculum: Animals were exposed to increasing numbers of *T. rhodesiense* organisms and the day of death recorded. Each point represents the mean  $\pm$  standard error of the days of death after infection in each group of 5 to 7 C57BL/6 mice.

parasites were observed, the animals invariably succumbed to the infection.

## 2. Studies on the Development of Resistance to Infection

Mice were inoculated with  $10^4$  -  $10^7$  irradiated organisms. They were challenged at varying times thereafter with  $10^3$  viable organisms. As shown in Table 4, the rapid development of resistance was seen following exposure to this wide range of immunizing inocula. Increased variation in the degree of augmented resistance was observed when lower immunizing doses were used. An injection of  $10^7$  irradiated organisms produced a highly predictable protection pattern and was chosen for the initial exposure inoculum in all future experiments. Protection was observed from 3 days after exposure until a minimum of 25 days after exposure.

## 3. Studies on the Specificity of Resistance

The specificity of immunity was next determined by the initial exposure of animals to either the Wellcome or a variant strain. Animals were

TABLE 4  
Development of Immunity Following Injection  
with Irradiated T. rhodesiense

# of Organisms <sup>a</sup>	<u>Day of Challenge</u> <sup>b</sup>			
	3	5	10	14
10 <sup>7</sup>	5 <sup>c</sup>	5	5	5
10 <sup>6</sup>	5	5	5	5
10 <sup>5</sup>	5	5	4	4
10 <sup>4</sup>	1	2	2	3

<sup>a</sup> Number of irradiated T. rhodesiense organisms injected at T<sub>0</sub>.

<sup>b</sup> Days after initial injection with irradiated T. rhodesiense.  
Animals challenged with 10<sup>3</sup> non-irradiated organisms.

<sup>c</sup> Number of mice surviving 30 days after challenge. Five mice per group.



TABLE 5  
Variant Specificity of Immunity to T. rhodesiense

Exposure Organism <sup>a</sup>	<u>Challenge Organism<sup>b</sup></u>	
	Wellcome	Variant
Wellcome	30 <sup>c</sup>	5
Variant	5	16.5
None	5	5

<sup>a</sup> Animals initially exposed to  $10^7$  irradiated organisms.

<sup>b</sup> Animals challenged with  $10^3$  non-irradiated organisms 10 days after immunization.

<sup>c</sup> Mean day of death following challenge. Five to seven mice per group.

subsequently challenged with either of the above strains. As shown in Table 5, immunity was observed only when homologous challenge was performed.

B. Specific Immunologic Studies on the Mechanisms of Resistance to T. rhodesiense

1. Studies of Humoral Mechanisms.

a. Transfer of resistance with serum - Animals were challenged with  $10^3$  organisms and injected 2 hours later with 0.5 milliliters of undiluted or diluted (1:20-V:V) serum obtained from normal animals previously inoculated with  $10^7$  irradiated trypanosomes. As shown in Figure 2, protection was maximal with serum which had been obtained from animals that were inoculated with irradiated organisms 5 to 7 days before sacrifice. The apparent drop in protection with undiluted serum from day 5 to days 7 and 10 is within the range of experimental variation for the experiments shown.

b. Correlation of in vivo transfer of protection and in vitro cytotoxic antibody activity - Previous studies have indicated that serum obtained from animals immunized against T. rhodesiense can inhibit the in vitro incorporation of 3H-Leucine into TCA precipitable material by trypanosomes. The cytotoxic effect is dependent on the presence of complement and the variant specificity of these reactions correlates with the results of in vitro neutralization tests. Because of the potential importance of developing an in vitro correlate to in vivo immunity, in vitro cytotoxic activity was compared to the in vivo protective capability of serum. As shown by comparing Figure 2 with Figure 3, the two activities, while initially appearing together and increasing in parallel, subsequently followed different courses. The waning of in vitro activity while in vivo protective capability remained may have a variety of explanations; however, differences in sensitivity of the assays make direct quantitative comparisons difficult.

c. Antibody dependent cytotoxicity against Trypanosoma rhodesiense mediated through an alternative complement pathway

Description. Recent studies in our laboratories have documented the toxicity of immune serum against T. rhodesiense. The toxicity was assessed in vitro by the extent of inhibition of incorporation of radio-labelled leucine, and was found to be completely dependent on the presence of heat labile serum factors. Although the system behaved in a fashion highly suggestive of a conventional antibody and complement-mediated cytotoxic reaction, no further information as to the character of the heat labile factor(s) involved was obtained. The present communication supplies evidence that the cytotoxic reaction is dependent on magnesium but not calcium ions, is abrogated by the anti-complementary factor of cobra venom, and proceeds in the absence of the fourth component of complement. These findings suggest that the activity can be mediated through an alternative complement pathway.

The Wellcome strain of *T. rhodesiense* was maintained in inbred adult male albino rats (CDF, Charles River Breeding Laboratories, Wilmington, MA) as previously described. The antiserum (IS) used for all the studies described was a hyperimmune pool obtained by prolonged immunization of inbred rats with irradiated trypanosomes derived from syngeneic donors. IS and control serum from unimmunized rats (NS) was decomplemented by incubation with sheep erythrocyte stroma sensitized with rabbit antibody. Pools of fresh frozen guinea pig serum from Hartley guinea pigs and NIH "multipurpose strain" guinea pigs were used as sources of C4 sufficient serum; (C4D) guinea pig serum was obtained from a strain of guinea pigs with a complete deficiency of C4. Cobra venom factor (CVF) was obtained from Cordis Laboratories, Miami, FL.

Progress. Support of antibody mediated cytotoxicity against trypanosomes by C4 deficient serum. Incorporation of leucine into trypanosomes was studied as a function of dose of immune or normal rat serum; in the presence of C4S or C4D guinea pig serum. The results are presented in figure 4. In the absence of, or with small amounts of immune serum, no significant difference can be detected between normal and immune serum reaction mixtures whether C4S or C4D serum is used. There appears to be a somewhat higher incorporation of isotope in the case C4D serum. At higher levels of immune serum, there is inhibition of the ability of the organisms to incorporate the isotope in the presence of both C4S and C4D serum. The efficiency of the serum in inhibiting isotope incorporation is comparable in the presence of the two sources of complement; there is essentially no effect of immune serum at .8 microliters and complete inhibition at 3 microliters.

Effect of EDTA on cytotoxicity. Since C4 is unnecessary for maximum inhibition of uptake of leucine, further evidence of involvement of the complement system was sought. Divalent cation requirements of the reactions were studied by the incorporation of EDTA into the reaction mixtures. Fig. 5 demonstrates that this compound blocks the cytotoxic effect of decomplemented immune serum in the presence of both C4S and C4D guinea pig serum. At concentrations of 25 or 50 mM EDTA, incorporation of isotope into trypanosomes was very similar whether in the presence of normal or immune serum. Immune hemolysis was also abolished by EDTA as expected, with no lysis at 13 mM EDTA or at higher levels of the compound. In the absence of EDTA or at concentrations of 6 mM there was complete hemolysis of EA in the presence of C4S guinea pig serum whereas C4D guinea pig serum supported hemolysis only to the extent of about 10%, an observation consistent with previous findings by other investigators.

Effect of cobra venom factor on cytotoxicity. To further explore the role of the complement system, the effect of cobra venom factor on the cytotoxic reaction was studied. Fig. 6 illustrates that the reaction can in fact be blocked by incorporation of 1.25U of CVF into the reaction mixture. Smaller amounts of CVF are ineffective. This blockade of cytotoxicity occurs in both C4D and C4S guinea pig serum. Immune hemolysis is also abolished in both situations by CVF.

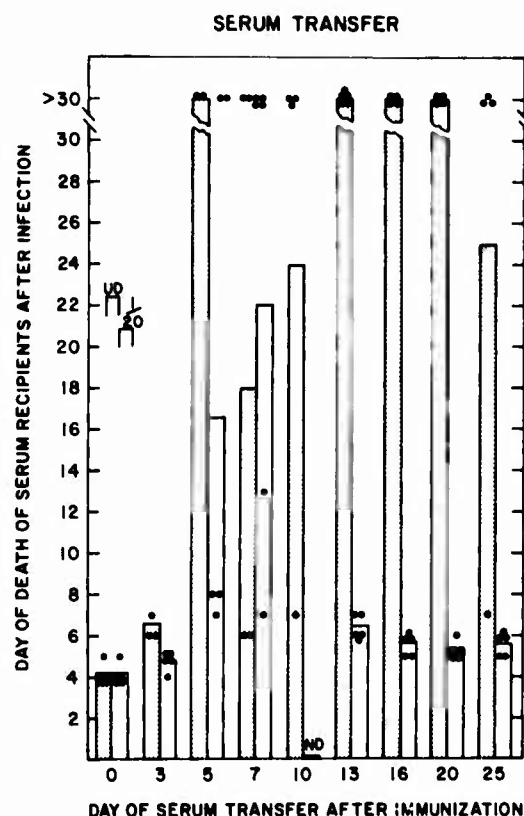


Figure 2. Adoptive transfer of Protection with Serum: Mice were challenged i.p. with  $10^3$  *T. rhodesiense* organisms two hours before i.p. injection of 0.5 ml of undiluted (o), or diluted (o) (1:20-V:V) serum. Serum obtained from animals exposed for 5-25 days previously to irradiated *T. rhodesiense* showed significant protection. The apparent dip in protection shown by serum obtained 7-10 days after exposure is not significant.

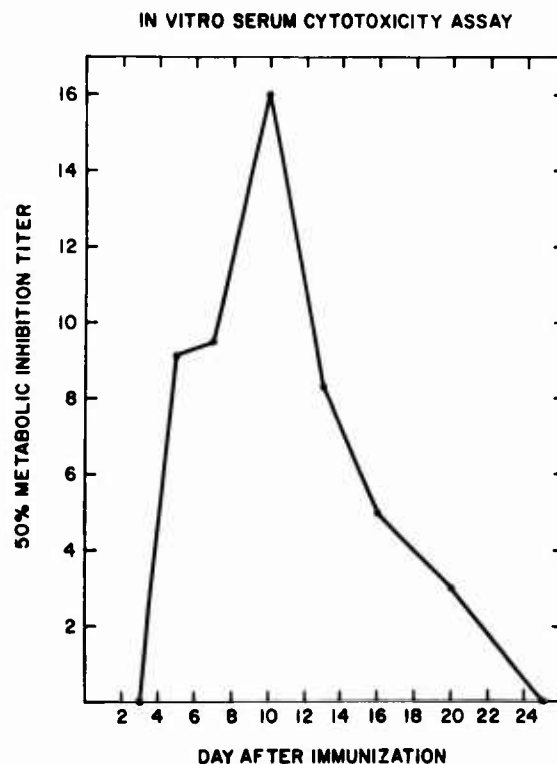
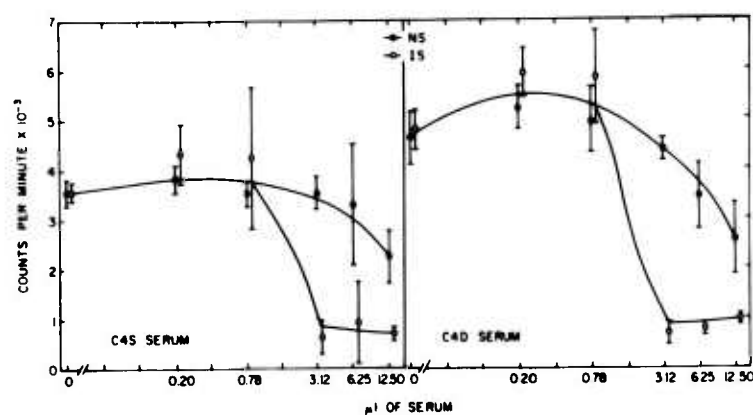
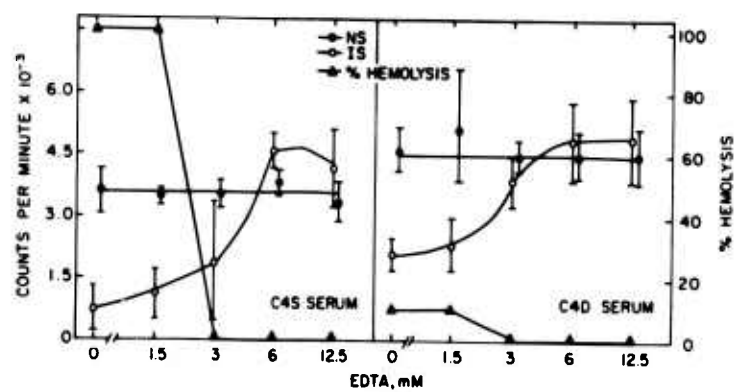


Figure 3. In Vitro Serum Cytotoxicity Assay: Each point represents the 50% metabolic inhibition titer of sera pooled from 10 mice. Serum was obtained from animals who were exposed from 3-25 days previously to irradiated I. rhodesiense. In vivo protective capacity and in vitro cytotoxicity originally appeared in parallel; however, the latter titer waned more rapidly.

Figure 4



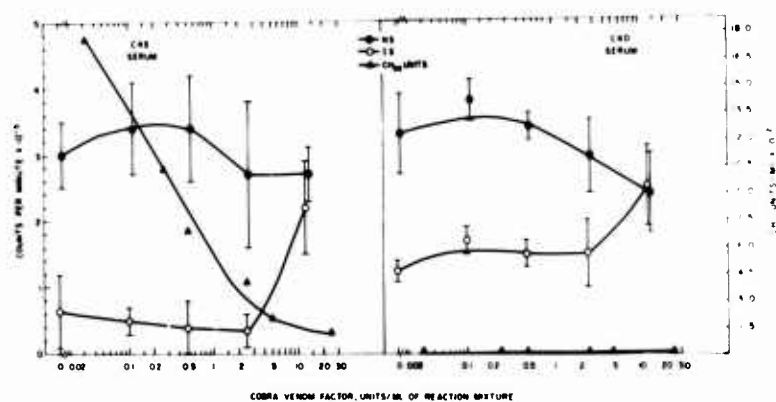
Support of Antibody Mediated Cytotoxicity Against *T. rhodesiense* by C4-Deficient and Normal Serum as Sources of Complement



Antibody Mediated Cytotoxicity Against *T. rhodesiense*: Inhibition by EDTA Treatment of C4-Deficient and Normal Serum

Figure 5

Figure 6



Antibody Mediated Cytotoxicity Against *T. rhodesiensis*: Inhibition by CVF Treatment of C4-Deficient and Normal Serum

Effect of EGTA on cytotoxicity. Table 6 illustrates the effect of EGTA on the anti-trypanosome cytotoxic reaction. It can be seen that there is little effect on the reaction of 16 mM EGTA whether C4D or C4S guinea pig serum is used. However hemolysis of EA is completely inhibited with this concentration of the compound. Addition of 16 mM calcium reconstitutes the ability of the system to support immune hemolysis, but has no perceptible effect on the anti-trypanosome cytotoxic reaction.

Discussion. The studies reported in this communication support the presumption that the complement system is involved in the cytotoxic reaction against trypanosomes mediated by immune serum. Blockage of the reaction by EDTA suggests that divalent cations are required, a prominent feature of the complement system. Similarly, the abrogation of the reaction by cobra venom factor, a highly specific reagent for the activation of C3, suggests that this component is required for activity. The fact that C4D guinea pig serum supports the reaction indicates that C4 is not essential to the reaction. Finally the blockade of immune hemolysis, but not cytotoxicity against trypanosomes, by EGTA suggests the possibility that the compound chelates sufficient calcium to prevent activation of C1, but insufficient magnesium to block C3-proactivator (C3PA) activation. This suggestion is supported by the fact that addition of calcium to the system restores its hemolytic capacity.

Although these studies strongly suggest a role for an alternative complement pathway in the anti-trypanosome reaction, little information of relevance to the possibility that the classical pathway is also operative is offered. Since the efficiency of the reaction as a function of immune serum dose is similar with both C4D and C4S guinea pig serum, it might be speculated that the same mechanism is operative in both cases. However, further studies involving blockade of alternative pathways, while leaving the classical sequence intact, will be required to clarify this point.

The mechanisms of activation of the alternative pathway in this reaction is not known. Relatively little information is available regarding the ability of rat immunoglobulins to fix complement by the various known mechanisms. In the case of human and guinea pig immunoglobulins, there is data to indicate that immune complexes composed of a variety of immunoglobulin classes can activate alternative pathways. Demonstration of the activation of C3PA by C3 has lead to the point of view that consumption of C3PA by immune complexes may be mediated through C3 activation via the classical pathway. In the present situation, there is apparently no requirement for either C1 or C4; the role of C2 has not been explored. Although rigorous evidence for the involvement of antibody, and thus of immune complexes, in the present system has not been presented, strong circumstantial evidence has appeared previously. Further insights into the pathways involved in this cytotoxic reaction will be forthcoming as information on the immunoglobulin classes involved is obtained.

The ubiquity or lack thereof of alternative complement pathway involvement in antibody dependent killing of African trypanosomes among the various model systems and naturally acquired infections is unknown. In particular it will be of great interest to determine whether antibody from patients



with African sleeping sickness will behave this way.

Table VI

EFFECT OF IMPURE AND NORMAL RAT SERUM ON INCORPORATION OF  $^{35}$ S-METHIONINE BY *T. BRUCEI* IN THE PRESENCE OF NORMAL AND C5-DEFICIENT GUINEA PIG SERUM TREATED WITH ESTA

TREATMENT	INCORPORATION OF $^{35}$ S-MET BY TRYPAVOSOMES (CPM)				% HEMOLYSIS
	C5S SERUM		C5D SERUM		
	IS	NS	IS	NS	
No ESTA	96 ± 16	1117 ± 79	194 ± 88	1641 ± 270	100
ESTA (15.6μM)	191 ± 179	1171 ± 101	217 ± 175	1994 ± 339	0
*Mg <sup>++</sup> (3μM)	221 ± 43	1297 ± 442	219 ± 165	2133 ± 727	0
*Ca <sup>++</sup> (16μM)	94 ± 37	1099 ± 179	189 ± 58	1991 ± 428	100
*Mg <sup>++</sup> (3μM), Ca <sup>++</sup> (16μM)	184 ± 59	1057 ± 185	258 ± 279	1265 ± 230	100

<sup>A</sup> MEAN OF QUADRUPPLICATE REACTION MIXTURES ± 95% CONFIDENCE LIMITS.

d. Studies on the mechanism of neutralization of Trypanosome infectivity.

Objective. The objective of these experiments is to determine whether or not complement components are required for the killing of trypanosomes by antibody in vivo.

Description. Studies described in previous annual reports have documented the inhibition of incorporation of leucine into trypanosomes by antibody in the presence of fresh serum as a source of complement. This inhibition of uptake is completely dependent on fresh serum under the conditions employed. To investigate the requirement for complement in vivo after injection of antibody sensitized organisms, two kinds of experiments were performed. C5 deficient mice were used to determine whether or not this component is required and cobra venom factor was used to deplete animals of C3 and the later components.

Progress. Table 7 illustrates the fact that neutralization can proceed in C5 deficient mice. Similarly, neutralization proceeds in animals pretreated with cobra venom factor, as demonstrated in table 8. The dose used to deplete these mice, 40 units per animal, was greater than that which completely abolished hemolytic activity as demonstrated in table 9.

Discussion. The experiments demonstrate a dichotomy between the

in vitro and in vivo requirements for complement in the killing of trypanosomes. It is quite conceivable that complement is sufficient to support killing of antibody sensitized organisms but is not necessary in the presence of an intact immune effector system under in vivo conditions. Studies by Dr. Ray Perry and Dr. Richard MacDermott suggest that antibody dependent cell mediated cytotoxicity can occur, thus offering an explanation for the present findings.

The fact that antibody titers in C5 deficient animals (Table 7) are somewhat lower than in the outbred complement sufficient animals, cannot be interpreted as being due to the absence of C5, since these two strains of animals differ in many other respects which might influence the efficiency of neutralization.

TABLE VII  
NEUTRALIZATION OF TRYPANOSOME INFECTIVITY IN COMPLEMENT  
SUFFICIENT<sup>1</sup> AND C<sup>5</sup> DEFICIENT (A/J)<sup>2</sup> MICE

μl immune serum/500 trypanosomes	DEAD/TOTAL (Day 13)	
	C Sufficient <sup>1</sup>	A/J <sup>2</sup>
0	5/5	5/5
0.3	5/5	5/5
0.6	2/5	5/5
1.3	0/5	1/5
2.5	0/5	1/5
No trypanosomes	0/5	0/5

<sup>1</sup> Walter Reed albino mice,  $36 \pm 17$  CH<sub>50</sub>/ml (mean  $\pm$  95% confidence limits)

<sup>2</sup> No hemolytic complement detectable

e. Class of immunoglobulin involved in the in vitro antibody mediated cytotoxicity assay.

Objective. These experiments were aimed at determining the specific class of immunoglobulin involved in the in vitro cytotoxicity assay.

Description. Immunoabsorbent columns were used for the isolation of specific classes of immunoglobulins from pooled rat anti T. rhodesiense serum. Details concerning the preparation of the immunoabsorbent columns can

TABLE VIII

LACK OF COBRA VENOM FACTOR<sup>1</sup> EFFECT ON LETHAL OR IMMUNE  
SERUM NEUTRALIZED TRYPANOSOME CHALLENGE

Mouse Pretreatment	Trypanosome treatment	Number mice dead/total (day 13)
Nil	Normal serum <sup>2</sup>	10/10
Cobra factor	" "	10/10
Nil	Immune Serum <sup>2</sup>	0/10
Cobra factor	" "	1/10
Nil	No trypanosomes	0/10
Cobra factor	" "	0/10

<sup>1</sup>  
40 units/mouse 4 hours before challenge

<sup>2</sup>  
2.5  $\mu$ l/500 trypanosomes (one mouse dose)

be found under work unit entitled "Immunological Mechanisms in Microbial Infections". Briefly, the procedure involves the covalent coupling of specific goat anti-rat immunoglobulins to cyanogen bromide activated Sepharose 4B. Goat antisera specific for IgG2a, IgG1, IgM, IgA, and IgE classes of rat immunoglobulin were prepared by Dr. Daniel Stechschulte of the University of Kansas. Pooled rat anti T. rhodesiense serum was percolated through a given specific immunoabsorbent column. The effluent contained all of the rat serum proteins except that immunoglobulin adsorbed to the homologous immunoabsorbent column. The adsorbed immunoglobulin was then eluted from the column with glycine at pH 3.0. This procedure allowed for the testing of rat anti T. rhodesiense depleted of a single immunoglobulin, as well as the activity of the isolated immunoglobulin.

Progress. Immuno-electrophoretic analyses was done to determine the purity of all depleted antisera and all eluates of specific immunoglobulin preparations. Using the "monospecific" goat anti-rat immunoglobulins, all treated rat anti T. rhodesiense sera lacked one specific immunoglobulin

Table IX  
EFFECT OF COBRA VENOM FACTOR<sup>1</sup> ON SERUM COMPLEMENT LEVELS  
IN WALTER REED FEMALE MICE

time post treatment	number mice acomplementenmic/total	CH <sub>50</sub> /ml $\pm$ 95%CL
0	1/8	36+17
2 hrs	8/8	0
1 day	8/8	0
2 days	7/8	77
3 days	5/8	20+8
4 days	1/8	30+10
5 days	0/8	39+9

<sup>1</sup>  
10 units/mouse

while retaining what appeared to be all of the other serum components. The "monospecific" goat antisera also indicated that all but the IgG1 preparation of the isolated immunoglobulins were not contaminated with any other immunoglobulins. The IgG1 immunoglobulin eluate contained IgG2a immunoglobulin. Although the preparations appear clean, it should be stressed that further tests are required to establish purity.

Depleted rat anti T. rhodesiense and isolated immunoglobulin was tested in the in vitro cytotoxicity assay. Table 10 contains the results from two representative experiments on hyperimmune sera. Data not shown also suggest activity in the IgM fraction .

Discussion. The data shown clearly indicate activity in the IgG2a fraction. Further studies will be required to clarify the roles of IgG1, IgM, IgA, & IgE.

## 2. Studies on Cellular Mechanisms.

a. Kinetics of the transfer of resistance with cells - Transfer of spleen cells and challenge of syngeneic recipients was performed utilizing

TABLE X

Cytotoxicity against Trypanosoma rhodesiense of sera depleted of immunoglobulins and of purified immunoglobulins

Expt #	Preparation	Leucine incorporation CPM $\pm$ 95% Confidence limits
1	Normal serum	2129 $\pm$ 122
	Immune serum	179 $\pm$ 144
	Immune serum Passed over "blank" Column	87 $\pm$ 87
	Immune serum depleted of IgG <sub>2a</sub>	1345 $\pm$ 741
	Immune serum depleted of IgM	166 $\pm$ 110
2	Normal serum	2156 $\pm$ 264
	Immune Serum	163 $\pm$ 60
	IgG <sub>2a</sub> fraction of Immune serum	488 $\pm$ 435
	IgM fraction of Immune serum	2928 $\pm$ 424

cells obtained from donors that were previously immunized with irradiated trypanosomes from 3 to 25 days prior to sacrifice. As shown in Figure 7, immunity was transferred most effectively utilizing cells obtained from animals which were exposed from 3 to 5 days before sacrifice. Survival for a period of 10 days after challenge is interpreted as an indication of immunity to the injected variant type organism since a single organism was able to kill mice in 6 to 7 days (Figure 1). Moreover, organisms, isolated from animals immediately before death on day 12 after challenge with  $10^3$  Wellcome trypanosomes, will kill mice that are completely resistant to the original Wellcome organisms (Table 11). This indicates that these delayed deaths are due to the emergence of variant strains.

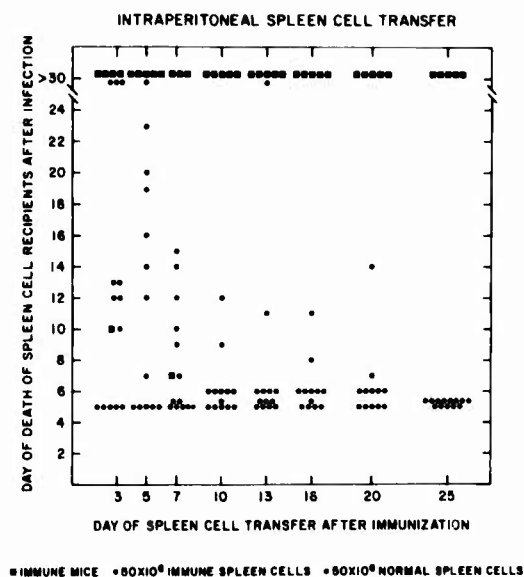


Figure 7. Time course of ability to transfer with spleen cells protection against challenge with  $10^3$  *T. rhodesiense* organisms: Maximum protection was observed when cells, obtained from animals exposed from 3-7 days before sacrifice, were utilized. Intact immune mice (■), normal mice receiving  $50 \times 10^6$  normal spleen cells (○) or  $50 \times 10^6$  immune spleen cells (●).

TABLE XI  
Variant Composition of "Late" Killing Trypanosomes

Initial Exposure <sup>a</sup>	Challenge Organism <sup>b</sup>	MST <sup>c</sup>
0	Day 12	6.8
Wellcome	Isolate #1	6.7
0	Day 12	4.8
Wellcome	Isolated #2	6.4
0	Wellcome	5.7
Wellcome	Strain	30

<sup>a</sup>Animals initially exposed to  $10^7$  irradiated Wellcome strain organisms.

<sup>b</sup>Animals challenged with  $10^3$  non-irradiated organisms, 10 days following initial exposure.

<sup>c</sup>Mean survival time in days following challenge. Five mice per group.

Varying numbers of immune cells were transferred to normal mice before challenge with trypanosomes (Table 12). One of 7 mice, injected with  $5 \times 10^6$  immune cells, and 4 of 7 injected with  $15 \times 10^6$  cells survived for 10 more days following challenge. Maximum transfer of resistance was obtained with  $50 \times 10^6$  cells.

b. Subpopulation analysis of the cells capable of transferring resistance - Spleen cells were obtained from animals inoculated with irradiated trypanosomes 5 days before sacrifice. These cells were subsequently

TABLE XII  
Adoptive Transfer or Resistance to T. Rhodesiense  
With Spleen Cells

Source	<u>Transfer<sup>a</sup></u>	<u>Survival<sup>b</sup></u>	
	Number	MST <sup>c</sup>	#A/#C <sup>d</sup>
Unexposed <sup>e</sup>	50x10 <sup>6</sup>	5.0	0/7
Exposed <sup>f</sup>	5x10 <sup>6</sup>	6.1	1/7
	15x10 <sup>6</sup>	8.7	4/7
	30x10 <sup>6</sup>	12.6	4/7
	50x10 <sup>6</sup>	14.2	7/8

<sup>a</sup>Source and number of spleen cells injected i.p. 4 hours before challenge.

<sup>b</sup>Survival of animals challenged with 10<sup>3</sup> Wellcome strain organisms.

<sup>c</sup>Mean survival time in days following challenge.

<sup>d</sup>Number of animals alive 10 days following challenge/number challenged.

<sup>e</sup>Cells obtained from animals previously unexposed to T. rhodesiense.

<sup>f</sup>Cells obtained from animals exposed to 10<sup>7</sup> irradiated T. rhodesiense organisms (Wellcome strain) 5 days before sacrifice.

fractionated utilizing the criteria of cytotoxic antibody sensitivity and column adherence. As shown in Figure 8, when cells were fractionated by nylon wool columns alone or nylon wool columns and anti-theta or anti-light chain sera, the non-adherent, anti-Ig treated population failed to transfer protection. The adherent, anti-theta treated population afforded prolonged survival. Two experiments are shown.



c. Variant specificity of transferred immunity - Spleen cells from animals immunized with the Wellcome strain organisms were transferred to normal recipients on day 5 after immunization. The animals were challenged with either homologous or heterologous variant organisms. Intact immune animals as well as animals adoptively immunized with spleen cells showed resistance to the homologous organisms (Figure 9). No augmented resistance to the heterologous strain was observed.

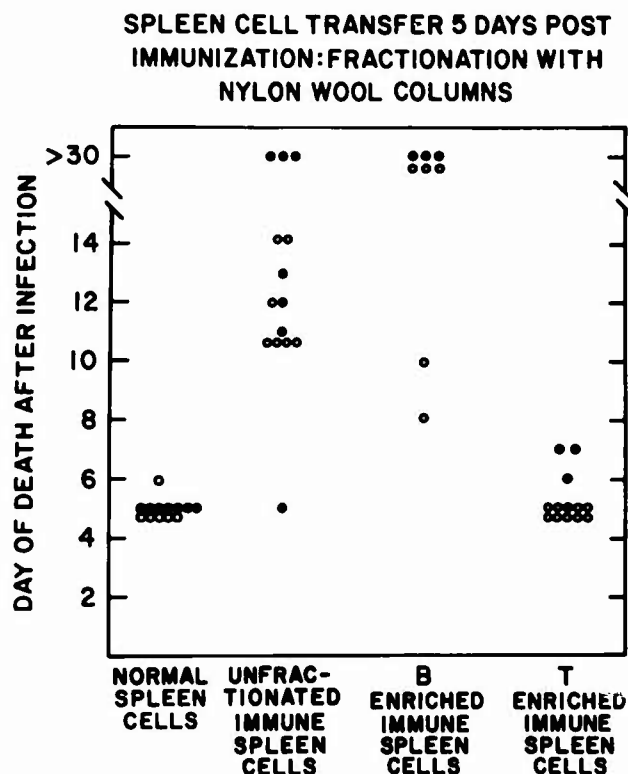


Figure 8. Adoptive transfer of protection to  $10^3$  *T. rhodesiense* organisms with nylon wool column fractionated subpopulations of spleen cells: Immune spleen cells were obtained 5 days post initial exposure to irradiated *T. rhodesiense*. Unfractionated immune spleen cells: untreated (●) or complement treated (○). B enriched immune cells produced by: column adherence (●) or column adherence plus anti-theta and complement treatment (○). T. enriched immune cells produced by: column non-adherence (●) or column non-adherence plus anti-light chain and complement treatment (○).  $50 \times 10^6$  cells were transferred in each group. ○ and ● represent separate experiments.

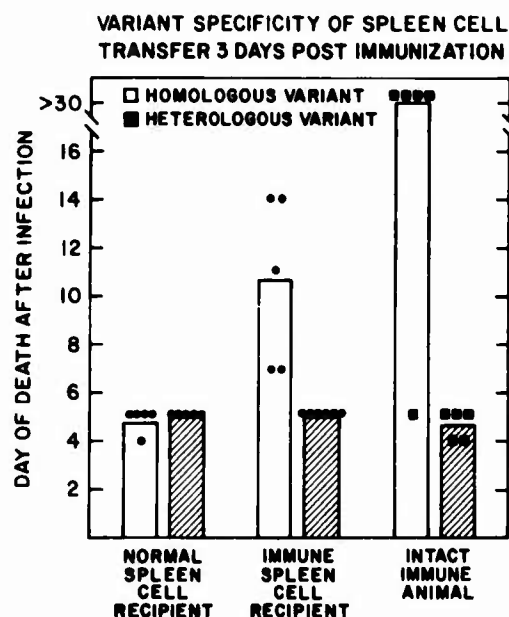


Figure 9. Variant specificity of spleen cell transfer three days post immunization: Mice were challenged with  $10^5$  Wellcome or  $10^5$  variant T. rhodesiense organisms. Mice were immunized with irradiated Wellcome organisms. Significant resistance was observed only when animals were challenged with organisms which were homologous to the initial irradiated exposure organisms.

These results demonstrate that protection is mediated by B lymphocytes in these cell transfer experiments. These experiments however do not show whether or not the T cell may be needed in a collaborative role with antibody forming B cells. To approach this question experiments were performed using athymic nude mice. Since these mice lack a functional thymus they can be used to study the necessity for T cells in controlling infection with trypanosomes or in producing protective immunity after immunization with irradiated organisms.

#### C. Studied of Resistance to T. rhodesiense in Athymic Mice

Athymic (nu/nu) mice backcrossed on the Balb/c line were used. Controls were their phenotypically normal heterozygote (nu/+) littermates. All mice were housed under sterile conditions. Athymic mice were reconstituted by placement of two newborn thymuses under the skin. These

mice were used 30 days later. The EATRO 1886 strain of T. rhodesiense was used for the infection experiments while the Wellcome strain was used for the immunization experiment.

1. Course of infection with T. rhodesiense in athymic or normal mice.

Parasite numbers reached a peak on day 6 in both normal and athymic mice and decreased slowly until day 15 (Fig 10). In normal mice after day 15, the mean number of trypanosomes in the peripheral blood increased more rapidly and steadily than in athymic mice. All normal mice were dead by day 28. The parasite numbers in athymic mice on the other hand rose more slowly in a cyclic fashion. The last athymic mouse died on day 45.

The ability of the athymic mice to control the parasite numbers as well or better than normal mice was seen in this experiment. Since the time of death and parasite numbers appeared different in the two groups of mice, the effect of thymic reconstitution was next studied.

2. Course of infection with T. rhodesiense in athymic, normal and thymic reconstituted mice.

$10^3$  organisms from the same stabulate of organisms used in the previous experiments were injected i.p. into groups of normal, athymic and thymic reconstituted mice. Again the peak number of parasites appeared on day 6 after infection. Parasite numbers were sharply reduced in all groups of mice (Fig. 11). By day 13 parasite numbers were increasing in all groups of mice. In this experiment some cycling of parasite numbers occurred in normal mice. The last normal mouse died on day 32. Survival times in thymic reconstituted mice were similar to those of normal animals with mean parasite numbers intermediated between normal and athymic mice. Parasite numbers in athymic mice again rose slowly in a cyclic fashion. The last athymic mouse survived 48 days. Thus the differences observed between athymic and normal mice can be narrowed by thymic reconstitution.

3. Infection, treatment and rechallenge of athymic and normal mice with T. rhodesiense.

To show that the ability of athymic mice to control the infection as well as normal mice was due to an active host response, mice were infected, cured, and rechallenged. Athymic and normal mice infected for 15 days were cleared of parasites by normal human serum and Berenil treatment (Fig. 12). From days 35 to 49 no parasites were observed. Both athymic and normal mice did not develop parasites after challenge with  $10^3$  organisms on day 49 after the original infection. A control group of 4 normal mice which had not been previously infected but had received NHS and Berenil had circulating parasites three days after challenge.

Serum taken from the athymic and normal mice on day 15 after infection, before NHS treatment, was examined for antibodies by an indirect fluorescent antibody procedure using the original trypanosome inoculum as antigen.

COURSE OF INFECTION OF *TRYPANOSOMA RHODESIENSE* (EATRO 1886)  
IN NUDE AND LITTERMATE MICE

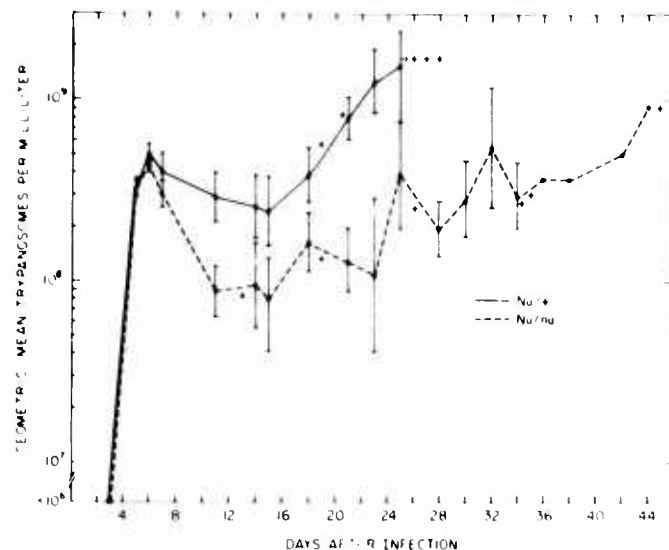


Figure 10. Comparison of geometric mean parasite numbers in athymic (nu/nu, ---) and normal (nu/+, —) mice infected with  $10^3$  *T. rhodesiense* organisms. The day of death of individual mice in each group of 6 mice is marked by +. Each point is the geometric mean  $\pm$  the standard error.

Normal mice showed both IgG and IgM antibody while only IgM antibody could be found in athymic mice (Fig. 13). The mean IgM titer in athymic mice was greater than that in normal mice.

#### 4. Immunization of athymic and normal mice with irradiated *T. rhodesiense*.

As a further indication of the development of active immunity in athymic mice, irradiated trypanosomes were used as immunogen in attempt to immunize normal or athymic mice. Both athymic and normal mice were protected against challenge with  $10^3$  live organisms 12 days later (Table 13).

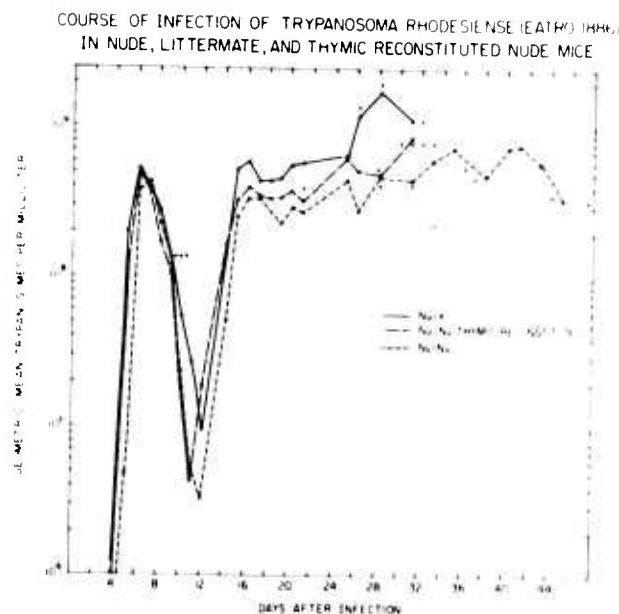


Figure 11. Comparison of geometric mean parasite numbers in athymic (nu/nu TR, --- ---), normal (nu/+, —) and thymic reconstituted (nu/nu TR, — —) mice infected with  $10^3$  *T. rhodesiense* organisms. The day of death of individual mice in each group of 6 mice is marked by +. Thymic reconstituted mice are nu/nu mice which received newborn thymic implants 5 weeks before infection with *T. rhodesiense*.

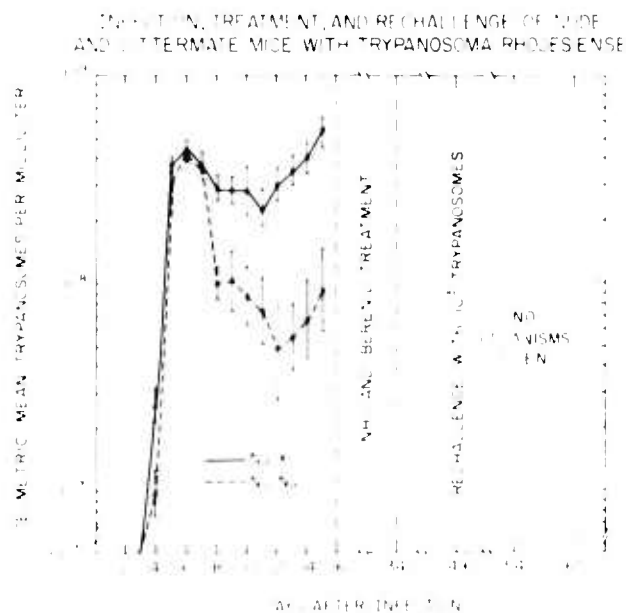


Figure 12. Infection, treatment and rechallenge of athymic (nu/nu, ---) and normal (nu/+, —) mice with *T. rhodesiense*. All mice received NHS on day 15 and Berenil on day 32 and 34. No organisms were present when mice were challenged on day 49. No organisms were seen after challenge. Control (nu/+) mice (not shown) which weren't previously infected but did get NHS and Berneil became parasitemic three days after challenge.

INDIRECT IgG AND IgM FLUORESCENT  
ANTIBODY TITERS 15 DAYS AFTER  
INFECTION WITH T. RHODESIENSE

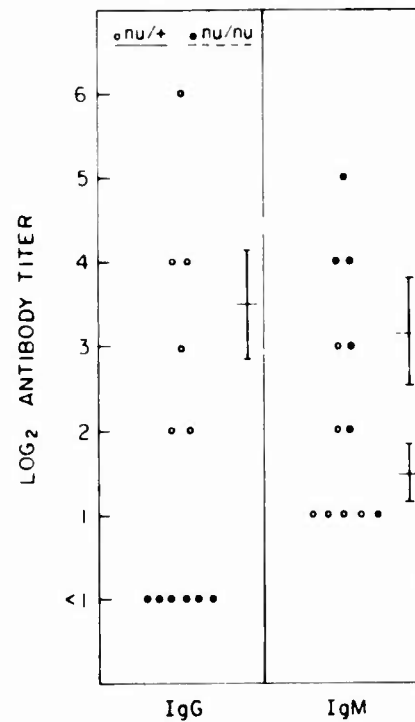


Figure 13. Indirect IgG and IgM fluorescent antibody titers 15 days after infection of athymic (nu/nu, ●---) or normal (nu/+, ○—) mice with  $10^3$  T. rhodesiense organisms. ● and ○ are log<sub>2</sub> titers of individual sera and  $\bar{I}$  or  $\bar{I}$  are the mean titers  $\pm$  standard errors.

TABLE XIII  
IMMUNIZATION OF NUDE AND LITTERMATE MICE  
WITH TRYPANOSOMA RHODESIENSE (WELLCOME)

Mice*	Immunization with Irradiated Organisms	Live Challenge**	When Day Of Death
Nude (Athymic)	--	$10^3$	5.0
	$10^7$	$10^3$	>30
Heterozygote (Normal Control)	--	$10^3$	5.2
	$10^7$	$10^3$	>30

\* 6 mice were included in each group.

\*\* All mice were challenged 12 days after immunization.



Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 202 Antigenic composition of trypanosomes

Publications:

1. Diggs, C., Flemmings, B., Dillon, J., Snodgrass, R., Campbell, G. Esser, K. Immune Serum-mediated Cytotoxicity Against Trypanosoma rhodesiense. J. Immunol. 116, 1005-1009, 1976.
2. Campbell, G.H. and Phillips, S.M., Adaptive Transfer of Immunity to Trypanosoma rhodesiense Infection with Immune Serum or Spleen Cells. Fed. Proc. 35, 415, 1976.
3. Diggs, C, Toussaint, A. and Dillon, J., Neutralization by Antiserum of Infectivity of Trypanosoma rhodesiense for Cobra venom Treated and C5 Deficient Mice. Fed. Proc. 35, 415, 1976.
4. Campbell, G.H., Phillips, S.M. and Esser, K.M. Course of Trypanosoma rhodesiense Infection in Athymic (Nude) mice. Abs. ASM, 86, 1976.
5. Flemmings, B.J., Diggs, C.L. and Powell, C.J. Jr., Alternative Pathway Activation in Antibody Mediated Cytotoxicity Against Trypanosoma rhodesiense. Abs. ASM, 77, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OC 6438	76 06 30	DD DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTR <sup>a</sup>	9. LEVEL OF SUMMARY a. SPECIFIC DATA - CONTRACTOR ACCESS <input type="checkbox"/> YES <input type="checkbox"/> NO	
75 07 01	H. Term	U	U	NA	NL	A. WORK UNIT	
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
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b. CONTRIBUTING							
c. CONTRIBUTING							
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(U) Selective Breeding of Rats for Response to Drugs and Other Agents							
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012600 Pharmacology 012900 Physiology 016200 Stress Physiology 016800 Toxicology							
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20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME <sup>a</sup> JOY, COL R.J.T.				NAME <sup>a</sup> Hunt, E.L.			
TELEPHONE <sup>a</sup> 202-576-3551				TELEPHONE <sup>a</sup> 202-427-5428			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME <sup>a</sup> Hawkins, T.D.			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Pharmacogenetics, (U) Alcohol, (U) Drugs, (U) Dependency, (U) Behavior, (U) Breeding, (U) Genetics, (U) Rats							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>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 bio-effects problems.</p> <p>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 drugs of abuse and models of physical dependence.</p> <p>25. (U) 75 07 - 76 06 Selective breeding of rat family lines was performed using two separate criteria: selection for differential alcohol intakes and selection for sensitivity to audiogenic seizure. Selection for barbiturate intakes was discontinued because of extremely low fertility of high drinker males. Very low susceptibility to audiogenic seizure thresholds was developed. In the seizure prone colony, an outbred subgroup was established. A family line with apparently enhanced sensitivity due to priming was detected. Experiments to document this priming were begun. Audiogenic seizure prone animals were used in several experiments to evaluate the CNS effects of microwave radiation. The selective breeding program is being discontinued. Maintenance and use of the more valuable breeding lines, for example the seizure prone lines, will be continued under other work units. (For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 75 - 30 Jun 76.)</p>							

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

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PII Redacted

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. Darvl Hawkins. M.S.

Description

Selective breeding of rat family lines continued during the FY76 reporting period in three separate areas. These were selection for (1) differential alcohol intakes, (2) differential barbital intakes and (3) sensitivity to audiogenic seizure. Concurrently, a new method for assessing audiogenic seizure sensitivity was developed.

Progress

1. Selection for differential alcohol intakes

Selective breeding to maintain separate inbred lines having either high or low alcohol preference was continued at George Washington University. Two additional filial generations were produced. Litters from each of the lines were assessed for alcohol preference. Other litters were tested for audiogenic seizure proneness. The seizure tests indicated that each of the alcohol lines was very resistant to seizures. In contrast to this result, those from experiments in FY75 showed that the low-alcohol drinker rats were very sensitive to audiogenic seizure during withdrawal. The results, collectively, support the proposition that the low drinker rats are very sensitive to the central nervous system effects of alcohol and this might account for the typical aversion of these animals to alcohol. Development of these lines is to be continued at the Indiana University Medical School under non-DoD funding.

2. Selection for barbital intakes

This work was discontinued mid-year because of the extremely low

fertility of the males that ingested very large amounts of barbital.

### 3. Selection for audiogenic-seizure proneness

Two filial generations were produced from several distinctive inbred lines. The lines exhibit different temporal courses of sensitivity following weaning. Rats from line C are maximally sensitive at three weeks of age, and their sensitivity declines in subsequent weeks. Line B exhibits a relatively constant frequency of audiogenic seizures from weaning to maturity (15 weeks). Line A shows increases in sensitivity from three to five weeks of age. This enhanced sensitivity might be due to an auditory "priming" effect. Experiments were begun to determine whether such a factor was operating.

It is anticipated that maintenance and use of the audiogenic-seizure sensitive rats will be continued for utilization under other work units (e.g., FY77 Work Units 057 and 070).

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)656	
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>	8. DOWN INSTR <sup>a</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM A. WORK UNIT
74 11 01	R. Comp.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	61101A	3A161101A91C		00	205		
B. CONTRIBUTING							
C. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup> (U) Continuation in the development of an experimental seminar program to improve inter-racial and inter-ethnic interaction for WRAIR							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup> 013400 Psychology 015400 Sociology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
74 11		76 06		DA		C. In-house	
17. CONTRACT GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER:				FISCAL YEAR		0.1	
C. TYPE:				CURRENT		00	
D. KIND OF AWARD:				76		0.1	
E. CUM. AMT.						00	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research ADDRESS: Washington, DC 20012				NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL NAME: JOY, R.J.T., COL TELEPHONE: 202-576-3551				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: Holloway, H.C., COL TELEPHONE: (202) 576-3556 SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21. GENERAL USE Foreign intelligence not considered				ASSOCIATE INVESTIGATORS NAME: NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Race relations; (U) Inter-racial training; (U) Inter-ethnic interaction; (U) Behavior							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code) (23) (U) Development of an experimental pilot program to improve racial and ethnic interactions, particularly among military medical personnel.  (24) (U) Preparation of documentary and visual materials; preparation of lesson plans and research into the perception of racial and ethnic differences.  (25) (U) 75 07 - 76 06 During the last year an appropriate bibliographic and conceptual base has been developed. Using this base, as expanded by WRAIR staff, a syllabus has been developed. These materials are being used in ongoing Race Relations courses at WRAIR and WRAMC. The Final Report and visual aid materials, including an annotated bibliography concerning Experience of Black Americans Serving in the American Armies, are nearing completion and should be made available in Final Report in Sep 1976. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 75-30 Jun 76.							

<sup>a</sup>Available to contractors upon on-memorandum approval

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 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 205 Continuation in the development of an experimental seminar program to improve inter-racial and inter-ethnic interaction for WRAIR

Investigator.

Principal: Harry C. Holloway, MD, COL, MC

DESCRIPTION

Development of an experimental pilot program to improve racial and ethnic interactions, particularly among military medical personnel.

PROGRESS

During the last year an appropriate bibliographic and conceptual base has been developed. Using this base, as expanded by WRAIR staff, a syllabus has been developed. These materials are being used in ongoing Race Relations courses at WRAIR and WRAMC. The final report and visual aid materials, including an annotated bibliography concerning Experience of Black Americans Serving in the American Armies, are nearing completion and should be made available in Sep 1976.



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)636	
3. DATE PREV. SUMMRY <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8a. DISSEM INSTRN <sup>a</sup>	8b. SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	9. LEVEL OF SUM <sup>a</sup>
75 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	61101A	3A161101A91C	00	206			
b. CONTRIBUTING							
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Factors Influencing Secretary States							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
003800 Life Support 016200 Stress Physiology							
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	
a. DATES/EFFECTIVE: NA				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER <sup>a</sup>				FISCAL YEAR		c. FUNDS (in thousands)	
c. TYPE				76		2	
d. KIND OF AWARD				77		75	
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. Academic Institution)			
NAME: Joy, COL Robert J. T.				NAME: Trout, MAJ Hugh H. III			
TELEPHONE: 202 576-3551				TELEPHONE: 202 576-3284			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Harmon, MAJ John W.			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Hyperacidity; (U) Stress; (U) Trauma; (U) Gastric Acid							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) The technical objective is to validate the hypothesis that antrectomy in rhesus monkeys will decrease the maximal gastric acid secretion in response to both pentagastrin and histamine. These studies are of military importance because they will enable the surgeon to make better clinical assessments as a basis for treating soldiers with increased gastric secretion secondary to trauma.							
24 (U) Monkeys will be prepared with gastric fistulas and dose-response data of gastric acid output will be collected using both histamine and tetragastrin as stimulants. Antrectomies will be performed and the dose-response studies repeated.							
25 (U) 75 07-76 06. The technique of studying monkeys has now been established. The antrectomy results are not yet completed but our preliminary findings indicate that, unlike all other species yet tested, the rhesus monkey responds more fully to histamine than to tetragastrin. This has significant importance since it may alter our current beliefs as to the mechanisms of gastric acid secretion in man. In addition, further studies are being planned to evaluate the effects of division of the vagus nerves in the monkey.							
Support in the amount of \$9,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75-30 Jun 76.							

\* Available to contractors upon originator's approval.

DD FORM 1498

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 206 Factors influencing secretory states

Investigators

Principal: Hugh H. Trout, III, MC

Associate: John W. Harmon, MAJ, MC

I. Antrectomy in Dogs

A. Background and Statement of the Problem. Resection of the antrum and duodenal bulb in man results in a decrease in maximal histamine stimulated gastric acid secretion. In marked contrast to these findings in man, it has been shown in dogs and cats with vagally innervated gastric pouches (Pavlov pouches) that antrectomy results in an increase in gastric acid secretion in response to either of the stimulants histamine or pentagastrin (1,2,3). The present study was undertaken to determine if these differences were species related or were due to the different types of preparation (essentially a gastric fistula in man as opposed to isolated gastric pouches in the experimental animal). In addition the investigations were modified in an attempt to explore possible causes for these differences.

B. Experimental Approach. Dogs were prepared with gastric fistulas and dose response data were collected using histamine and tetragastrin as stimulants and acid output as response. The vagal nerves to the antrum of the stomach were divided and the studies were repeated. Finally the antrum was removed and the studies were once again repeated.

C. Results and Discussion. Division of the vagal fibers to the antrum had little effect on acid output in response to either histamine or tetragastrin. Antrectomy increased acid response to both stimulants. These results indicate that the earlier work with pouches is a true reflection of the acid secreting response following antrectomy in the dog or cat. It demonstrates that these differences are species related and are not due to alterations created by the construction of fundic pouches. The results showing no significant change after division of the vagal nerves to the antrum show that the increased acid secretion seen after antrectomy are not due to any interference with vagal innervation of the stomach or small bowel when the antrectomy is performed.

II. Histamine and Gastrin in Monkeys

A. Background and Statement of the Problem. There is a continuing controversy over substances ultimately responsible for gastric acid



secretion. Gastrin, histamine and acetylcholine have each been proposed as being the primary stimulant. In cat, dog and human, gastrin and its analogs elicit the same maximal gastric acid output as does histamine. The monkey, however, has not yet been carefully studied (4). Since the monkey is phylogenetically closely related to the human, a more thorough comprehension of the factors regulating gastric acid secretion in the monkey may prove to be of benefit in better understanding human gastric secretory mechanisms.

B. Experimental Approach. Rhesus monkeys were prepared with gastric fistulas; dose-response data in conscious non-sedated animals were collected using histamine, tetragastrin, pentagastrin, and gastrin as stimulants and acid output as response. Other studies were performed using histamine and tetragastrin in combination with a continuous infusion of carbachol, and acetylcholine analog.

C. Results and Discussion. Histamine elicited a peak acid output response which was double that achieved by gastrin or any of its analogs. The addition of carbachol did not significantly alter the acid response to either histamine or tetragastrin. The meaning of these results is unclear but suggests that there are serious gaps in our understanding of the regulatory mechanisms of gastric acid secretion at least as they apply to the Rhesus monkey. Further studies involving resection of the antrum or transection of the vagus nerves are being performed.

### III. Antrectomy in Monkeys

A. Background and Statement of the Problem. An earlier investigation indicated that antrectomy in the monkey completely abolished gastric secretory response to histamine (5). Since our previous work has indicated that the doses used in the other investigation were not sufficient for maximal acid response and since, if the earlier work is correct, this finding would be of major importance in understanding gastric secretory physiology in the monkey we are repeating these studies using a slightly more sophisticated model and a more adequate dose schedule.

B. Experimental Approach. Rhesus monkeys were prepared with gastric fistulas; dose-response data were obtained in conscious nonsedated animals using histamine and tetragastrin as stimulants and gastric acid output as response. Antrectomies were performed.

C. Results and Discussion. Sufficient time for testing has not yet elapsed following antrectomy.

### IV. Partial Vagotomy in Dogs

A. Background and Statement of the Problem. Highly selective vagotomy which consists of dividing the vagal nerves to the acid

secreting portion of the stomach is an operation which is becoming increasingly popular. The advantages of this operation are that the gastrointestinal tract does not need to be opened and there are fewer postoperative side effects. Essentially the operation consists of two parts - first the division of vagal fibers along the lesser curvature of the stomach which innervate the acid secreting portion of the stomach and second the division of fibers around the esophagus. This study was designed to determine what the relative effect of each of these two components of the operation have in acid secretion.

B. Experimental Approach. Two groups of 4 dogs were prepared with gastric fistulas. Control studies were undertaken testing with histamine, tetragastrin and 2-deoxy-d-glucose. One group then had the vagal fibers to the less curvature divided and the other group had the fibers around the esophagus transected. The dogs were then retested and then had the remainder of their fibers divided thus completing a highly selective vagotomy in each dog. Final repeat testing was then carried out.

C. Results and Discussion. The final operations and repeat testing have not yet been completed.

## V. Duodenal Mucosa

A. Background and Statement of the Problem. The duodenal mucosa has recently been shown to have a significant capacity to dispose of acid without contribution from the pancreas or liver. A defect in duodenal disposal of acid could result in increased gastric acidity by slowing the gastric emptying of acid. This mechanism could be important in the pathogenesis of gastric as well as duodenal ulceration.

B. Experimental Approaches. An experimental preparation has been developed using a duodenal pouch in a chronic dog with 2 Gregory cannulas and a recirculating system through a reservoir with a pH stat titrator system. This allows minute to minute measuring of duodenal acid loss at varying rates of perfusion and at varying acid concentrations. The questions of the relative influence of rate and concentration, the role of bicarbonate in the system as determined by the influence of acetazolamide, the effects of aspirin and bile salts and the effects of cyanide are all being studied.

C. Results and Discussion. Initial results show that the rate of acid loss is dependent on both the concentration of acid and the rate of perfusion (6,7). Diamox inhibits acid loss and this varies at different acid concentrations. Cyanide and aspirin do not seem to affect acid disposition by the duodenum.

## VI. Estrogen-like Effects of Cannabinoids

A. Background and Statement of the Problem. The possibility that marijuana could have estrogen-like effects was raised by our initial report of gynecomastia in chronic marijuana users (8) and by a subsequent report of an animal model in which  $\Delta^9$  tetrahydrocannabinol (THC) caused breast stimulation in rats. Marijuana may reduce serum testosterone levels and reduce sperm counts in humans. Marijuana increases uterine weight in rats.

B. Experimental Approach. The effect of  $\Delta^9$  THC on rat testicular development was studied in Walter Reed rats. The rats were given THC orally to avoid the stress of injection and the controls were pair-fed with the THC animals. Testicular weight, body weight, kidney weight, serum testosterone, testicular testosterone, serum follicle stimulating hormone, serum luteinizing hormone, and serum prolactin were all determined. The reversibility of any effects were determined by allowing a THC treated group a 60 day recovery from THC before study.

C. Results and Discussion.  $\Delta^9$  THC was shown to specifically delay testicular development as evidenced by lower testicular weights in THC treated animals as compared with controls (9). Serum and testicular testosterone were also decreased in THC treated animals. FSH, LH and prolactin were unchanged. The effects were reversible in 60 days off THC. The tests of the THC treated animals had histologic defects which allowed them to be distinguished from controls.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 206 Factors influencing secretory states

Literature Cited.

References:

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2. Anderson, S., and Grossman, M. I.: Effect of antrectomy on gastric secretion of acid and pepsin in response to histamine and gastrin in dogs. *Gastroenterology* 49:246, 1965.
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6. Gurll, N. J., and Harmon, J. W.: Canine duodenal mucosal neutralization of acid. *The Physiologist* 1976, in press.
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8. Harmon, J. W., and Aliopoulos, M. A.: Gynecomastia in marijuana users. *N. Eng. J. Med.* 287:936, 1972.
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Publication

Harmon, J. W., Trout, H. H., Gurll, N. J., and Carner, J. W.: Effect of proximal gastric vagotomy on gastric emptying of semisolid food slurry. *Gastroenterology* 70: A35/893, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL	
					DA OC 6441	76 07 01	DD-DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISSEM INSTRN	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM	
75 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	61101A	3A161101A91C		00		207		
B. CONTRIBUTING								
C. CONTRIBUTING								
11. TITLE (Precede with Security Classification Code)								
(U) Model System for Antiparasitic Drugs								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS								
002600 Biology								
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		A. PROFESSIONAL MAN YRS		B. FUNDS (in thousands)		
NA		PRECEDING						
A. DATES/EFFECTIVE:		EXPIRATION		FISCAL YEAR				
B. NUMBER		C. TYPE:		CURRENT				
D. KIND OF AWARD:		E. CUM. AMT.		7/		2.30		
19. RESPONSIBLE DOD ORGANIZATION		20. PERFORMING ORGANIZATION		NAME		Walter Reed Army Institute of Research, Div, Medicinal Chemistry		
NAME: Walter Reed Army Institute of Research		NAME: Walter Reed Army Institute of Research, Div, Medicinal Chemistry		ADDRESS		Washington, DC 20012		
ADDRESS: Washington, DC 20012		PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)		NAME		Davidson, David E. LTC		
RESPONSIBLE INDIVIDUAL		NAME		TELEPHONE		202-576-2292		
NAME: Joy COL R.		NAME: Davidson, David E. LTC		SOCIAL SECURITY ACCOUNT NUMBER				
TELEPHONE: 202-576-3551		ASSOCIATE INVESTIGATORS		NAME		Loizeaux, P. S. LTC		
21. GENERAL USE		NAME		Ketterling, L. L. CPT				
Foreign Intelligence not considered								
22. KEYWORDS (Precede EACH with Security Classification Code)								
(U) Leishmaniasis; (U) Schistosomiasis; (U) Drug Development; (U) Anti-parasitic; (U) Animal Models; (U) Trypanosomiasis								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
<p>23. (U) To develop new test systems and improve existing ones for identification and development of drugs to prevent and treat parasitic diseases in military personnel.</p> <p>24. (U) Development of animal model test systems capable of rapidly screening large numbers of chemical compounds for their anti-parasitic activity.</p> <p>25. (U) 75 07 - 76 06 From 250 compounds tested by topical application in a mouse model, two chemical classes have been identified which provide complete protection against skin penetration by <i>Schistosoma mansoni</i> cercariae. The best of these compounds afford up to 72 hours of protection in concentrations as low as 0.3 percent, and cosmetically acceptable, and resist removal by washing. Studies of 1A-3-N-oxide and 1A-4-N-oxide, promising antischistosomal drugs, are being conducted in the Rhesus Monkey-Schistosoma mansoni model.</p> <p>A Leishmania tropica mouse model and a Leishmania donovani monkey model are under development, and early trials with experimental and reference drugs are in process.</p> <p>An in vitro system for mass screening of antitrypanosomal drugs is also under development. One promising antitrypanosomal drug has been partially evaluated in the rhesus monkey model. For technical report, see Walter Reed Army Institute of Research Annual Progress Report July 75 - Jun 76.</p> <p>Support in the amount of \$26,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>								

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 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 207 Model systems for antiparasitic drugs

Investigators.

Principal: LTC David E. Davidson, Jr., VC

LTC Peter S. Loizeaux, VC

Associate: MAJ Robert E. Desjardins, MC

CPT Lyle L. Ketterling, MSC

Marie M. Grenan; Gloria P. Willet

1. Description

Schistosomiasis, Leishmaniasis, and African Trypanosomiasis are parasitic diseases which pose a serious threat to U.S. military personnel who may be required to operate in tropical and subtropical areas of the world. All drugs which are currently available for use against these diseases are, without exception, less than fully effective or have serious side effects. Accordingly, efforts to develop drugs against these parasitic diseases have been initiated. Initial efforts are being directed toward developing laboratory methods to screen and evaluate chemotherapeutic and chemoprophylactic agents.

In-house research is supported by and coordinated with contract research.

2. Progress

a. Schistosomiasis

1.) Primary Antischistosomal Drug Screens

Primary screening of drugs for antischistosomal activity is performed by the Medical Research Unit, Brasilia, Brazil, using Schistosoma mansoni in the laboratory mouse. The Division of Medicinal Chemistry, WRAIR, selects and provides the drugs for evaluation, analyzes test results, maintains the computerized data base of test information, reports results, and makes recommendations for the synthesis of new compounds. During FY 76, results were received and evaluated for 1498 compounds tested prophylactically. Ten commercially discreet compounds showed activity worthy of further investigation. Therapeutic testing was initiated late in the fiscal year. No active compounds were found among the 16 drugs tested.

## 2.) Cercarial Antipenetration Tests

During this period a test system was established to determine the ability of drugs to prevent skin penetration by schistosome cercariae. The test system utilizes S. mansoni in the Walter Reed strain ICR mouse. Test drugs are routinely prepared for topical administration in an appropriate vehicle (usually 95% Ethanol) in concentrations of 5% and 2.5%. The tail of each mouse is cleaned and placed in the drug solution for 5 minutes. Twenty-four hours later mice are exposed to  $100 \pm 10$  infective S. mansoni cercariae for one hour by tail immersion. Forty-nine days after cercarial exposure the mice are anesthetized with sodium pentobarbital-heparin solution and worms are collected from the liver and mesentery by perfusion. The number of worms present in treated mice is compared to the number found in control mice treated with vehicle only. Worm burdens less than 50% of the control burden are considered significant.

Compounds found active in the initial screen are subjected to a "wash" test. Drug treatment is unchanged; however, one hour prior to cercarial exposure the tails of treated and control mice are washed by placing them in a continuous flow of warm tap-water for 30 minutes. The wash test indicates the resistance of drug and vehicle to removal through water solubility or by the mechanical action of flowing water. Following washing, the tails are air-dried and the test continues as described above. Compounds active in the wash test are evaluated further to determine the duration of drug effectiveness, optimal and minimal effective concentration, and optimal vehicle.

During the reporting period 250 compounds were tested, of which 32 significantly inhibited cercarial penetration following the wash test. Of these, 7 compounds representing 4 chemical classes completely prevented penetration. Compounds WR 17,018 and WR 22,537 were completely protective for 24 hours at concentrations as low as 0.63%.

## 3.) Antischistosomal Drug Studies in Subhuman Primates

WR 223,630, a chloroindazole analog of hycanthone, was evaluated against S. mansoni infections in rhesus monkeys. Ten monkeys were divided into 5 groups of 2 animals each. Groups I and II received prophylactic treatment; groups III and IV received therapeutic treatment. Groups I and III received the drug via the intramuscular route; groups II and IV were treated by gavage. Group V served as untreated controls. Results indicated that WR 223,630 was more effective when administered therapeutically. Fecal egg outputs from these animals fell to zero immediately after treatment, although pre-treatment

counts were as high as 900 eggs per gram of feces (EPGF). No more than 3 adult worms were recovered from any treated monkey by perfusion, and all of these worms were severely stunted. Prophylactic treatment was not totally effective. The onset of patency, as determined by the appearance of eggs in the feces, was delayed by 2-3 weeks and never reached more than 72 EPGF, compared with a peak of 624 EPGF for the corresponding control monkey. Few worms (maximum of 40) were recovered from the IM treated animals, and these were stunted and abnormal. Worms recovered from the orally treated animals appeared normal. Pathologic manifestations correlated well with worm burdens and egg output data. Of the treated animals, Group II was the most severely affected as evidenced by active, multiple lesions of the liver and colon. In other groups, lesions ranged from moderate or mild granulomatous reactions representing healing lesions (groups I and III) to essentially no lesions (group IV) that could be considered characteristic of schistosome infection.

b. Leishmaniasis

1.) Visceral leishmaniasis

The primary anti-leishmanial screen uses male golden hamsters as the host for the Khartoum strain of L. donovani. Seven animals per group are injected intracardially with 10 amastigotes obtained from spleens of donor hamsters. Beginning on the third day after parasite inoculation, test compounds are administered twice daily for four consecutive days. Animals are killed on day 7 following infection, livers removed and individually weighed, and the total parasite numbers per liver determined from impression smears. Data are compiled and evaluated with the aid of a computer. The percent suppression compared to infected, untreated controls is calculated. Percent suppression is also compared to that observed in infected, glucantime treated control hamsters. Glucantime is greater than 90% suppressive at 104 mg/kg/day. A glucantime index is calculated at the 90% suppressive level.

During this reporting period 522 compounds were screened. Of these, 117 were found to be active, of which 42 received multiple additional tests. Thirty-nine compounds showed antileishmanial activity equal to or greater than glucantime, the reference drug. All but two of these compounds belong to a single chemical class. Seven compounds of this class have activity more than 100 times that of glucantime.



Special testing was performed on WR 6026, one of the most promising drugs. The compound was administered at various time intervals following infection to determine the effect on later stages of the disease. WR 6026 is considerably less effective against established infections than against early infections, but it is superior to glucantime at any stage of infection.

## 2.) Cutaneous leishmaniasis

A drug screen has been developed to identify compounds having activity against the cutaneous form of leishmaniasis, using the BALB/C mouse as the host for the Seidmann strain of L. tropica. Approximately 500,000 amastigotes harvested from donor mice are injected into a footpad of the test animal. In 3 weeks a tumorous lesion develops which contains massive numbers of multiplying amastigotes. Drug treated animals receive test compounds on each of 4 successive days starting 3 days after parasite inoculation. Antileishmanial drug activity is indicated by inhibition of the size and rate of development of the tumor, which is measured and compared with the contra-lateral uninfected foot pad and with the tumor in untreated control mice. Drug screening was initiated with 12 "standard" antiparasitic drugs, but none exhibited more than marginal activity against L. tropica. Screening is currently being performed at the rate of 6 drugs per month.

## c. African Trypanosomiasis

### 1.) Primary Mouse Screen

A total of 1750 compounds were screened in vivo in the primary mouse test utilizing the Wellcome CT strain of Trypanosoma rhodesiense. Compounds were administered subcutaneously to mice which had been lethally infected by intraperitoneal injection of 0.5 ml of a 1:50,000 dilution of heparinized blood from heavily infected donor mice. Active compounds extend survival time beyond the  $4.5 \pm 0.3$  days observed in untreated infected controls. Treated mice surviving 30 days are considered "cured."

Since late in 1972, approximately 7000 compounds have been treated in this system, and approximately 250 have exhibited activity. The active compounds fall into more than 10 chemical classes including: 1) diamidines, 2) terephthalanilides, 3) tri-phenyl-phosphoniums, 4) bis-quinolines, 5) arsenicals, 6) triazines, 7) nitrofurans, 8) nitrothiazoles, 9) phenanthiridines, 10) puromycin analogues, 11) miscellaneous.

### 2.) Development and Assessment of an in vitro Screen for Potential Antitrypanosomal drugs

a. Objective

During the past 12 months pilot studies were undertaken to examine the feasibility of using an in vitro test for primary screening of potential antitrypanosomal drugs in a microtiter plate system. While the system is not yet functional as a drug screen the following progress has been made.

b. Selection of horse serum as a media supplement

Initial culture techniques were similar to those employed by the Department of Immunology for the study of antisera and involved the use of a glucose enriched medium TC199 with added rat serum. Substitution of fetal calf serum for rat serum in the medium was not successful. Since the use of rat serum was tedious and too expensive for large scale use in a screening assay, several alternate sources of serum were tested. Human, bovine, rabbit and rhesus monkey sera were unsatisfactory, i.e., they would not support growth; however, commercially available decomplexed horse serum (Microbiologic Associates) was found to support uptake of  $^3\text{H}$ -thymidine and  $^{14}\text{C}$ -isoleucine as well as or better than rat serum. Horse serum has been used in all subsequent cultures.

c. Comparison of BGJ, TC199 and RPMI 1640

These three commercially available tissue culture media were tested for their ability to support growth of trypanosomes as indicated by  $^3\text{H}$ -thymidine uptake. As predicted from its nutrient composition, BGJ medium proved most efficacious, with the parasite remaining morphologically unchanged (long-slender blood form) during 3 hours of incubation at  $37^\circ\text{C}$ . The optimum period of growth and incorporation of isotope was 2 hours.

d.  $^{14}\text{C}$ -glucosamine incorporation

In BGJ and other glucose-containing media, even in the absence of supplemental glucose, the uptake of  $^{14}\text{C}$ -glucosamine was too low for practical measurement. This finding, retrospectively, is not surprising in view of the active transport mechanism for all hexoses and hexosamines in most cell membranes. For like reason  $^{14}\text{C}$ -mannose was also poorly incorporated. Initial attempts to culture the organism in glucose-free solutions (without serum) were unsuccessful.

Recently, exploring the possibility of enhancing hexose transport by brief preincubation in the absence of hexose, it was discovered that the organism remains viable (motile) and morphologically

unchanged during 3 hours of incubation in a phosphate buffered saline solution with 30% horse serum, and readily incorporated thymidine and glucosamine.

$^{14}\text{C}$ -glucosamine and  $^3\text{H}$ -thymidine transport in this PBS + horse serum medium at  $37^\circ\text{C}$  peaks at 2 hours of incubation and is quite reproducible. In a recent experiment, *T. rhodesiense* was cultured in this medium for 2 hours at  $37^\circ\text{C}$  with added  $^{14}\text{C}$ -glucosamine ( $0.5\ \mu\text{Ci/ml}$ ) and  $^3\text{H}$ -thymidine ( $1.5\ \mu\text{Ci/ml}$ ), then harvested on a MASH III Automated Cell Harvester. The mean  $\text{DPM} \pm \text{S.E.}$  of 24 replicate wells was  $8704 \pm 200$  for  $^3\text{H}$  and  $1232 \pm 30$  for  $^{14}\text{C}$ . The MASH III is capable of washing the harvested trypanosomes with a cold  $40^\circ\text{C}$  TCA 10% solution. In this way it was demonstrated that the  $^{14}\text{C}$  present had been incorporated into TCA insoluble material, i.e., DNA or protein. It remains to be demonstrated whether the label is present in the glycoprotein coat of the trypanosome as anticipated.

e. Incorporation of amino acids

The difficulty initially encountered with  $^{14}\text{C}$ -glucosamine incorporation prompted testing of a variety of  $^{14}\text{C}$  labeled amino acids. In BGJ + glucose + horse serum  $^{14}\text{C}$ -leucine,  $^{14}\text{C}$ -isoleucine,  $^{14}\text{C}$ -methionine and  $^{14}\text{C}$ -threonine were all readily transported. None, however, could be demonstrated, by pronase digestion, to have localized in the glycoprotein coat.

f. Ability of the dual label system to detect different drug effects on DNA and protein synthesis.

Using  $^{14}\text{C}$ -isoleucine uptake as an indication of protein synthesis and  $^{14}\text{C}$ -thymidine uptake as an indicator of DNA synthesis theophylline was added to the culture in multiple dilutions. A sigmoid dose-effect curve was obtained with suppression of  $^3\text{H}$ -thymidine incorporation between  $0.0195\ \text{mM}$  and  $1.25\ \text{mM}$  theophylline and no depression of  $^{14}\text{C}$ -isoleucine uptake. The presumed mechanism of action (enzyme inhibition of cyclic AMP phosphodiesterase) was tested by repeating the culture with added dilutions of N $^6$ -2'-O-Dibutyryl Cyclic AMP. Identical differential dose-effect curves were obtained.

g. Optimization of concentration of organisms in culture.

In the interest of optimizing efficiency, it was considered desirable to use the highest concentration of organisms in culture that would sustain growth. In practice, it was found that with initial concentrations higher than  $2.5 \times 10^7/\text{ml}$  the reliability of the automated harvesting system decreases; however, this has proved to be a convenient and adequate starting concentration in the culture system.

#### h. Summary

It is possible to demonstrate incorporation of  $^{14}\text{C}$ -glucosamine into protein and of  $^3\text{H}$ -thymidine into DNA simultaneously in short term cultures of blood-form T. rhodesiense. The medium employed is an inexpensive solution of phosphate buffered saline and 30% horse serum. At  $37^\circ\text{C}$  maximum incorporation occurs at 2 hours of incubation, at which time the parasites remain morphologically intact and actively motile. The isotope is added in reasonably small quantities of  $0.5\ \mu\text{Ci/ml}$   $^{14}\text{C}$  and  $1.5\ \mu\text{Ci/ml}$   $^3\text{H}$  with final counts of  $\sim 1200\ \text{DPM}$  ( $^{14}\text{C}$ ) and  $\sim 8000\ \text{DPM}$  ( $^3\text{H}$ ) and S.E. of  $\pm 2.5\%$ .

Additional work to complete the feasibility studies requires demonstration of the  $^{14}\text{C}$  label in the glycoprotein coat and the application of known antitrypanosomal drugs into the generation of dose-effect curves.

#### 3. Summary

Existing animal models for Schistosomiasis, Leishmaniasis and Trypanosomiasis have been adapted to the screening of drugs for antiparasitic activity, and in some instances new methodology has been developed. These models have been utilized in the screening and evaluation of potential chemoprophylactic and chemotherapeutic agents. An in vitro trypanosomiasis screening system is under development.

Project 3A161102B71P  
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01  
Biomedical Sciences

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL DD FORM 1 (APR 65)	
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75 07 01	D. Change	U	U	NA	NL	8. SPECIFIC DATA CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF WORK UNIT
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NAME: Joy, COL R.J.T.				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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				ASSOCIATE INVESTIGATORS			
				NAME: Watts, Dr. D. M.			
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22. KEYWORDS (List in order of importance with Security Classification Code) (U) Arboviruses; (U) Ecology; (U) Mosquitoes; (U) Disease Vectors; (U) Control; (U) Taxonomy							
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) 75 07 - 76 06. Infection process of 2 strains of California encephalitis group viruses - Jamestown Canyon (JC) and Keystone (KEY) were studied in whitetail deer. Only the former strain produced a viremia, but both strains produced infection. Prior infection with JC interfered with subsequent KEY infection. Aedes atlanticus mosquitoes were found to be much more susceptible to KEY infection than were Aedes canadensis mosquitoes. Experimental transmission was not accomplished with either species. Natural infection of gray squirrels with KEY was demonstrated in the field. No St. Louis encephalitis infected mosquitoes were found among 1,116 overwintering Culex pipiens mosquitoes collected. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$52,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORM 1490A, 1 NOV 75 AND 1490B, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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

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Description

This task involves field and laboratory studies of the relationships 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. Ecological Studies of Keystone and Jamestown Canyon Viruses in the Pocomoke Cypress Swamp, Worcester County, Maryland

a. Introduction. Since the discovery that Keystone (KEY) and Jamestown Canyon (JC) viruses were enzootic in the coastal plains of Maryland, studies have been aimed at defining their ecologies. The woodland mosquito, Aedes atlanticus has been implicated as the primary vector and reservoir of KEY virus on the basis of (1) repeated isolations of KEY virus from field collected A. atlanticus each year since 1971, (2) occurrence of KEY virus infection in sentinel rabbits that correlated in time and space with the adult A. atlanticus population, (3) demonstration of A. atlanticus feeding on small forest-dwelling mammals, and (4) demonstration of transovarial transmission of KEY virus by A. atlanticus. The vertebrate species involved in the natural cycle have not been determined; however, limited serological surveillance studies suggest either gray squirrels and/or cottontail rabbits serve as the principal vertebrate host. In addition, host preference studies have shown A. atlanticus to feed frequently on whitetail deer, thus posing a question regarding this species' role in the ecology of KEY virus. Attempts to identify the mode of transmission of JC virus have not been very successful; however, serological data strongly implicate whitetail deer as being the primary vertebrate host. Even though the virus has been isolated twice from A. canadensis collected in the Pocomoke Cypress swamp environ, the very low minimal field infection rate fails to support a primary vector role for this species. Major research objectives were:



(1) Assessment of the vector potential of Aedes atlanticus, Aedes canadensis and Psorophora ferox following their ingestion of various concentrations of KEY and JC virus.

(2) Determination of the ecological role of selected vertebrates in the maintenance cycle of KEY and JC virus.

(3) Determination of the relationship of gray squirrels to the maintenance cycle of KEY virus in the Pocomoke Cypress Swamp (PCS) study area.

(4) Determination of the seasonal distribution and relative population density of A. atlanticus and the dynamics of KEY virus infection in this mosquito.

#### b. Materials and methods

##### (1) Stock viruses

KEY virus used in this study was isolated in 1972 from a pool (#1736) of A. atlanticus that was collected in the PCS study area. This virus had received 4 passages in baby hamster kidney cell culture (BHK-21, Clone 13). JC virus was isolated from a pool (#4) of A. canadensis that were collected in the same study area in 1971. This virus had received 2 mouse brain passages and 2 subsequent passages in BHK-21 cells. The viruses were identified in plaque reduction neutralization tests (PRNT) in which known prototype CE virus and homologous as well as heterologous antisera were employed. Similar viruses had been used previously at WRAIR in the Department of Virus Diseases as reference stocks.

Virus stocks were prepared by inoculating 1.0 ml aliquots of approximately  $10^{3.0}$  suckling mouse lethal dose<sub>50</sub>/1.0 ml of each virus onto medium free monolayers<sup>1/</sup> of BHK-21 cell cultures. After 1 hour at 35 C with 15 minute interval agitation, 25 ml of medium 199<sup>2/</sup> was added to each cell monolayer. Each day cell cultures were examined microscopically for cytopathic effect (cpe). Once cpe approached 75%, the remaining cells were removed from their glass substrate and the contents of each bottle were transferred to a chilled erlenmeyer flask. The FBS concentration was increased to 20% and the suspension was centrifuged for 1 hour at 8000 rpm in a refrigerated centrifuge. The supernatant was transferred in 0.5 ml aliquots to sterile 1 dram vials that were stored at -70 C. Each virus stock was tested for sterility according to standard bacteriological technique. The identity of virus stock was confirmed by PRNT employing virus specific mouse hyperimmune ascitic fluids.

<sup>1/</sup> A 1 to 2 log<sub>10</sub> increase in infectivity titers of stock viruses resulted by inoculating cell monolayers that were 50 to 75% confluent as opposed to inoculating confluent monolayers.

<sup>2/</sup> Medium 199 contained 2% fetal bovine serum (FBS), 1% NaHCO<sub>3</sub>, penicillin (200u/ml) and streptomycin (150 ug/ml).



## (2) Antisera

Adult New Zealand white rabbits were inoculated intravenously (IV) with  $10^5$  to  $10^6$  SMLD<sub>50</sub>/0.03 ml of the desired stock virus. On day 14 post inoculation (PI) approximately 25 ml of blood was drawn as a source for specific antiserum. A second virus inoculum equal to or greater than the infectivity titer of the first was administered IV on day 21 PI. On day 30 rabbits were exsanguinated and the blood was allowed to clot for expression of serum.

Mouse hyperimmune ascitic fluids were prepared in 7 to 8 week old female mice as described by Brandt et al. (1967)<sup>1</sup> and Chiewslip and McCown (1972)<sup>2</sup>.

Virus neutralizing titers and specificity of each antiserum was determined in PRNT employing homologous and heterologous viruses.

## (3) Cell Cultures

Cell culture lines considered for use in plaque assay and PRNT were BHK-21, clone 13, BHK-21 clone 15<sup>3</sup>, Vero cells and LLC-MK<sub>2</sub> cells. BHK-21 cells were grown in Eagle basal medium containing Hanks'<sup>2</sup> salts and 10% FBS. The LLC-MK and Vero cells were cultured in medium 199 plus 20% FBS. Each type of medium was supplemented with 0.75% NaHCO<sub>3</sub> (1 ml/100 ml of medium) and penicillin (200 µ/ml) and streptomycin (150 µg/ml). Cell lines were maintained in 32 oz. prescription bottles and were dispersed for subcultivation with 0.25% trypsin.

Of the cell lines studied, BHK-21, clone 15 was selected for use in plaque assay and PRNT. The results presented in Table 1 indicate that the plaque forming unit titer for both JC and KEY virus was highest in BHK-21, clone 15 cell line. Furthermore, in comparison to BHK-21, clone 13, the plaques were more distinct and the cell's monolayer remained confluent much longer.

## (4) Plaque assay

Tissue culture flasks (30 ml) were seeded with 5 ml aliquots of a suspension of BHK-21, clone 15 cells and incubated for 2 days at 35°C. The medium was drained from the cell cultures and 0.2 ml of log<sub>10</sub> dilutions of virus were inoculated onto 2 or more cell cultures. After 1 hr. incubation at 35°C with 15 minute interval agitation, 5.0 ml of an agar overlay<sup>1/</sup> was added to each cell culture.

<sup>1/</sup> Agar overlay contained 1.0% Difco purified Agar, 10% FBS, 10% medium 199 (10X), 4% NaHCO<sub>3</sub>, 1% diethylaminoethyl (Deae) dextran, 0.5% vitamins (100X), 0.5% essential amino acids and penicillin (200 µ/ml) and streptomycin (150 µg/ml).

Cells plus inoculum were incubated at 35 C for 3 days for BHK-21 cells, and 4 days for Vero and LLC-MK<sub>2</sub> cells. A second overlay<sup>1/</sup> in aliquots of 5 ml was then added to each culture. Plaques were read 12 to 24 hours later.

#### (5) Plaque Reduction Neutralization Test

All sera were incubated at 56 C for 30 minutes. Sera dilutions of 1:5 and serial 2-fold dilutions were made in medium 199-H that contained 20% FBS, 1% NaHCO<sub>3</sub> and antibiotics. A 0.5 ml volume of a virus suspension that was diluted to yield approximately 100 plaque forming units/0.2 ml was added to a similar volume of each serum dilution. The virus-serum mixtures were incubated at room temperature for 1 hr and then inoculated onto medium free monolayers of BHK-21, clone 15 cultures, 0.2 ml/culture. Cells plus inoculum were incubated at 35 C for 1 hour with agitation at 15 minute intervals. Each culture received 5 ml of an agar overlay similar to that described in the plaque assay section of this report. A second overlay having neutral red incorporated as referred to above was added 3 days later and plaques were read 12 to 24 hrs thereafter. The mean plaque count from 2 or more cultures that received the virus dose plus diluent was taken as 100% virus infectivity. The percentage virus infectivity reduction was calculated on this basis and then plotted on log probit paper as described by Cutchens *et al.* (1960)<sup>4</sup> to arrive at the 50% plaque reduction endpoints. A reduction of 50% or more of the test virus plaque dose for sera tested for antibody at 1:5 dilution was used as the criterion for neutralization. Controls for each test included cell monolayers with and without an agar overlay and initially, known positive sera specimens were assayed to assess the reproducibility of the PRNT.

The identity of virus that was recovered from experimentally infected mosquitoes and vertebrates was confirmed in mouse neutralization tests employing virus specific antisera.

#### c. Vertebrate susceptibility studies

##### (1) Whitetail deer

Serological surveillance and mosquito host preference studies in the PCS environ suggest that deer may serve as a host for KEY virus. Since deer frequently become infected with JC virus, the effectiveness of this animal as a host of KEY virus would appear to depend on the ability of deer to support KEY virus replication and whether or not KEY virus could replicate in deer previously infected with JC virus. To address this question we attempted to define the course of (1) JC and KEY virus infection in deer after primary inoculation and (2) infection in deer following heterologous challenge inoculation with JC and KEY virus.

<sup>1/</sup> Agar overlay contained 1% Difco purified agar, 2% FBS, 10% 199 medium (10X), and neutral red (1:1200 dilution).

Orphaned whitetail deer fawns were obtained during June and July <sup>1/</sup> of 1975 from the Virginia State Commission of Game and Inland Fisheries. A total of 18 deer ranging from approximately 3 to 8 weeks of age were transported from Cumberland, Virginia to the WRAIR Experimental Farm at Ft. Meade, MD. Three deer that were ill when picked up died within 3 days after arrival at the WRAIR farm. The 15 remaining deer were marked with ear tags and placed in two 15 x 15 ft arthropod-proof rooms. Deer were fed a liquid mammal and human infant formula diet twice a day with 8 oz plastic baby bottles. After approximately 2 months, deer were transferred from the liquid to a solid diet of calf starter and maple tree leaves and twigs. Two additional deer died of unknown causes while being maintained on the liquid diet.

Blood was drawn via jugular vein puncture from each deer to determine if they possessed antibody to KEY and/or JC virus. At approximately 6 months of age, deer were inoculated subcutaneously in the right scapular region with either KEY or JC virus. Control deer were inoculated similarly with virus-diluting medium. The complete inoculation schedule of the deer is presented in Table 2. The deer were physically restrained for injection of the inocula and for blood sample collections. Blood was drawn on days 1, 2, 3, 4, 5, 7, 10 and at 10 day intervals P.I. for varying periods of time depending on the objectives of the study. Approximately 5 ml of blood was drawn from each deer, 2 ml for virus studies and 3 ml for serology. Blood for virus studies was transferred to 1.0 dram vials, sealed with Parafilm, quick frozen in a dry-ice-ethanol mixture and stored at -60 C for assay by intracerebral inoculation in suckling mice. The blood for serology was left at 4 C overnight, then centrifuged at 1500 rpm for 30 minutes for clarification of serum. The serum was assayed for antibody in PRNTS described previously in this report.

All 6 whitetail deer fawns, except number 188 developed a viremia following SC inoculation with 2.0 ml of an inoculum that contained  $10^{5.0}$  SMLD<sub>50</sub>/1.0 ml of JC virus (Table 3). Virus was first detected in the blood of 3 deer on day 1 P.I. and by day 3, the other 2 deer had detectable levels of virus. The duration of viremia ranged from 2 to 4 days and virus titers ranged from a trace at a 1:5 dilution of blood to  $10^{5.0}$  SMLD<sub>50</sub>/1.0 ml. No virus was recovered from deer on days 5 and 7 P.I. Neither JC nor KEY virus neutralizing antibody were detected in sera of deer prior to or on the inoculation date. Blood of control deer no. 182 taken on days 2 and 4 P.I. did not cause recognizable illness in mice.

Homologous antibody titers detected in serum of deer no. 188, 195 and 180 following inoculation with JC virus are presented in Table 4. Antibody was present in serum of deer no. 195 on day 5 and on day 10 for deer no. 188 and 195. Peak titers prior to challenge of deer with KEY virus on day 30 were observed on day 10 for deer no. 180 and on day

<sup>1/</sup>Our sincere appreciation and thanks are extended to Dr. Chester F. Phelps and staff, especially Mr. R.H. Cross Jr. and Mr. G. Goin for donation of deer.

30 for deer nos. 188 and 195. No serological reactors were detected in serum of control deer no. 182. Deer no. 108, 109 and 185 were challenged with KEY virus on day 80 PI. PRNTS are being conducted to define the pattern, magnitude and specificity of the immune response.

On day 30 PI, deer no. 188, 195 and 180 were challenged with 2.0 ml of an inoculum that contained  $10^{6.6}$  SMLD<sub>50</sub>/1.0 of KEY virus. (Table 1) A similar virus dose was injected into control deer no. 182 that did not possess serum neutralizing antibody. Virus was not detected in blood taken from deer on day 1, 2, 3, 4, 5 and 7 PI and there was no apparent evidence of a secondary immune response. All challenged deer exhibited a decrease in antibody titer that was followed by a gradual increase to previous levels by day 7 PI for deer no. 188 and by day 10 and 30 PI for deer no. 195 and 180, respectively. The control deer (#182) developed detectable antibody to KEY virus (Table 5) however, no cross reaction was noted to JC virus. The failure to demonstrate a secondary immune response on challenging deer with the heterologous antigen is consistent with the findings reported for California encephalitis group (CE) viruses and feral animals in Wisconsin.<sup>5</sup>

Neutralizing antibody produced in deer after a single inoculation with JC virus cross-reacted with KEY virus; but heterologous peak titers were 4- to 6-fold lower (Table 5). Subsequent to the heterologous challenge on day 30 PI, cross reactivity titers dropped slightly on day 2 PI, but by day 4 PI titers were comparable to those observed on the challenge date.

A second group of deer, (nos. 183, 186, 191 and 196) was inoculated with  $10^{4.5}$  SMLD<sub>50</sub>/1.0 ml of KEY virus. Virus could not be detected in blood of deer on days 1, 2, 3, 4, 5 and 7 days PI.

Homologous neutralizing antibody were detected in serum of all deer on day 5 PI (Table 6). Peak antibody titers of 1 to 320 or greater were detected for deer nos. 186, 196 and 191 by day 40 PI. Antibody titer for deer no. 183 failed to reach 1:80. Studies aimed at determining the specificity of homologous antibody are being performed in PRNT employing JC virus. In addition, studies are underway to describe the immune response of deer following challenge inoculation with JC virus.

## (2) Cottontail rabbits

Convincing evidence implicating cottontail rabbits in the natural cycle of KEY virus in the PCS study area is lacking. Antibody to the virus was detected in 1 of 3 rabbits taken at Snow Hill, MD, 3/30 rabbits captured at Assateague Island, VA and 0/9 rabbits captured at Little Orleans, MD in 1973. In the present investigation the aim has been to determine the prevalence rate of KEY and JC infection in field-collected rabbits and to define the course of infection following inoculation with JC and KEY virus.

Cottontail rabbits were captured in live traps on Assateague Island, Virginia for a 2 week period in July and in December of 1975. Apples were used to prebait the traps and as a bait during the trapping period. In addition, 3 rabbits were captured while trapping for gray squirrels in the PCS study area. Rabbits were bled via cardiac puncture and retained for transfer to WRAIR for use in laboratory experiments. Sera obtained by centrifuging blood for 30 minutes at 1500 rpm were stored at -20 C for serological studies employing PRNTs. Blood specimens were stored at -70 C for virus assay. PRNT and virus assay methods employed were similar to that described previously.

In the laboratory, rabbits were inoculated SC with either JC or KEY virus. Blood was drawn for viremia studies on days 1,2,3,4 and 5 PI. Assay of blood for virus was according to procedures previously described for whitetail deer. Subsequent to the inoculation of some of the rabbits with KEY virus, field-collected A. atlanticus mosquitoes were allowed to ingest blood from them on days 2 and 3 PI. Mosquitoes were transferred to separate holding cages that were labeled to correspond with the rabbit upon which the mosquitoes fed. Cages with mosquitoes were placed in an insectary that was maintained at 80 F and 80% relative humidity. The mosquitoes were to be retained for a period of 25 to 30 days during which time they would be sacrificed at various intervals for virus assay. In addition, 100 mosquitoes were placed in individual 2.5 X 15 cm plexiglass cages where they were to remain until the completion of the gonodotrophic cycle. Mosquitoes were to be sacrificed for virus assay and the eggs originating from each infected female were to be hatched to provide specimens for determining the frequency of transovarial transmission of KEY virus and the progeny infection rate.

Each of six rabbits that were captured during July of 1975 were inoculated with 0.5 ml of an inoculum that contained  $10^{5.5}$  SMLD<sub>50</sub>/1.0 ml of KEY virus. Three of the rabbits died on the eve of the inoculation date and virus was not recovered from blood taken from the 3 surviving rabbits on days 1,2,3 and 4 PI. These rabbits were sacrificed on day 5 PI because of findings that 2 of the 3 rabbits that died had gross histopathological lesions consistent with those produced by Francisella tularensis.

Approximately 75 A. atlanticus fed on each of the 3 rabbits on days 2 and 3 PI. These mosquitoes were sacrificed immediately on receiving the report that 2 of the 3 rabbits that died appeared to be infected with tularemia.

In December 1975 each of 6 rabbits were inoculated with 0.5 ml of an inoculum that contained  $10^{4.8}$  SMLD<sub>50</sub>/1.0 ml of KEY virus. Two rabbits developed a viremia by day 2 PI and another rabbit was viremic on day 3 PI. The duration of viremia ranged from 1 to 4 days and virus titers ranged from trace levels to  $10^{4.7}$  SMLD<sub>50</sub>/1.0 ml. Preinoculation sera from the 3 rabbits that did not develop a viremia contained serological reactors to KEY and JC virus.

A group of 4 rabbits were each inoculated with 0.5 ml of an inoculation that contained  $10^{4.0}$  SMLD<sub>50</sub>/1.0 ml of JC virus. Attempts to recover virus from blood taken on days 1 and 2 PI were unsuccessful. On day 3 PI, 3 of 4 rabbits were dead, thus resulting in the termination of the study. Two of the 3 rabbits that died had gross pathological lesions suggestive of a tularemia infection. In addition, preinoculation sera of 2 rabbits contained serological reactors of KEY virus.

JC and KEY virus serological reactors were detected in rabbits captured in the Pocomoke Cypress Swamp and from rabbits obtained from Indian Springs, which is located in the western area of Maryland. Of the 10 sera taken from rabbits captured at Assateague Island, VA, 5 reduced KEY virus infectivity 75% or more and 2 sera reduced JC virus infectivity 50% or more. Four rabbits were captured in the Pocomoke Cypress Swamp, MD during July of 1975. One of these sera reduced JC virus infectivity 100% but did not react with KEY virus. A second one reduced both KEY + JC virus infectivity 100% and the other one reduced infectivity of KEY by 100% but no reduction was noted with JC virus. Of the 7 sera taken from rabbits captured at Indian Spring, MD, only one reduced KEY virus infectivity more than 50%. None of the sera cause a reduction of JC virus infectivity.

#### d. Vector competence studies

Of the mosquito species that are active during the period that KEY virus circulates in the PCS environ, the most abundant are Aedes atlanticus, Aedes canadensis and Psorophora ferox. Attempts to isolate KEY virus from P. ferox have not been successful and only 1 isolate of this virus was obtained from 63,216 A. canadensis in 1972. On the other hand, the KEY virus isolation rate from A. atlanticus is very high as indicated by approximately 1 isolate per 300 mosquitoes.

As mentioned earlier in this report, the arthropod vector of JC virus has remained unknown. The only clue originating from studies conducted in the PCS area has been the isolation in 1971 of JC virus from 42,434 A. canadensis and a second isolation in 1972 from 62,216 A. canadensis.

The purpose of these experiments was to incriminate potential vectors of JC and KEY virus and to determine relative vector competence of each mosquito species. In addition, emphasis was placed on the development of techniques to employ in addressing questions relative to transovarial transmission of KEY virus in A. atlanticus and other mosquito species.

Mosquitoes employed in virus transmission experiments included A. canadensis, A. atlanticus and P. ferox. The Aedes species were collected as adults using CDC light traps in the PCS study area during the 1975 mosquito season. (June-Sept). Mosquitoes were transferred to screened holding cages and transported to WRAIR. Wet towels were draped around the transporting cages in order to reduce mortality associated with low humidity and high temperature. At WRAIR, the mosquitoes were allowed to ingest blood from hamsters and chickens to stimulate development of eggs. A 2nd bloodmeal was made available after the first

gonadotrophic cycle to obtain a 2nd batch of eggs. The eggs were deposited on multiple layered cheesecloth which was sealed in petri dishes and stored at 25 C until the eggs were needed for hatching to provide adults for virus transmission experiments. P. ferox was from a self-sustaining colony that was in its first generation. Mosquitoes were maintained at 27 C and 80% relative humidity prior to and during the virus transmission experiments. A 5% sucrose solution and sliced apples were provided to the mosquitoes as a source of carbohydrate. Mosquitoes ranged from 5 to 12 days of age when given the infective bloodmeal.

JC and KEY viruses employed were 2nd or 3rd passage seeds of the strain described previously. The infectious bloodmeal was prepared by mixing the desired dose of virus with guinea pig or chicken defibrinated blood. The mixture was transferred to a feeding apparatus that allowed the mosquitoes to feed through a lamb skin membrane. After engorgement, mosquitoes were transferred to 2.5 X 15 cm plexiglass holding cages where they were retained for virus transmission trials and infectivity assay. Transmission trials were conducted by allowing individual mosquitoes to feed on a 3 to 5 day old mouse. After feeding on mice, mosquitoes were sacrificed for virus assay. Mosquitoes were triturated individually and inoculated intracerebrally into 3 to 5 day old mice, 0.03 ml per mouse. Estimates of the quantity of virus ingested by mosquitoes were determined by infectivity assay employing 3 to 5 day old mice.

A. canadensis failed to become infected after ingesting  $10^{3.0} \text{SMLD}_{50}/1.0 \text{ ml}$  of JC virus. On increasing the virus dose to  $10^{4.5} \text{SMLD}_{50}/1.0 \text{ ml}$ , 1 of 9 and 1 of 8 mosquitoes became infected after 14 and 21 day incubation periods, respectively. Transmission of a lethal dose of virus was not accomplished by either infected mosquito. After mosquitoes ingested a virus-blood mixture containing  $10^{6.0} \text{SMLD}_{50}/1.0 \text{ ml}$ , a substantial increase in the infection rate occurred and the transmission of virus to mice was accomplished by mosquitoes after an incubation period of at least 9 days.

Attempts to demonstrate vertical transmission of JC virus by A. canadensis were unsuccessful. Virus was not recovered from 21 pools that consisted of 502 eggs laid by 15 infected mosquitoes. In addition, negative results were obtained on assaying 19 pools that contained 170 fourth stage larvae that were reared from eggs laid by 10 infected mosquitoes. No evidence of virus was detected in a total of 122 pools or 1250 field collected A. canadensis larvae.

A. atlanticus readily became infected after ingesting defibrinated blood that contained  $10^{5.2} \text{SMLD}_{50}/1.0 \text{ ml}$  and  $10^{6.0} \text{SMLD}_{50}/1.0 \text{ ml}$  of KEY virus (Table 7 & 8). Attempts to demonstrate transmission of the virus to mice have not been successful. Preliminary findings suggest that an alternative virus recipient animal or an indicator other than mortality will be required to detect virus transmission.



The pattern of virus recovery in A. atlanticus following ingestion of defibrinated blood that contained  $10^{5.2}$  SMLD<sub>50</sub>/1.0 ml was indicative of virus replication. Virus titers determined immediately after mosquitoes ingested virus averaged  $10^{4.0}$  SMLD<sub>50</sub>/1.0 ml. A slight decrease in virus content was observed on day 3 as indicated by an average titer of  $10^{3.6}$  SMLD<sub>50</sub>/1.0 ml. On day 5 and 7 virus titer had increased to an average of  $10^{4.6}$  and  $10^{5.0}$  SMLD<sub>50</sub>/1.0 ml and persisted at comparable levels on days 14 and 21 post feeding.

Experiments with A. canadensis indicate that this species became infected after ingesting blood containing  $10^{3.4}$  and  $10^{5.2}$  SMLD<sub>50</sub>/1.0 ml of KEY virus. (Tables 9 & 10) The overall infection rate for mosquitoes after ingesting the lower dose of virus was 5 of 15 (33%) while 13 of 30 (43%) became infected after ingesting the higher dose. Attempts to demonstrate virus transmission to mice using mortality as an indicative system were unsuccessful.

The amount of KEY virus recovered from individual A. canadensis was determined after this species ingested  $10^{5.2}$  SMLD<sub>50</sub>/1.0 ml. Virus titers on days 0, 2, 4, 7, 14, 21 and 34 post feeding averaged  $10^{2.6}$ ,  $10^{2.3}$ ,  $10^{2.5}$ ,  $10^{3.2}$ ,  $10^{2.5}$  and  $10^{3.0}$ , respectively.

P. ferox was allowed to ingest blood containing  $10^{3.6}$  SMLD<sub>50</sub>/1.0 ml of KEY virus. Attempts to recover virus and to demonstrate virus transmission to mice were unsuccessful (Table 11).

e. Field studies of populations of gray squirrels and Aedes atlanticus. Evidence of KEY virus infection in gray squirrels was revealed during serological surveillance studies that were conducted during 1972 and 1973 on the eastern shore of Maryland. Attempts to capture squirrels during 1972 in the PCS were unsuccessful, however, 8 of 27 squirrels from Snow Hill, Maryland and 1 of 27 squirrels from Wallops Island, Virginia possessed KEY virus antibody. Subsequent laboratory experiments indicated that squirrels develop a viremia following inoculation with KEY virus. In addition, mosquito host preference studies showed A. atlanticus to feed on squirrels in nature.

This study was initiated to define the seasonal distribution and the incidence of KEY virus infections in gray squirrels in relation to the dynamics of KEY virus infection in the A. atlanticus population of the PCS area.

Gray squirrel trapping stations were established at various intervals in the oak-pine upland forest that is located along the western edge of the PCS. The stations were selected in areas that were judged to provide food and cover. One or two 15 X 48 X 15 cm national live traps were placed at each of 36 stations during March of 1975. In order to supplement the trap captures and to increase the chances of capturing squirrels, 40 squirrel nesting boxes and 15 squirrel feeders were placed



on trees <sup>4</sup> to 5 m above the ground at various intervals in the forest. Cob-Corn<sup>1/</sup> was maintained in feeders continuously and also used to pre-bait and to bait trap. Nesting boxes were checked during the latter months of spring and summer for young squirrels. The location of the squirrel trapping stations, and squirrel feeders are shown in Figure 1.

Captured gray squirrels were anesthetized with metaphane and examined for determination of sex and age. Each squirrel was then marked<sup>6</sup>, bled, and released at the site of capture. The age of squirrels were determined on the basis of weight and external characteristics of teats and scrotum as described by Brown and Yeager<sup>7</sup> in Table 12. Approximately 1.0 ml of blood was obtained from each squirrel by cardiac puncture. Blood was transferred to Wasserman tubes and placed in a styrofoam container. Wet ice was used during the A. atlanticus activity period to keep the blood chilled until it arrived in the laboratory. Blood specimens were centrifuged for 15 minutes at 1500 rpm and serum was then transferred to sterile 1-dram vials and stored at -70 C. Pelleted red blood cells were stored at -70 C. Serum specimens were assayed in PRNT initially at a 1:5 dilution employing JC and KEY viruses. Since many of the squirrels were bled several times, the last serum specimen from each squirrel was selected to be assayed first. The next serum specimen to the last was selected for assay, only if the last one contained antibody. This selection scheme continued until the seroconversion date was determined for each squirrel that was captured more than once. Some of the serum specimens that caused reduction of both JC and KEY virus dose were diluted in serial 2-fold increments and tested against the 2 viruses.

Blood specimens were diluted 1:5 and inoculated in 0.03 ml aliquots intracerebrally into 3-5 day old mice. Mice were observed daily for sign of morbidity and mortality.

Chloroform sensitivity test was performed according to standard virological procedure.

Aedes atlanticus female mosquitoes were collected with CDC light traps, supplemented with dry ice, every other night from 22 July 1975 through 24 October, 1975. Traps were secured to tree limbs approximately 1.5 m above the ground at 6 sites in the upland area and 2 sites in the swamp (Figure 1). Traps were placed in the field at approximately 1800 hours and picked up and returned to the laboratory between 0800 and 0900 hours of the following day. After immobilizing mosquitoes by a brief exposure to -20 C temperature, they were transferred according to capture date and location to chilled 8-dram sterile screw cap vials and stored at -70 C. At a later date the contents of each vial were examined on a chilled surface, and the A. atlanticus were removed and transferred to sterile chilled Wasserman tubes in pools of 25 or less. In addition to pooling mosquitoes for virus isolation studies, 25 A. atlanticus from each trap period were stored at -70 C for parity rate studies. Engorged mosquitoes were saved for blood meal identification studies.

<sup>1/</sup> Corn was donated by Mr. Orville Autin, Pocomoke City, MD

Mosquitoes were prepared for virus assay by triturating each pool in a Tenbroeck tissue grinder. A 2 ml volume of medium 199-H that contained 20% heat treated FBS, 0.75%  $\text{NaHCO}_3$  (1 ml/100) and antibiotics (500  $\mu$ /ml of penicillin and 500  $\mu$ g/ml of streptomycin) was added to pools containing 11 to 25 mosquitoes and 1 ml volume were added to pools that contained 10 or less. Mosquito suspensions were poured into Wasserman tubes and then centrifuged for 30 minutes at 2500 rpm at 4 C. Supernatant from each suspension was inoculated intracerebrally into a half litter of 3 to 5 day old mice, 0.03 ml per mouse. All mice that exhibited signs of illness or that died during a 12 day observation period were placed at -70 C. Later the brain tissue was aspirated from mice and suspended in medium similar to that used to triturate mosquitoes such that the final concentration was 20 per cent. This suspension was inoculated intracerebrally into a litter of 3 to 5 day old mice, 0.03 ml/mouse. A 20% brain-medium suspension was prepared from ill or dead mice for use in PRNTS employing KEY and JC virus mouse hyperimmune ascitic fluid. All original mosquito suspensions that contained virus were reinoculated in 0.03 ml aliquots into 3 to 5 day old mice.

#### (1) Gray squirrel trapping results

A total of 90 gray squirrels consisting of 72 (80%) adults and 18 (20%) juveniles were captured from 24 March through 5 September, 1975 in the PCS (Table 13). The sex and age class distribution was 56 males (48 adults and 8 Juveniles) and 34 females (24 adults and 10 juveniles). Juvenile squirrels first appeared in traps during the 15 to 17 July trapping period. Of the 90 squirrels, 26 were single captures and 64 were captured 2 to 14 times each. Mean recapture was 4.2 times for males and 4.4 times for females. The recapture frequency for male squirrels was 42 of 56 (75%) while 22 of 34 (64%) females were re captured at least once. The proportion of new to recaptured squirrels and the sex and age comparison of squirrels for each trapping period is shown in Table 14.

A method for calculating the population density of gray squirrels is being considered; however, it is not likely that a valid estimate can be determined due to the bias associated with the trapping procedure and the period of trapping. As a possible means of dealing with squirrel trap proneness and shyness, a marked-unmarked ratio was obtained by conducting a hunter bag check during October and November 1975. On the basis of these data employing standard mark-recapture method, the population density was 247 squirrels for the study area.

Only those gray squirrel blood specimens taken on and after 16 July, the beginning of the A. atlanticus activity period, were assayed for virus. Of 251 specimens assayed, 1 virus isolation was obtained from an adult female that was captured on 3 September. Primary inoculation of 3 to 5 day old mice via the intracerebral route with a 1:5 dilution of blood caused 1 of 5 mice to die on day 8 PI. Subsequent inoculation (I.C.) of mice with an aliquot of a 20% brain-diluent suspension prepared from the

mouse caused 100% mortality of mice on day 6 P.I. An attempt to re-isolate the virus from the original blood specimen was successful. Characterization and identification studies employing a 20% seed virus preparation indicated that the infectivity titer via the IC route of inoculation for 3 to 5 day old mice was  $10^{5.2}$  SMLD<sub>50</sub>/0.03 ml. The infectivity titer obtained in BHK-21, C-15 (plaque assay) was shown to be  $5 \times 10^6$  PFU/0.2 ml. After treatment with chloroform, the infectivity of the virus was reduced by  $10^{3.5}$  SMLD<sub>50</sub>/0.03 ml. Attempts to demonstrate a reduction of infectivity of the virus in PRNTs using JC, KEY and St. Louis encephalitis virus (SLE) antisera were unsuccessful. Similar studies employing antisera of other viruses enzootic in the PCS are in progress.

Of 88 squirrels captured, 48(56%) possessed serum neutralizing antibody that caused 50% or more reduction of KEY virus infectivity. (Table 15). Analyses of the data did not reveal a significance difference in antibody prevalence rates between adults and juveniles or between sexes of either age class. Statistical tests of significance were done on data compared in contingency tables using  $\chi^2$  test. The seasonal distribution of KEY virus antibody detected in sera of gray squirrels in relation to the A. atlanticus activity period is presented in Figure 2. Although KEY virus antibody was detected in gray squirrels during the spring months and during the first part of July, antibody in response to active KEY virus infection was not detected until 27 July (13th week of the study) or 5 days after the emergence of A. atlanticus. Seroconversions continued through the 19th week of the study, thus resulting in an increase in antibody prevalence rates from 10% on 27 July to 70% or greater for the 16th, 17th, 18th and 19th week of the study. Of the 25 squirrels that seroconverted, 15 were adult males, 4 were adult female, 2 were juveniles males and 4 were juvenile female. A summary of data pertaining to KEY virus seroconversion in gray squirrels is presented in Table 16.

Evidence of JC virus infection on the basis of serological reactors was not detected in the gray squirrel population. Of 138 sera that contained KEY antibody, 86 caused reduction of JC virus infectivity. The magnitude of reduction ranged from 23 to 100%, however, the percentage of reduction was far greater during the period of active infection of gray squirrels. In addition, only 1 of 19 sera that contained KEY antibody caused reduction of JC virus prior to the period of active virus circulation as opposed to 71 of 119 that caused reduction thereafter. A number of the sera that caused reduction of both JC and KEY virus were assayed in order to ascertain their identity. Results presented in Table 17 indicate that all were KEY virus antibody.

## (2) Aedes atlanticus population

A total of 75,000 adult female A. atlanticus were collected in the PCS during a period extending from 22 July to 24 October, 1975. The seasonal distribution of A. atlanticus in relation to mean daily temperature and weekly precipitation is presented in Figure 3. The first adult

A. atlanticus was collected on 17 July, 6 days after observing 1st stage larvae of this species. On 18 July, 4 females were collected and by 22 July the average number per trap period had increased to 156. An abrupt increase followed as indicated by peak average of 2,513 mosquitoes per trap period on 26 July. Subsequent population indices decreased very rapidly, reaching an average of 308 mosquitoes 6 days later. The number of mosquitoes collected continued to show a gradual downward trend and by 16 September the average had dropped to 13 mosquitoes. A second hatch of A. atlanticus eggs occurred subsequent to rain that fell during the last week of August. As a result, a second population of adults emerged as indicated by an increase from an average of 13 mosquitoes collected on 18 September to an average of 141 on the 20th of September. The number of A. atlanticus collected gradually decreased thereafter, and by 24 October the average number had dropped to 3.0 mosquitoes.

During 1975, 64,720 A. atlanticus (3263 pools of 25 or less mosquitoes) were assayed for virus. To date, preliminary results show 194 possible virus isolations (Table 18). This value is based on the number of suckling mouse brain (SMB-1) suspensions that have on subsequent inoculation into mice produce illness or mortality after an incubation period of at least 36 hours. Aliquots of SMB-2 suspensions are being prepared for virus screening studies and for identification in PRNTs. Counter-electrophoresis and radio-immune assay techniques are being considered for the former. Preliminary titrations of 5 SMB-2 suspensions yielded comparable infectivity titer (PFU) suggesting that PRNT can be performed without having to titrate each suspension for calculation of virus dose. One of these isolates was shown by PRNT to be KEY virus. The progress of virus isolation studies has been hampered considerably by nonspecific illness and mortality of mice.

## 2. Bionomics of Floodwater Mosquitoes

### a. Effect of environmental factors on breaking of diapause in floodwater mosquito embryos

This work is a continuation of studies reported last year. Soil samples were collected in the PCS study area and divided into 7 approximately equal parts. One part was flooded immediately on collection. The remaining 6 samples were divided equally and subjected to 2 different photoperiods: 3 aliquots at L:D 15:9 and 3 at L:D 9:15. Temperature for all subsamples was 25 C. Collections were made in February and April, 1976. Results for the February collections are presented in Table 19. No eggs were collected in the April samples. The results of tests on the February collections did not differ markedly from those of the 1975 collections for the same time period, i.e. virtually 100% of the eggs from all 3 species under investigation (Aedes atlanticus, A. canadensis, and Psorophora ferox) hatched when exposed to 25 C, regardless of the photoperiod they were exposed to.

The fact that no eggs were collected in April points out one of the problems inherent in a test of this sort which depends on random sampling i.e., the numbers of eggs tested for each species under consideration may not be constant, and eggs from each species may not even be present in a collection. A third problem is that the age of the eggs is unknown and un-determinable. Their ages may be estimated by circumstantial evidence.

b. Influence of dissolved oxygen upon hatching of eggs of flood-water mosquitoes.

Laboratory studies were initiated to determine the effect of dissolved oxygen content (D.O.) as a hatching stimulus for eggs of flood-water mosquitoes. Techniques for controlling the D.O. of water were developed and refined. Basically 2 systems were tested: the addition of nutrient broth (1:1000) to the water and secondly, passage of  $N_2$  gas through the water at controlled flow rates. Each system was evaluated in 250 ml of distilled water which had been fully aerated by bubbling air through the water for 30 minutes prior to testing. Preliminary tests have shown that 30 minutes of aeration will produce the maximum D.O. reading. Magnetic stirrers were used to produce continuous agitation and provide even dispersal of nutrient broth or nitrogen throughout the volume of water being tested. All D.O. readings were made using a Beckman model D2 oxygen analyzer. Readings are expressed as mg/l of dissolved oxygen. All tests were conducted at a temperature of 22-24 C.

Nutrient broth reduced the D.O. at a much slower rate than did  $N_2$  passage. It was not possible to maintain the D.O. at constant levels using nutrient broth. The average time required to reach minimum D.O. levels was approximately 24 hours. The 2.90 mg/l level was maintained for several hours, then began a gradual increase. After 48 hours, the mixture was approaching full oxygenation again (7.6 mg/l D.O.).

Use of gaseous  $N_2$  bubbled through the water at controlled flow rates was found to be a much more satisfactory method for controlling D.O. By using  $N_2$ , the D.O. could be reduced more rapidly and to lower levels than was possible using nutrient broth. D.O. rates as low as 1.5 mg/l can be obtained in 40 minutes using  $N_2$ , whereas this level was unobtainable using nutrient broth. In addition, the D.O. could be held within virtually any range desired between 1.5 and 9.0 mg/l. This was achieved by placing the water in a petri dish, bubbling the  $N_2$  through it until the desired D.O. was obtained, then sealing the dish so it was air tight.

This system has not been evaluated on mosquito eggs due to the lack of reproducing colonies of floodwater mosquitoes in this laboratory. Efforts are currently underway to establish a reproducing colony of P. ferox. In addition, field collected Aedes canadensis females are being held in the laboratory and fed blood meals so that they will produce sufficient numbers of eggs for testing.

c. Measurement of differences in metabolic activity between diapausing and non-diapausing culicine mosquitoes

The purpose of this study is to quantify the differences in metabolic rates between diapausing and non-diapausing culicine mosquitoes, particularly Culex tritaeniorhynchus and C. pipiens. C. tritaeniorhynchus was chosen because it is currently colonized at WRAIR, can be induced to diapause relatively easily, and can be reared in large numbers. C. pipiens is of interest because it has been identified as a vector of St. Louis encephalitis, and the mechanisms this mosquito uses to survive the winter are currently being investigated in this department. Information gained from this study will yield quantitative data regarding metabolic rates during initiation, continuation and termination of diapause which can be used as a baseline for evaluating the effects of various environmental conditions on diapause in C. pipiens. Future studies will include an investigation of the role juvenile hormone plays in diapause in C. pipiens, using the data currently being gathered as a baseline. Establishment of a baseline metabolic rate under defined conditions will provide a means for evaluation of the diapause condition in field populations of C. pipiens, providing further insight into the physiological mechanisms this mosquito uses to survive the winter months.

Respiration rates of diapausing and non-diapausing C. tritaeniorhynchus and C. pipiens of known ages are being recorded using standard Warburg manometric apparatus, except that instead of the usual 15 ml reaction vessel, specially designed precalibrated 4-6 ml micro-reaction vessels are being used to measure the small volumes of oxygen consumed. Diapause is controlled in C. tritaeniorhynchus by placing the 3rd and 4th stage larvae at photoperiods of 15:9 L:D or 9:15 L:D and a temperature of 25 C. Adults emerging from the 15:9 photoperiod will not enter diapause, whereas those emerging from the 9:15 regime will enter diapause. Preliminary results indicate that the onset of diapause occurs during the first 3 days post emergence. Respiration rates for 1-day old C. tritaeniorhynchus from each photoperiod are not significantly different when compared (2.52 ul O<sub>2</sub>/mg/hr for long day adults vs. 3.20 ul O<sub>2</sub>/mg/hr for short day adults). After day 3 post emergence, however, the respiration rate for non-diapausing adults is approximately twice that for diapausing adults (5.82 ul O<sub>2</sub>/mg/hr for long day adults vs. 2.82 ul O<sub>2</sub>/mg/hr for short day adults. This 2-fold difference in respiration rates remains constant at least through day-22 post emergence (3.90 ul O<sub>2</sub>/mg/hr long day adults vs 1.98 ul O<sub>2</sub>/mg/hr short adults day), indicating that while approximately 3 days are required for diapause to become entrenched, once it is established, the degree of intensity remains fairly constant.

Evaluation of the diapause condition in C. pipiens is still in the preliminary stages, however; initial tests indicate that differences in metabolic activity between diapausing and non-diapausing adults will be similar to those for C. tritaeniorhynchus. Diapause is induced in C. pipiens by placing the 4th stage larvae and pupae at a photoperiod of 10:14 L:D and a temperature of 15 C. Non-diapausing adults are maintained at 14:10 L:D and 25 C.



### 3. St. Louis Encephalitis Virus Studies

#### a. Summer virus isolations from mosquitoes

According to Maryland State Health Department records, there were 8 confirmed and 2 suspected human cases of SLE during the 1975 epidemic. All patients exhibited clinical signs and symptoms compatible with encephalitis. The confirmed SLE virus infections were based on either a 4-fold change in serum hemagglutination inhibition (HAI) titers, or a single titer equal to or greater than 1:80.

The SLE virus infections involved 2 females and 6 males that ranged from 8 to 64 years of age. Signs and symptoms most frequently observed were headaches, fever, diminished mentation and confusion. In addition, diarrhea, vomiting and nuchal rigidity were reported less frequently. Two of the cases suffered residual neurological deficits ranging from periodic confusion to total disorientation. There were no fatalities.

The history of the human cases indicated that SLE virus was contracted in Maryland. None of the patients had traveled or resided outside of the state within 20 days prior to the onset of illness. The first 3 confirmed SLE virus infections and one suspected infection involved individuals that had not traveled outside of Prince George County. One of 3 confirmed SLE cases reported from Baltimore City had been employed as a field worker in Ocean City, Worcester County and Westminster, Carroll County. The other confirmed cases involved residents of the counties Anne Arundel and Talbot. The second suspected case of SLE was associated with a resident of Queen Anne County, Maryland.

Studies aimed at determining the mosquito species involved in the SLE epidemic were conducted by entomologists of the Maryland Department of Agriculture and of the Walter Reed Army Institute of Research, Washington, D.C. CDC light trap (supplemented with dry ice) collections were made at night on September 10, 11, 12, 13, 16 and 17 in the northwestern section of Prince George's County, Maryland. Mosquitoes were transported to the laboratory, immobilized by a brief exposure to -20 C and then transferred to sterile screen-capped vials and placed at -70 C for subsequent virus isolations studies. Identification and assay of mosquitoes for virus was performed as described earlier in this report.

A total of 769 mosquitoes of the genera Culex, Aedes, and Anopheles were collected at 18 sites in Prince George's County, Maryland. The capture rate per trap night was 9 Culex, 12 Aedes, and 0.5 Anopheles. All mosquitoes except one Culex sp. individual were unengorged.

A summary of the Culex sp. mosquitoes assayed for virus is presented in Table 20. No virus was isolated from 23 mosquitoes collected on September 10. SLE virus was, however, isolated from a pool of 12 mosquitoes collected September 11 and from a pool of 25 mosquitoes collected September 12. Subsequent attempts to isolate virus from mosquitoes

collected on 13, 16 and 17 September were unsuccessful. The minimum field infection rate (MFIR) was 1 per 163 mosquitoes collected from all sites.

SLE virus was isolated from a pool of 7 Aedes mosquitoes collected September 11. Attempts to isolate virus from 35 other pools of 419 Aedes species were unsuccessful. The MFIR was 1 for 58 mosquitoes collected on September 11 or an overall MFIR of 1 per 436 mosquitoes overall. The isolation of SLE virus from an individual of the genus Aedes is not surprising in view of the very high MFIR observed for Culex sp. mosquitoes.

b. Attempted winter virus isolations from mosquitoes

The occurrence of the SLE virus epidemic in the Washington, D.C.-Maryland area during 1975, prompted investigation to test the hypothesis that the virus overwintered in hibernating Culex pipiens. Mosquitoes were collected from underground bunkers at a number of abandoned Army forts located in Delaware, Maryland, New Jersey, and Pennsylvania during January and February of 1976. In the laboratory, mosquitoes were identified and assayed for virus as described previously in this report. As shown in Table 21, a total of 116 (109 pools) of C. pipiens were assayed for virus. No virus was isolated from mosquitoes; however, the results were not clear due to what appeared to be nonspecific mouse mortality.

4. Studies of trombiculid mite vectors of rickettsioses

a. Introduction

(1) General

Laboratory studies were initiated in October 1975 to determine the chigger-rickettsial interactions necessary for transmission of Rickettsia tsutsugamushi within the vector mite and from mite to vertebrate host. This program is being conducted in collaboration with the US Army Medical Research Unit (Malaysia). Since scrub typhus is known to be transmitted exclusively by one-host vectors (the larvae of certain Leptotrombidium spp.) vertical (transovarial) transmission has long been recognized as essential to maintenance of the infection in nature. The fluorescent antibody technique has been used previously to identify R. tsutsugamushi in organs of transovarially-infected Leptotrombidium fletcheri; however, sites for rickettsial replication in the vectors are as yet unknown. Previously negative mites have been shown to acquire R. tsutsugamushi during larval feeding on an artificially infected host, but with one exception, the exposed mites failed to transmit the agent to their progeny. The fate of R. tsutsugamushi taken up by negative mites is unknown. If a vertical transmission model such as that proposed by Fine<sup>8</sup> is applied to the available data on R. tsutsugamushi-infected mites, vertical transmission alone seems incapable of supporting the infection indefinitely in natural populations. It is apparent that additional data



are needed to understand the dynamics of R. tsutsugamushi in vector mites. The information gained from the present program will be of value in predicting levels of infection in natural populations of vectors and in formulating effective control strategies.

Initial goals are to identify the mite tissues and organs that are sites for rickettsial replication, and to establish a transovarially passed R. tsutsugamushi infection in previously negative mites.

## (2) Sources of chiggers

A colony of Eutrombicula alfreddugesi was established from specimens collected in October 1975 by the USDA Insects Affecting Man Laboratory in Gainesville, Florida. This species is being used in perfecting techniques and defining anatomical targets for detailed electron microscopic examination. Three known or suspected vector species presumably free of R. tsutsugamushi infection were obtained from the University of Maryland (Baltimore) courtesy of Dr. Robert Traub. The species and countries of origin are: L. intermedium-Japan; L. deliense-Malaysia; and Gahrliepia ligula-Pakistan. Recently, infected and noninfected L. arenicola and L. fletcheri mites were obtained from the USAMRU (Malaysia). These species are being used in histologic comparisons of transovarially infected-, R. tsutsugamushi exposed-, and R. tsutsugamushi-negative specimens.

## (3) Maintenance of chigger colonies

Chigger colonies are being maintained by mass rearing techniques similar to those described by Traub et al.<sup>9</sup>. One problem has been the synchronization of infected and noninfected colonies to obtain noninfected males. Spermatophore uptake is essential for offspring production in the 2 infected colonies, since the infected female mites produce almost exclusively female progeny.<sup>10</sup> By placing individual mites in separate holding containers, spermatophores can be acquired without mixing infected and noninfected lines of mites.

All stages are held in a dual program incubator set to provide 39 C during 12 hours of light and 25 C during 12 hours of dark. Under these conditions, the period from egg to adult in the vector species has averaged about 9 weeks. Nymphs and adults are fed once per week on eggs of the collembolan, Sinella curviseta, and egg rafts of Culex spp.

### b. Histological studies

Preparations were made for light and transmission electron microscopy to identify the organs and tissues of significance to R. tsutsugamushi transmission. Difficulties in fixation resulted from the small size, buoyancy and relatively impervious exoskeleton of vector mites. Initially, mites were immobilized in paraffin, and the exoskeleton of the opisthosomal region was teased away with a finely-honed minuten pin. The mites were then fixed in a gluteraldehyde solution. A more effective method was to

place the mites on a cold (5 C) microscope slide and coat them with a drop of 2% liquid agar or 10% gelatin USP, which hardened on the slide. The exoskeleton of each mite was punctured with a minuten pin, and the entire preparation was placed in 10% buffered formalin. To enhance fixative penetration, the specimens and fixative were sealed in a 15cc vaccine vial and a vacuum was created with a 50cc syringe. The specimens were sectioned and stained with hematoxylin and eosine. Specimens for transmission em examination were placed in Karnovsky's fixative for 48 hours and embedded in epon. A dorsal, longitudinal section through a noninfected L. intermedium adult is shown in Fig. 4. Salivary glands, gut, supraesophageal ganglion, epidermis and muscles are readily identified. A transmission em photomicrograph of salivary gland cells is shown in Fig. 5. Horseradish peroxidase-labeled antisera will be used to identify R. tsutsugamushi in em preparations of exposed or transovarially infected specimens.

c. Establishment of R. tsutsugamushi infection in negative mites

Although there is only one reported instance of a transovarially passed infection resulting from exposure during larval feeding, rickettsiae are imbibed by larval mites and persist through the post-larval stages<sup>9</sup>. Over an 8 week period, approximately 2,000 L. deliense larvae were mass-fed on rickettsemic mice to study the fate of the agent in previously negative mites and, if possible, to establish a vertically-transmitted infection. The host mice had been inoculated intraperitoneally with 1,000 MLD<sub>50</sub> of the Karp strain of R. tsutsugamushi. The mice normally died 10-11 days post-inoculation.

It was noted that few of the chiggers fed to repletion on the sick mice, and this incomplete feeding apparently caused the higher than expected mortality rates observed in the nymphal stage. Comparative feedings on infected and noninfected hosts will be made incident to the other studies described.

Although data are lacking at present, it has been suggested that a "gut barrier" or similar structure(s) may prevent transovarial passage of rickettsiae imbibed by previously negative mites<sup>9</sup>. Since barriers to infection are known in vector systems of other arthropod-borne diseases, a micro-inoculation technique was developed in an attempt to establish a transovarially-passed infection in negative mites. Capillary tubes were heated over a Bunsen burner and pulled to produce a very fine glass needle. The needle was fitted in a 100 µl syringe mounted on a micro-manipulator. Twenty G. ligula adults immobilized in gelatin were inoculated by inserting the needle into the dorsal opisthosomal region. All but 2 of these mites died, apparently from the injury caused by the inoculation. To reduce this injury, a sharpened minuten pin was used to puncture the exoskeleton at the inoculation site. Two of 10 mites inoculated in this manner have survived for 4 weeks post-inoculation. During FY77, suspensions of triturated, infected L. arenicola and L. fletcheri will

be inoculated into negative adult and nymphal mites. The micro-inoculation apparatus will also be used to inject fixative solutions into specimens for histologic studies.

#### Conclusions and recommendations

1. The development of a viremia in deer following inoculation with JC virus and the failure to detect virus in the blood of deer following inoculation with a comparable dose of KEY virus strongly implies that deer are not directly involved in the maintenance of KEY virus. The data do, however, support and extend previous findings that deer are important vertebrate host of JC virus in the coastal plains of Maryland. Results concerning the immune response suggest that deer previously infected with JC virus are refractory to KEY virus infection and that deer infected with KEY virus failed to develop a detectable viremia after challenge inoculation with JC virus. Although deer appear to be dead end hosts for KEY virus, it is conceivable that this species contribute to transovarial passage of this virus by serving as a blood source for KEY virus infected A. atlanticus.

2. Presumptive serological evidence of KEY virus infection in cottontail rabbits captured on the eastern shore of Maryland (6/13) and in western Maryland (1/7), and the development of a viremia following experimental inoculation of rabbits with KEY virus suggest that this vertebrate may be an important host of KEY virus. The occurrence of natural KEY virus infection in rabbits on Assateague Island is of interest since gray squirrels do not inhabit this island, nor are there any records of A. atlanticus being collected there. Previous isolation of KEY virus from salt marsh mosquito species on the island suggests the maintenance cycle of KEY here involves a mosquito species other than A. atlanticus. Further investigation regarding cottontail rabbits as host of KEY and JC virus are needed.

3. Experimental vector competence studies indicate that Aedes atlanticus became infected after ingesting KEY virus. The 50% threshold level of infection remains to be determined; however, available data suggest that this species could readily become infected by feeding on viremic gray squirrels and cottontail rabbits. Although A. canadensis became infected after ingesting KEY virus, the high threshold level of infection suggests that this species would seldom become infected through feeding on viremia gray squirrels and cottontail rabbits. Attempts to demonstrate KEY virus transmission to mice by A. atlanticus and by A. canadensis have not been successful. Preliminary studies suggest that an alternative indicator system may be necessary to detect virus transmission. Preliminary studies involving P. ferox and KEY virus suggest that this species may be refractory to infection or else a high threshold level of infection precludes a vector role. Additional studies are needed to accurately establish the threshold level of KEY virus infection in P. ferox and A. atlanticus.

Although A. canadensis became infected after ingesting JC virus, the threshold level of infection suggest that this species could not serve as a primary vector on the basis of viremia levels observed in whitetailed deer. Other arthropods including biting flies should be considered as possible vectors of JC virus.

4. The gray squirrel data clearly show this vertebrate to become infected with KEY virus and for the first time show KEY virus infection in gray squirrels to be associated in time and space with KEY virus infection in the A. atlanticus populations. KEY virus infection occurred in adults and juveniles squirrels, but majority of seroconversion involved adult males. The tendency of squirrel to frequent the same trap and to exhibit territorial behavior suggest that these data do not reveal a valid estimate of the extent of KEY virus infection in different age and sex class of the gray squirrel populations.

The contribution of KEY virus infected gray squirrels to the maintenance of KEY virus is pending further investigation; however, available data strongly imply an amplifying role. This role is somewhat questionable due to the failure to isolate virus from blood of gray squirrels, especially those squirrels that were negative for virus 4 days prior to seroconverting to KEY virus. Laboratory studies are being performed to clarify these findings. In addition, consideration will be given to the possible influence of KEY virus infection in the gray squirrels population on the seasonal distribution of KEY virus infection prevalence rates in the A. atlanticus population.

5. Field collections of floodwater mosquito eggs coupled with laboratory respiration studies of diapausing and non-diapausing eggs offer an excellent opportunity of discovering the environmental factors inducing diapause in eggs in nature. An understanding of these factors would be extremely valuable in forecasting mosquito population trends and in designing control strategies.

6. St. Louis encephalitis virus was prevalent throughout the United States and parts of Canada during the summer of 1975. The extension of its geographical range to the more northern section of the U.S. including the mid-Atlantic states prompted much speculation concerning the endemicity of this virus. Although attempts to isolate St. Louis encephalitis virus from mosquitoes collected during the wintry season in Maryland were unsuccessful, further studies are needed to clarify ecology. This should include more intensive collections during the winter season and mosquitoes should be provided with a bloodmeal before subjecting them to virus assay.

7. Techniques are being developed which will enable study of Rickettsia tsutsugamushi infections in trombiculid mite vectors. Eventually, the use of these techniques should help define the epidemiological significance of mites and rodents in the natural maintenance of the disease, and thus permit the development of better focused control strategies.

TABLE 1

Infectivity titer based on plaque-forming units of Jamestown Canyon  
and Keystone virus in continuous cell lines

Virus	Cell Lines			
	LLC-MK <sub>2</sub>	BHK-21,C-13	BHK-21,C-15	VERO
Jamestown Canyon	$2 \times 10^{5 \frac{1}{2}}$	$2 \times 10^6$	$1 \times 10^8$	$5 \times 10^7$
Keystone	$4 \times 10^6$	$3 \times 10^7$	$8 \times 10^7$	$6 \times 10^6$

1/ Titer based on 0.2 ml. of inoculum

TABLE 2

Inoculation and challenge scheme for whitetail deer-Jamestown Canyon  
and Keystone virus study

Deer No.	Sex	Weight(kg)	Primary Inoculum	Challenge Inoculum	Days (P.I.)
188 <sup>2/</sup>	M	20.5	JC Virus	Key Virus	30
195	M	12.7	" "	" "	"
180	M	13.6	" "	" "	"
182(C) <sup>2/</sup>	F	16.9	Medium 199-H	" "	30
185	F	16.4	JC Virus	" "	80
108	F	19.1	" "	Key Virus	"
109	M	17.3	JC Virus	" "	80
183	F	16.9	Key Virus	JC Virus	80
186 <sup>2/</sup>	F	13.6	" "	" "	"
191	M	13.2	" "	" "	"
196	M	18.2	Key Virus	JC Virus	80
187(C) <sup>2/</sup>	M	15.5	Medium 199-Y		
189(C) <sup>2/</sup>	F	18.2	Medium 199-H		

<sup>2/</sup>

Deer no. 188 ill on 5 Dec and died on 18 Dec or 38 days P.I.

Deer no. 186 ill on 14 Dec and sacrificed on 22 Dec or 8 days P.I.

Deer no. 187 ill on 10 Nov and died on 16 Nov or 6 days P.I.

Deer no. 189 ill on 28 Nov and submitted immediately for diagnostic studies

TABLE 3

Duration and magnitude of viremia in whitetail deer fawns following subcutaneous inoculation with  $10^{5.0}$  SMLD<sub>50</sub>/1.0 ml of Jamestown Canyon virus

Deer Number	Days Post Inoculation					
	1	2	3	4	5	7
180	- <sup>1/</sup>	3.4 <sup>2/</sup>	2.9	1.8	-	-
195	3.2	5.0	-	-	-	-
188	-	-	-	-	-	-
185	-	-	2.3	2.2	-	-
108	T <sup>3/</sup>	4.1	3.9	2.6		
109	2.4	3.9	3.4	T	-	-
189 (control)	-	ND <sup>4/</sup>	-	ND	-	ND
182 (control)	-	ND	-	ND	-	ND

<sup>1/</sup> Virus not recovered from 1:5 dilution of blood

<sup>2/</sup> Log<sub>10</sub> SMLD<sub>50</sub>/1.0 ml

<sup>3/</sup> Trace of virus

<sup>4/</sup> Not done

TABLE 4

Homologous antibody titers of deer inoculated subcutaneously with  $10^{5.0}$  SMLD<sub>50</sub>/1.0 ml of Jamestown Canyon virus and challenged on day 30 PI with  $10^{6.6}$  SMLD<sub>50</sub>/1.0 ml with Keystone virus

Deer No.	<u>Days Post Inoculation</u>									
	5	10	20	30 <sup>4/</sup>	32	34	37	40	60	100
188	0	<80 <sup>1/</sup>	80	160	20	90	80	D <sup>2/</sup>		
195	10	280	640	>640	500	320	500	>640	600	640
180	0	250	200	160	100	100	80	80	160	225
182(C) <sup>3/</sup>	0	0	0	0	0	0	0	0	0	0

<sup>1/</sup> Reciprocal antibody titers

<sup>2/</sup> Dead

<sup>3/</sup> Control

<sup>4/</sup> KEY Challenge

TABLE 5

Cross reaction of antibody raised to Jamestown Canyon virus with Keystone virus (Days 10, 20, 30) and heterologous antibody titers produced after challenging Jamestown Canyon virus-inoculated deer with Keystone virus

Deer No.	<u>Days Post Inoculation</u>								
	10	20	30 <sup>3/</sup>	32	34	37	40	60	100
188	0	10 <sup>1/</sup>	10	0	10	10	D <sup>2/</sup>		
195	30	80	100	40	80	80	40	60	30
180	20	10	10	5	10	20	40	10	<10
182(C)	0	0	0	0	0	25	50	160	90

<sup>1/</sup> Reciprocal antibody titers

<sup>2/</sup> Dead

<sup>3/</sup> KEY Challenge



TABLE 6

Homologous antibody titers detected in serum of whitetail deer following subcutaneous inoculation with  $10^{4.5}$  SMLD<sub>50</sub>/1.0 ml of Keystone virus

Deer No.	<u>Days Post Inoculation</u>					
	5	10	20	40	60	80
186	15 <sup>1/</sup>	150	ND <sup>2/</sup>	540	Dead	
183	15	26	<80	<1/80	<1/80	<1/80
196	10	80	80	320	470	340
191	40	170	520	350	320	220

<sup>1/</sup> Reciprocal antibody titers

<sup>2/</sup> Not done

TABLE 7

Infection and transmission rates for Aedes atlanticus after ingesting  $10^{6.0}$  SMICLD<sub>50</sub>/1.0 ml. of Keystone virus<sup>1/</sup> through a membrane

Incubation (Days)	No. of Mosquitoes	Infection Rates	Transmission Rates
3	3	3/3(100%)	ND <sup>2/</sup>
5	5	5/5(100%)	0/5(00%)
7	ND	ND	ND
14	4	4/4(100%)	0/4(00%)
21	3	3/3(100%)	0/1(00%)
TOTAL	15	15/15(100%)	0/10(00%)

<sup>1/</sup> Keystone virus had been through 4 cell culture (BHK-21) passages

<sup>2/</sup> Not done

TABLE 8

Infection and transmission rates for Aedes atlanticus after ingesting  $10^{5.2}$  SMICLD<sub>50</sub>/1.0 ml. of Keystone virus<sup>1/</sup> through a membrane

Incubation (Days)	No. of Mosquitoes	Infection Rate	Transmission Rate
3	5	4/5 (80%)	ND <sup>2/</sup>
5	5	5/5(100%)	0/5(00%)
7	5	5/5(100%)	0/5(00%)
14	7	7/7(00%)	0/7(00%)
21	5	4/5(80%)	0/4(00%)
TOTAL	27	25/27(93%)	0/21(00%)

<sup>1/</sup> Keystone virus had been through 4 cell culture (BHK-21) passages

<sup>2/</sup> Not done

TABLE 9

Infection and transmission rates for Aedes canadensis after ingesting  $10^{3.4}$ SMICLD<sub>50</sub>/1.0 ml of Keystone virus<sup>1/</sup> through a membrane

Incubation (Days)	No. of Mosquitoes	Infection Rate	Transmission Rate
8	10	1/10(10%)	0/10(00%)
14	5	1/5(20%)	0/5(00%)
21	5	1/5(20%)	0/5(00%)
33	5	2/5(40%)	ND <sup>2/</sup>
TOTAL	25	5/15(33%)	0/20(00%)

<sup>1/</sup> Keystone virus had been through 2 mouse brain passages

<sup>2/</sup> Not done

TABLE 10

Infection and transmission rates for Aedes canadensis after ingesting  $10^{5.2}$ SMICLD<sub>50</sub>/1.0 ml of Keystone virus<sup>1/</sup> through a membrane

Incubation	No. of mosquitoes	Infection Rate	Transmission Rate
7	10	6/10(60%)	0/10(00%)
14	10	4/10(40%)	0/10(00%)
21	10	3/10(30%)	0/8(00%)
TOTAL	30	13/30(43%)	0/28(00%)

<sup>1/</sup> Keystone virus had been through 2 mouse brain passages

TABLE 11

Infection and transmission rates for *Psorophora ferox* after ingesting  
 $10^{3.6}$  SMLD<sub>50</sub>/1.0 ml of Keystone<sup>1/</sup> virus through a membrane

Incubation (Days)	No. of Mosquitoes	Infection Rate	Transmission Rate
5	6	ND <sup>2/</sup>	0/6
7	7	ND	0/7
14	10	0/10	0/10
21	10	0/10	0/7
28	10	0/10	ND
	43	0/30	0/30

<sup>1/</sup> Keystone virus had been through 4 cell culture (BHK-21) passages

<sup>2/</sup> Not done

TABLE 12

Criteria for distinguishing between adult and juvenile gray squirrels

Adults	Juveniles
Males	
1. Ventral surface and posterior end of scrotum blackened, and generally free of hair.	1. Posterior end only of scrotum with smooth skin, brown to black and free of hair.
2. Cowper's glands one-half inch or more in diameter from November to following July (in other months about same size as in juveniles).	2. Cowper's glands undeveloped.
Females	
1. Mammary glands large and noticeable, not hidden by hair growth; teats (in bred fox squirrel females) black tipped.	1. Teats inconspicuous, more or less hidden in growth of hair.
2. Uterus contracted in posterior position of coelom, horns about 2 mm. wide and flattened.	2. Uterus threadlike, extending forward toward kidneys.
Males and Females	
1. Body length (tip of nose to anus) over 280 mm. for fox squirrels and over 250 mm. for gray squirrels.	1. Body length (tip of nose to anus) under 280 mm. for fox squirrels and under 250 mm. for gray squirrels.
2. Tail rectangular, block shaped; sides parallel or nearly so.	2. Tail pointed, triangular; sides not parallel.
3. Tips of guard hairs on tail rufous to red (fox squirrels only).	3. Tips of guard hairs on tail silvery until first tail molt in fall (fox squirrels only).

TABLE 13

Population structure of the gray squirrel population in the Pocomoke  
Cypress Swamp, Worchester County, Maryland in 1975

Date of Trap Period	Males Adults	Male Juveniles	Females Adults	Female Juveniles
24 Mar	2	0	2	0
3-4 Apr	5	0	5	0
16-17 Apr	5	0	5	0
27-28 Apr	5	0	1	0
6-7 May	3	0	2	0
20-21 May	4	0	3	0
15-17 Jul	12	3	2	3
26-28 Jul	4	1	1	0
30-31-1 Aug	2	2	1	0
04-06 Aug	1	1	2	0
11-13 Aug	2	1	0	1
20-22 Aug	1	0	0	2
26-29 Aug	2	0	0	3
03-05 Sept	0	0	0	1
TOTAL	48	8	24	10

TABLE 14

Gray squirrel capture and recapture data for each trap period in the  
Pocomoke Cypress Swamp study area, Worcester County, Maryland, 1975

Date of Trap Period	No. of Runs/ Period <sup>1/</sup>	No. of Squirrels		Sex and Age			
		New	Recaptures	Males		Females	
				Adults	JUV	Adults	JUV
24 Mar	1	04(100%)	00(00%)	02	00	02	00
3-4 Apr	1	10(100%)	00(00%)	05	00	05	00
16-17 Apr	2	10(77%)	03(23%)	07	00	06	00
27-28 Apr	1	06(60%)	04(40%)	07	00	03	00
06-07 May	1	05(44%)	06(56%)	08	00	03	00
20-21 May	2	07(100%)	00(00%)	04	00	03	00
15-17 Jul	4	20(59%)	14(41%)	21	03	07	03
26-28 Jul	3	06(20%)	24(80%)	20	04	04	02
30-01 Aug	3	05(22%)	18(78%)	13	05	05	00
04-06 Aug	4	04(15%)	23(85%)	15	02	09	01
11-13 Aug	3	04(15%)	22(85%)	14	04	07	01
20-22 Aug	3	03(22%)	22(88%)	13	03	07	02
26-29 Aug	6	05(14%)	30(86%)	15	03	13	04
03-05 Sep	6	01(03%)	30(97%)	13	03	08	07
16-18 Sep	5	00(00%)	13(100%)	06	00	02	05
24-25 Sep	3	00(00%)	01(100%)	00	00	01	00

<sup>1/</sup> Run refers to each time traps were checked for squirrels.

TABLE 15

Distribution of Keystone virus antibody in gray squirrels by age and sex

Sex	Adults	Juveniles	Total
Male	28/47 (60%)	2/9 (22%)	30/56 (54%)
Female	13/24 (54%)	5/8 (63%)	18/32 (56%)
TOTAL	41/71 (57.7%)	7/17 (41%)	48/88 (56%)



TABLE 16

Appearance of KEY virus antibody in sera of the 25 gray squirrels that become infected during the study

Gray Squirrel No.	Sex/Age	Seroconversion Date <sup>1/</sup>		No. Seroconversion Per Trap Period	Time Interval <sup>2/</sup> Days
		Negative	Positive		
25	M/A	7 May	27 Jul	2	48
144	F/I	16 Jul	27 Jul		11
66	M/A	27 Jul	31 Jul	1	04
121	F/I	31 Jul	04 Aug	4	04
60	M/A	31 Jul	04 Aug		04
08	M/A	27 Jul	05 Aug		09
36	M/A	31 Jul	06 Aug		06
58	M/A	31 Jul	12 Aug	6	12
38	M/A	31 Jul	12 Aug		12
57	M/A	04 Aug	12 Aug		08
31	F/A	06 Aug	12 Aug		06
35	M/A	06 Aug	12 Aug		06
03	F/A	24 Mar	13 Aug		142
01	M/A	21 Aug	21 Aug		45
43A	M/I	01 Aug	21 Aug	4	20
50A	M/I	04 Aug	21 Aug		17
21A	M/A	12 Aug	22 Aug		10
56	M/A	27 Jul	27 Aug	4	31
54	F/A	16 Jul	28 Aug		43
147	F/I	27 Jul	28 Aug		32
80	F/A	06 Aug	28 Aug		22
70	M/A	27 Jul	3 Sept	3	61
64	F/I	17 Jul	3 Sept		48
71	M/I	21 Aug	05 Sept		15
16	M/A	28 Aug	17 Sept	1	14

<sup>1/</sup> Seroconversion occurred during period between dates.

<sup>2/</sup> Period of time from date gray squirrels were negative for KEY virus antibody to date KEY virus antibody were first detected in squirrel sera.

TABLE 17

Identification of Keystone virus antibody in selected gray squirrel sera that inhibited plaque formation of both Keystone and Jamestown Canyon virus

Gray Squirrel Serum No.	Date Collected	% Reduction <sup>1/</sup>		PRNT 50% Endpoints	
		KEY	JC	KEY	JC
169	05 Aug	100%	100%	640	20
199	12 Aug	100%	71%	110	<40
206	12 Aug	100%	100%	1280	40
257	28 Aug	100%	100%	>320	130
264	28 Aug	100%	100%	>320	<40
272	29 Aug	100%	100%	>320	<40
273	29 Aug	100%	100%	>320	100
281	03 Sept	100%	100%	>320	<40
290	04 Sept	100%	100%	40	< 5
323	25 Sept	100%	100%	>640	40

<sup>1/</sup> Percentage JC and KEY virus dose reduced by sera when screened at a 1:5 dilution for JC and KEY virus antibody.

TABLE 18

Presumptive virus isolations and minimum field infection rates  
(MFIR) for Aedes atlanticus

Collection Date (1975)	No. Mosquitoes Assay for Virus	No. of Isolations	MEIR
22 Jul	1,250	05	1/250
24 "	1,275	09	1/142
26 "	15,925	36	1/442
28 "	9,300	24	1/388
30 "	6,250	16	1/390
01 Aug	1,850	05	1/370
03 "	2,750	07	1/393
05 "	4,300	16	1/269
07 "	5,875	17	1/346
09 "	1,625	04	1/406
11 "	825	02	1/413
13 "	1,300	05	1/260
15 "	1,550	05	1/310
17 "	1,660	04	1/400
19 "	475	00	0/475
21 "	625	03	1/208
23 "	225	04	1/156
25 "	175	00	0/175
27 "	500	01	1/500
29 "	225	00	0/225
31 "	200	02	1/100
02 Sep	225	01	1/225
04 "	200	01	1/200
06 "	200	02	1/100
08 "	400	00	0/400
10 "	175	00	0/175
12 "	150	00	0/150
14 "	100	00	0/100

TABLE 18 (Continued)

Collection Date (1975)	No. Mosquitoes Assay for Virus	No. of Isclations	MEIR
16 Sep	100	00	0/100
18 " *	375	00	0/375
20 "	1125	03	1/375
22 "	575	01	1/575
24 "	400	02	1/200
26 "	525	03	1/175
28 "	175	01	1/175
30 "	175	02	1/88
02 Oct	250	01	1/250
04 "	75	00	0/75
06 "	200	04	1/50
08 "	200	00	0/200
10 "	150	00	0/150
12 "	125	04	1/31
14 "	125	01	1/125
16 "	174	00	0/175
18 "	125	00	0/125
20 "	50	00	0/50
22 "	00	00	0/0
24 "	25	01	1/25
TOTAL	64,520	194	1/332

\* Second emergence of A. atlanticus population

TABLE 19

Hatch rates for eggs of A. atlanticus, A. canadensis, and P. ferox in soil samples subjected to 2 photoperiod regimes at 25 C

Species	Photoperiod (L:D)	Intial Flooding	Day 7	Day 14	Day 24	Total Eggs
<u>A. atlanticus</u>	15:9	0/2	34/34	0/1	0	34
	9:15		3/3	40/40	1/1	44
<u>A. canadensis</u>	15:9	50/51	123/123	45/45	0	168
	9:15		3/3	81/82	7/8	91
<u>P. ferox</u>	15:9	0	401/401	201/201	220/220	822
	9:15		12/12	166/167	2/2	180

TABLE 20

Summary of SLE virus isolations from Culex sp. mosquitoes collected  
in Prince Georges County, Maryland, 1975

Collecting Date	No. <u>Culex</u> sp. captured/tested	Collecting Sites	Mosq. Pools/Virus Isolation
10 Sep	23/23	1-6	4/0
11 Sep	88/88	1-6	6/1*
12 Sep	133/133	1-6	9/1**
13 Sep	0/0	7-12	-
16 Sep	31/31	7-12	2/0
17 Sep	32/52	13-18	5/0
TOTALS 327***			26/2

\* SLE virus isolated from a pool of 12 mosquitoes

\*\* SLE virus isolated from a pool of 25 mosquitoes

\*\*\* Minimum field infection rate, 1 SLE isolate/163 Culex sp.

TABLE 21

St. Louis encephalitis virus isolation attempts from Culex pipiens  
collected during January and February of 1976

Collection Site	Collection Date	No. Mosquitoes Assayed/ No. of Pools
Ft. Armistead (Baltimore, MD)	3 Feb	72/8
Ft. McHenry (Baltimore, MD)	3 Feb	141/11
Ft. Mott (Salem Co., N.J.)	17 Feb	487/49
Ft. Mifflin (Delaware Co., PA)	22 Jan	151/15
Ft. Dupont (New Castle Co., DE)	23 Jan	213/21
Ft. Washington (Prince Georges Co., MD)	12 Feb	52/5
TOTAL		1116/109

Captions for Figures 1 - 5, following:

Figure 1. Location of gray squirrel traps and feeders and mosquito light traps in the Pocomoke Cypress Swamp area, Worcester Co., Maryland.

Figure 2. KEY virus antibody prevalence rate in gray squirrels in relation to Aedes atlanticus activity period.

Figure 3. Seasonal distribution and estimated population density of Aedes atlanticus in relation to temperature and rainfall.

Figure 4. Dorsal longitudinal section through a noninfected Leptotrombidium intermedium adult.

Figure 5. Electron photomicrograph of salivary gland cells from adult Leptotrombidium intermedium.



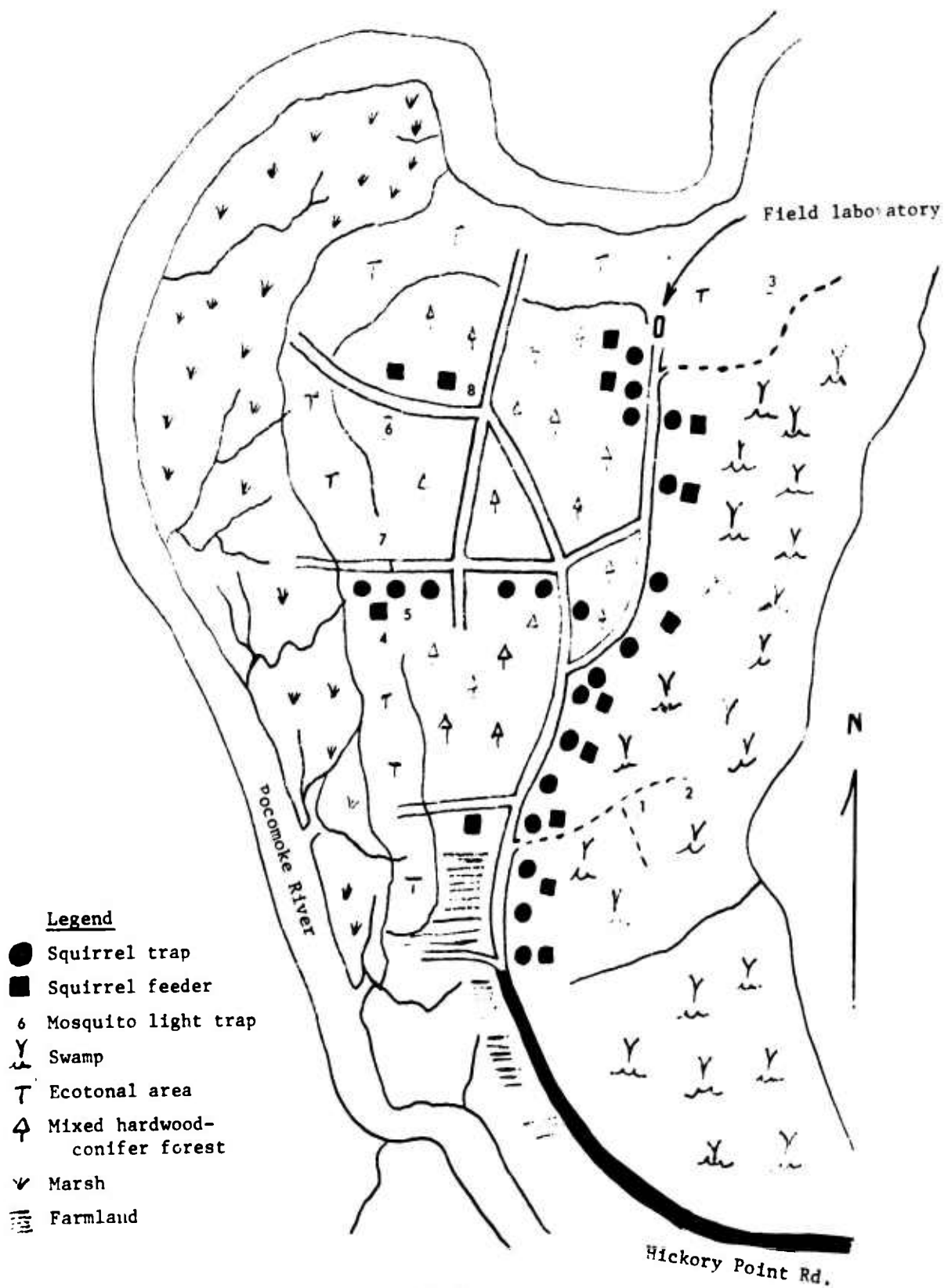


Fig. 1

Fig. 2

Fig. 3

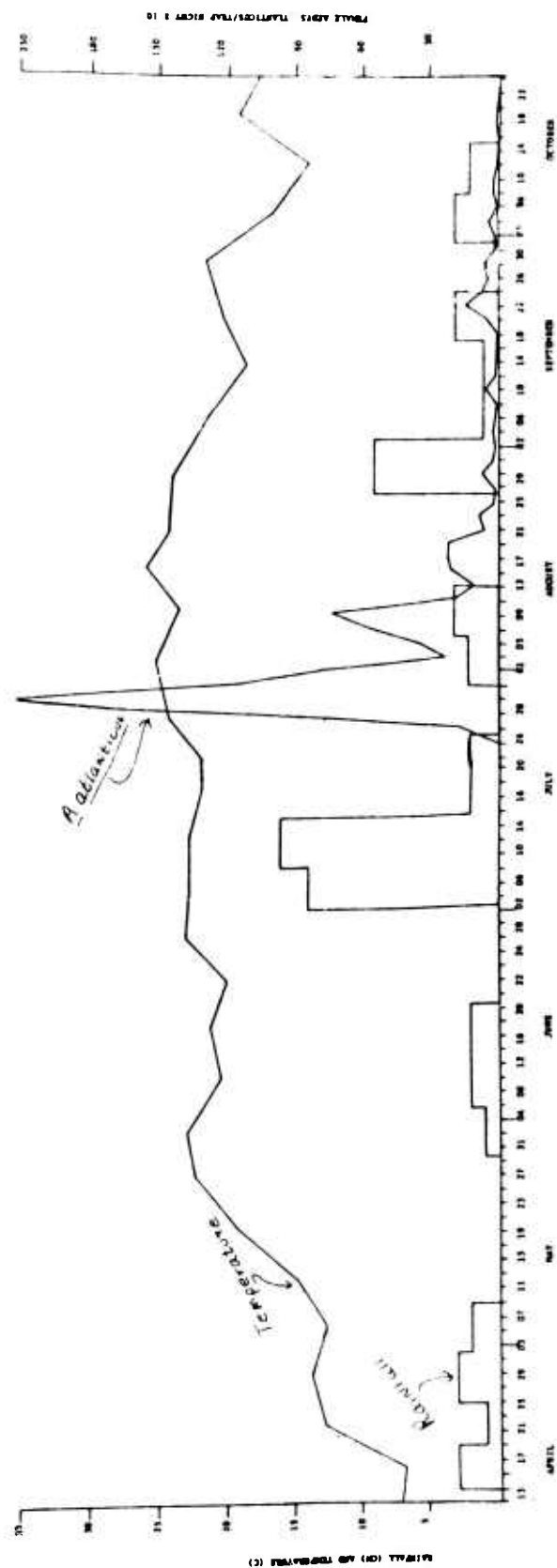
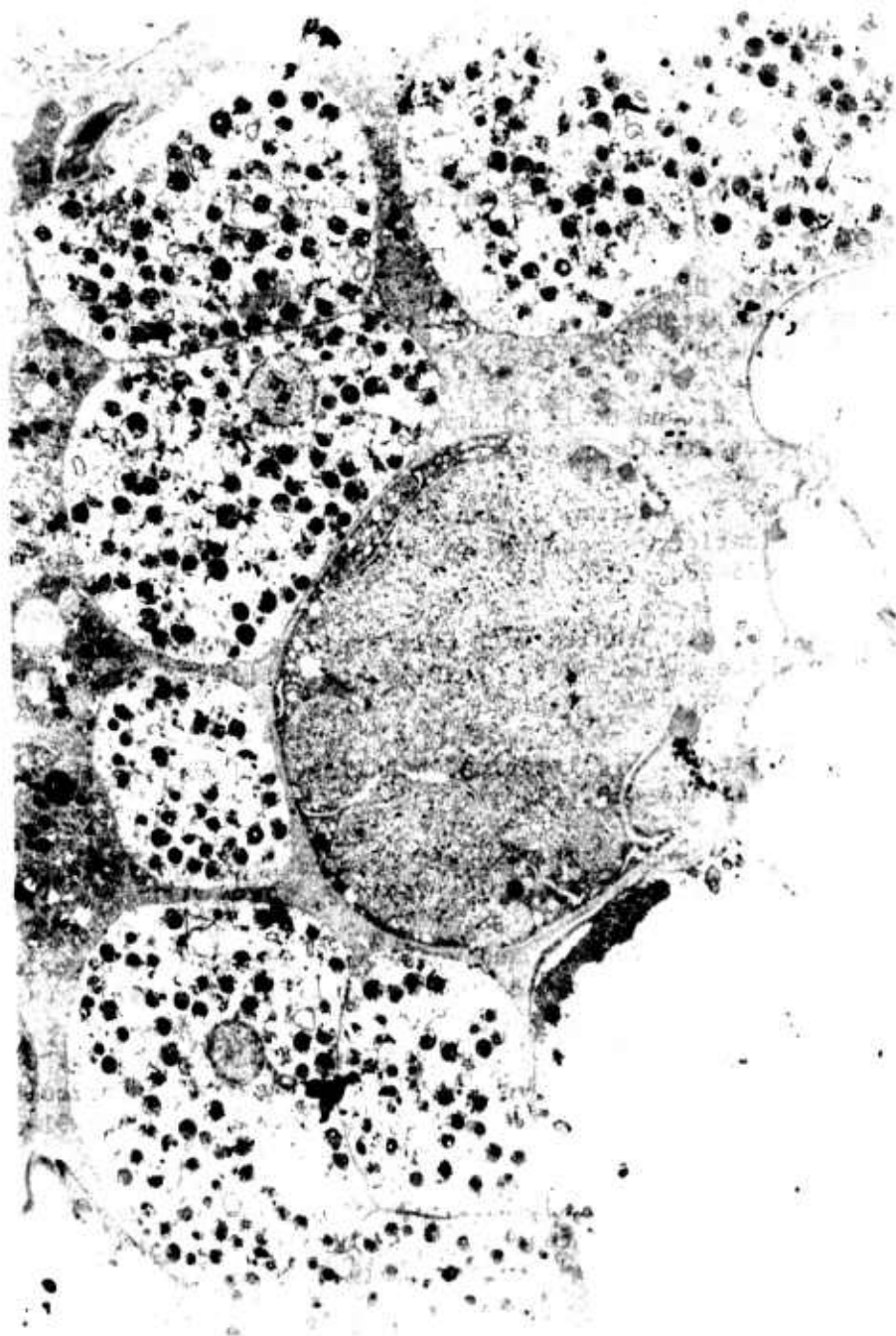


Fig. 4



Fig. 5



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		2. DATE OF SUMMARY		REPORT CONTROL SYMBOL	
				DA DB 6453		76 06 30		DD DR&E(A)636	
3. DATE PREVIOUS		4. KIND OF SUMMARY		5. SUMMARY SCTY		6. WORK SECURITY		7. REGRADING	
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8. SPECIFIC DATA- CONTRACTOR ACCESS		9. LEVEL OF SUM		10. NO. CODES		PROGRAM ELEMENT		PROJECT NUMBER	
<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		A. WORK UNIT							
11. TITLE (Precede with Security Classification Code)									
(U) Molecular Basis of Biological Regulation and Chemotherapeutic Drug Pharmacology									
12. SCIENTIFIC AND TECHNOLOGICAL AREAS									
002300 Biochemistry									
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ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012					
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)					
NAME: Joy, COL R.J.T.				NAME: Hahn, F. E., PhD					
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3657					
21. GENERAL USE				ASSOCIATE INVESTIGATORS					
Foreign intelligence not considered				NAME: Krey, Anne K., M.S.					
				NAME: Wolfe, Alan D., PhD				DA	
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(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)									
<p>23. (U) The scientific objective is to study the molecular pharmacology of elimination of R-factor-mediated bacterial multiresistance 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.</p> <p>24. (U) Approaches are fundamental laboratory investigations using advanced microbiological, biochemical and biophysical methods and concepts.</p> <p>25. (U) 75 07 - 76 06 R-factor-eliminating drugs: ethidium, quinaerine, acridine orange, quinine, chlorpromazine, chloroquine and methylene blue are intercalated into DNA as was proved by viscometric titrations of closed circular DNA. At low ionic strength, the antimalarial drug, primaquine, is also intercalated. - Triphenylmethane dyes form complexes with DNA and act as template poisons in the DNA polymerase I reaction; they also inhibit the hydrolysis by the same enzyme of its DNA polymerization products. - Electrical dichroism studies on complexes of DNA with distamycin A, quinaerine, chloroquine and methylene blue yielded precise determinations of the placement of these molecules relative to linear DNA. - R-factor DNA was isolated by lysis of <i>E. coli</i>, containing a <i>Pseudomonas</i> R-factor, on top of a linear sucrose gradient, followed by centrifugation and collection of fractions. - By order of Commanding General, USA Medical R&amp;D Command, the work on 074 is being discontinued. For final technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75-30 Jun 76.</p>									

DD FORM 1498

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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. This is a terminal report. The R&D Command has judged the Work Unit 074 "not (to) have a direct or supporting military application" and has ordered it abolished. The Principal Investigator, on scientific and epidemiological grounds, wishes to be on record as being in considered professional disagreement with this judgement and decision. The Work Unit 074 is an unqualified scientific success and the military importance of it will become apparent when troops are sent to areas of the world where multiresistant enteric infections are endemic or epidemic.

Progress and Results.

R-factor replication in vitro. First experiments employed a bacterial extract (*E. coli*) which contained both R-factor and a group of enzymes able to catalyze the incorporation of  $^3\text{H}$ -dCTP into a molecular polymeric form, precipitable by cold 5% trichloroacetic acid. The synthetic activity of the extract was dependent upon the presence of DNA and of both the ribo- and deoxyribonucleoside triphosphates. From sucrose gradient analyses it was apparent that parts of R-factors were being replicated. However, the activity of these extracts was variable. Therefore, isolation of the individual enzymes and proteins known to participate in DNA replication was undertaken.

Simultaneously, a series of experiments were performed to determine the influence of R-factor eliminating drugs and inhibitors of DNA synthesis on the three bacterial enzyme reactions which polymerize DNA. In an article submitted for publication in Biochemistry, we have reported that cationic triphenylmethane dyes inhibit DNA polymerization

carried out by DNA polymerase A. Consistent with biophysical studies (Müller and Gautier, 1975), these dyes, including crystal violet, methyl green and malachite green, inhibited polymerization of AT-rich DNA more effectively than polymerization of GC-rich DNA. Triphenylmethane dyes bind to the outside of DNA double helix, in contrast to planar drugs such as proflavine which bind internally (intercalate) between DNA base pairs. Intercalants possess a greater affinity for GC-rich DNA than for AT-rich DNA. Such AT or GC affinity differences were also observed in our work by comparing the inhibitions of polymerizations of AT and GC-rich DNAs.

DNA polymerase A also catalyzes the hydrolysis at the 3' termini of DNA; therefore, the influence of drugs and dyes upon this activity was determined, since one possible mode of R-factor elimination might be plasmid destruction by biosynthetic enzymes which are unable to carry out their anabolic functions in the presence of inhibitors of DNA synthesis. Both inside and outside binders inhibited the nuclease activity of DNA polymerase A. Hence, polymerase A will probably not destroy plasmids in the presence of eliminating agents.

Currently, polymerases B and C are being isolated in order to test the influence of R-factor eliminators and DNA binders upon their catalytic activities, as well as to lay the groundwork for in vitro R-factor replication.

Molecular biology of R-factor elimination in vivo. Prior to initiating studies on the in vivo fate of R-factor DNA upon exposure of R-factor harboring bacterial strains to "curing" agents, it was necessary first to show biosynthetic intermediates of plasmid replication in normally growing bacteria. A procedure for analyzing plasmid replication has been developed in Staphylococcus aureus (Sheehy and Novice, 1975). The method consists of enzymatic lysis of radioactively pulse-labeled bacteria on top of a neutral sucrose gradient followed by centrifugation under conditions such that the chromosomal DNA forms a pellet at the bottom of the centrifuge tube while smaller DNA molecules such as plasmids form discrete bands at appropriate locations in the gradient. This methodology was applied to the W3110 R<sup>+</sup> strain of Escherichia coli.

Since the bulk chromosomal DNA in these gradients forms a viscous mass at the bottom of the centrifuge tube, a fractionation device, mentioned in a previous Annual Report, was perfected to permit accurate and reproducible collection of fractions from the top or upper surface of gradients. Accuracy and reproducibility were checked by preparing a discontinuous "sandwich gradient" and a velocity centrifugation gradient of a radioactive-labeled ribosomal preparation from Bacillus megaterium and comparing the top collecting method to a bottom tube puncturing fractionation. A discontinuous "sandwich gradient" was prepared by filling a centrifuge tube with a number of equal portions

of sucrose solutions whose concentrations decrease in a stepwise fashion. Every alternate layer (four such layers) contained Blue Dextran 2000, a high molecular weight absorbancy marker, in a concentration that increased linearly from bottom to top or from top to bottom. Parallel tubes were fractionated by the upper withdrawal device and by the bottom puncture method; four symmetrical peaks whose heights increase (or decrease) linearly were obtained in the gradients and, when plotted to permit direct comparison, the profiles obtained by both methods were identical and superimposable. Gradient profiles of ribosomes, labeled with  $^{14}\text{C}$ -uracil and prepared from B. megaterium, were, likewise, identical and superimposable.

The demonstration of plasmid DNA by enzymatic lysis of  $^{14}\text{C}$ -thymidine-labeled W3110  $\text{R}^+$  cells on top of a neutral sucrose gradient was accomplished in the following manner. Cells were labeled by adding  $^{14}\text{C}$ -thymidine to a culture of cells in the early exponential phase of growth (cell density of  $8 \times 10^8/\text{ml}$ ). Deoxyadenosine was added to facilitate the incorporation of radioactive thymidine. After 90 min, the metabolic activity of the cultures was rapidly stopped by pouring them onto crushed ice containing KCN and EDTA. All subsequent steps, unless noted, were performed at 0 C. The cells were immediately pelleted by centrifugation and suspended in a small volume of solution consisting of 0.02 M KCN, 0.15 M NaCl and 0.1 M EDTA, pH 7.0. This concentrated cell suspension was mixed with an equal volume of lysozyme dissolved in a solution of 0.1 M NaCl plus 0.05 M EDTA (pH 7.0) to give a final enzyme concentration of 200  $\mu\text{g}/\text{ml}$ . An aliquot (0.5 ml) of this mixture, as well as a sample of  $^3\text{H}$ -thymidine-labeled cells of the isogenic plasmid-negative parent strain (control) was immediately layered on a 4 ml 10 to 30% linear neutral sucrose gradient which had been overlaid with 0.1 ml of 4% BRIJ-58 in 5% sucrose. Both the gradient and the intermediate BRIJ-58 layer contained 0.01 M EDTA and 1.0 NaCl. After permitting lysis to proceed for 5 min at room temperature, the gradient was centrifuged at 45,000 rpm for 105 min at 5 C, using a Beckman SW 50.1 swinging bucket rotor in a Beckman L2-65B ultracentrifuge.

Collection of the gradient was accomplished by removal of 2 drop fractions (approximately 0.1 ml) from the top by using the nylon plug fractionation device. Samples were processed for the determination of radioactivity by adding to all fractions 0.1 ml of ice-cold 10% trichloroacetic acid (TCA) containing 5  $\mu\text{g}/\text{ml}$  unlabeled thymidine. Bovine serum albumin was added to serve as a carrier; the acid-insoluble precipitates were collected on membrane filters (Millipore, 0.45  $\mu\text{m}$ ). The filters were pre-soaked in, and the acid-soluble precipitates that were formed were washed extensively with, ice-cold 5% TCA containing 5  $\mu\text{g}/\text{ml}$  unlabeled thymidine. Washed filter membranes containing radioactive material were placed directly into vials containing 10 ml of dioxane-based scintillation fluid. Radioactivity was determined by counting in a scintillation spectrometer (Nuclear-

Chicago, 720 series).

The only significant peak (with a heavy shoulder suggestive of replicative intermediates) in the gradient was labeled with  $^{14}\text{C}$  only and so represents the plasmid DNA; the lack of significant  $^3\text{H}$ -label in the gradient indicates the efficiency of separation of plasmid DNA from chromosomal DNA and also suggests that there is little breakage of chromosomal DNA to smaller and lighter fragments.

After the initial apparent success in demonstrating plasmid DNA by this method, a number of difficulties were encountered in reproducing these experiments. The following were found to cause variations in the degree of recovery of plasmid DNA: (1) the temperature of the prepared gradients and the temperature at which the centrifugation was performed; (2) length of exposure to, and temperature of treatment with, lysozyme; and (3) the temperature during enzymatic lysis in the presence of the intermediate layer of non-ionic detergent, BRIJ-58. Lower temperatures resulted in incomplete lysis and poor recovery of plasmid DNA; higher temperatures caused extensive lysis resulting in breakage of chromosomal DNA. Longer times of exposure to lysozyme (prior to layering of sample on gradient) produced a viscous chromosomal DNA which tended to float up from the bottom of the centrifuge tube during the fractionation and collection of samples; shorter times are insufficient to lyse the cells resulting in low yields of plasmid DNA. Systematic experiments to standardize these variables, thereby assuring reproducibly high and accurate yields of plasmid (R-factor) DNA, were begun just prior to, but were discontinued as a result of, abolishment of Work Unit 074.

Replication of R-factor in a DNA polymerase I-deficient mutant. The major, if not the only, role of DNA polymerase I is to function in DNA repair. The enzyme has an editing function which is one of removing defective regions and resynthesizing the correct DNA sequence. E. coli mutants have been isolated that on extraction prove to have less than two per cent of the normal level of DNA polymerase I enzyme. These can be used as a model system in studying both replication and repair. The mutants grow satisfactorily but are very sensitive to agents that damage DNA, for example, ultraviolet radiation.

Studies were undertaken to see if such a mutant was capable of accepting and replicating an R-factor. R-factor R1 was transferred by conjugation from E. coli RS-2 into E. coli N211 which is deficient in the DNA polymerase I enzyme. Only the resistance determinants for kanamycin, chloramphenicol and ampicillin could be studied because the recipient (E. coli N211) was naturally insensitive to streptomycin and sulfadiazine. Colonies which were resistant to the three antibiotics and exhibited the biochemical characteristics of the mutant strain were selected for polymerase assays. Log cultures, grown in Difco Penassay Broth, were harvested by concentrating the bacteria

with polyethylene glycol (PEB). The cells were lysed with lysozyme and EDTA, followed by a mixture of BRIJ-58, EDTA and sodium deoxycholate in sucrose Tris buffer. Conventional DNA polymerase assays were carried out on these clarified extracts. Strains which had greatly reduced levels of DNA polymerase activity, similar to the mutant, were selected for verification of the antibiotic resistance properties. Plasmid DNA was extracted from logarithmically grown cells labeled with  $^3\text{H}$ -thymidine as previously described in the 1975 Annual Report. The presence of closed circular plasmid DNA was shown in these extracts by assaying fractions of CsCl-ethidium bromide gradients for radioactivity.

Elimination experiments were carried out to determine whether higher frequencies of elimination of R-factors would occur in the mutant since compounds which bind to DNA by intercalation act as template poisons in the replication of plasmid DNA. Concentrations that were used were lower than our standard  $10^{-4}$  M because the growth inhibitory action of these compounds was greater in the mutant strain. Compounds were: quinacrine and ethidium bromide at  $2.5 \times 10^{-5}$  M, nitroacridine II at  $3 \times 10^{-6}$  M, acridine orange at  $5 \times 10^{-5}$  M and chloroquine at  $10^{-4}$  M. The elimination potencies of acridine orange, chloroquine, ethidium bromide, nitroacridine II and quinacrine were not significantly different for kanamycin and chloramphenicol resistance determinants in the two strains. However, the ampicillin determinant was eliminated at a high frequency by all the compounds in the mutant *E. coli* N211. In *E. coli* RS-2, the ampicillin determinant was eliminated only by ethidium bromide and quinacrine at a low frequency.

Distamycin A. The antibiotic distamycin A (DMC) continues to be of interest because a variety of DMC-produced biochemical and biological effects are consequences of the interaction of the antibiotic with DNA, in particular with A-T rich regions of this nucleic acid or with synthetic duplexes, devoid of G. A preference of DMC for A-T duplex regions has previously been discovered in optical studies on the binding of distamycin to native DNAs and to synthetic duplex polydeoxynucleotides of different base composition; it is also suggested by our present results on the electric dichroism of complexes of the antibiotic with native calf thymus DNA and with the duplex polymers poly d(A-T) and poly dG·dC.

DNA and poly d(A-T) produced large dichroic effects for distamycin which yielded for the composite transition moment of DMC's N-methylpyrrole chromophores an orientation of 39 degrees, if base pairs are assumed to be perpendicular to the helical axis in the B-form of DNA. In contrast, poly dG·dC, a duplex which prefers the DNA A-form of G-rich DNA-like polymers with a 20 degree tilt of bases, caused only a small dichroic effect yielding a 57 degree orientation. This suggests, in conjunction with the earlier optical indicators for distamycin's



binding, a preference of the antibiotic for polydeoxynucleotide duplexes devoid of the G-determined A-conformation. A B-conformational preference of DMC is also suggested by the large bathochromic shift which B-form poly d(A-BrU) produced for distamycin while only a small shift occurred with A-form poly dA·rU and poly rA·dT. Titration with calf thymus DNA, monitored spectrophotometrically, indicated more than one optical species of bound antibiotic; such titration, when monitored by electric dichroism, yielded a binding of 1 DMC per 6 base pairs of A-T rich regions of DNA, i.e. a stoichiometry which was likewise derived from (1) bathochromic shifts of the antibiotic by equimolar mixtures of A + T-containing oligodeoxynucleotides and from (2) biphasic melting profiles of poly d(A-T) with distamycin A. These results suggest for DMC's binding to DNA, a preference of the antibiotic for regions on which an abundance of A-T pairs imposes the B-form and a second and weaker mode of distamycin attachment to conformationally different regions in the A-form as determined by G.

Electric dichroism of DNA-bound drugs and dyes. Binding to DNA was studied for several chemotherapeutic drugs and dyes. To interpret their biological activities, the mode of DNA-interaction of some of these chemotherapeutic agents had been studied before by a variety of biophysical means, but the electro-optical results reported here contribute additional important information on the structure and electronic properties of complexes that such substances form with their bio-receptor DNA.

The antimalarial quinacrine, whose acridine chromophoric ring system has been shown by flow dichroism of the dye-DNA complex to intercalate by the stronger of two binding processes between the base pairs of the nucleic acid, exhibited dichroic effects which agreed with quinacrine-ring-system intercalation and yielded additionally transition-moment orientations for individual contributions to the bound-aminoacridine absorption. The moments for the two, perhaps three, longest-wavelength bands of that absorption appeared perpendicular to the DNA axis, i.e. parallel to the planes of the base pairs of the nucleic acid and therefore in the plane of the intercalated acridine ring system. This indicates involvement in the intercalation process of quinacrine's 9-imino moiety whose attachment to the dye's chromophore gives rise to the in-the-acridine-plane moments of the long-wavelength absorption bands of the drug. Quinacrine's near UV transitions seem to possess somewhat out-of-plane orientations while effects in the DNA absorption region also agreed with acridine intercalation.

The antimalarial chloroquine, also known to bind by maximum intercalation, appeared to possess two long-wavelength in-the-quinoline-plane transitions and an extrachromophoric shorter-wavelength contribution while the antitumor agent daunomycin, which intercalates with a lesser stoichiometry, also exhibited two intrachromophoric longest-wavelength

absorption moments but several transitions with an-out-of-the-anthracycline-ring orientation.

Titration of quinacrine with DNA, monitored by electric dichroism, yielded increasing dichroic effects which attained their maximum value at a DNA phosphorus to drug ratio of 5, i.e. at the stoichiometry of maximum intercalation. This suggests contribution to the dichroic effects of the drug only by this, the stronger, mode of dye-nucleic acid interaction. Electric dichroic titration also confirmed the known maximal intercalation stoichiometry in the case of chloroquine while daunomycin could not be titrated because it precipitated DNA.

The nonintercalating antibiotic distamycin A revealed an absorption with three different contributions when bound to DNA and exhibited in the complex with this nucleic acid dichroic effects which suggested orientations slightly different for the transition moments of the three individual N-methylpyrrole chromophores of DMC with a composite moment directed more nearly parallel than perpendicular to the helical axis of DNA.

Titration of DMC yielded binding of 1 antibiotic per 6 DNA base pairs suggesting in connection with spectrophotometric indications of DMC's binding, strong pseudoirreversible attachment to 6 DNA base pairs of 1 molecule of distamycin A. Finally, the thiazine dye methylene blue revealed, in the presence of large excesses of DNA, absorbances indicating the binding of single molecules of the dye while low nucleic acid concentrations accommodated attachment of dimers or dye aggregates. Methylene blue exhibited, as the single bound molecule which probably binds like chlorpromazine, by intercalation, dichroic effects of two in-the-intercalated-ring-system transitions while aggregates displayed effects which suggested orientation of externally attached methylene blue, different from that found for intercalation. Our results show heterogeneity of orientations for individual transition moments of composite drug absorptions. We have, hence, established a new method for studying the interaction of chemotherapeutic agents with their bioreceptor DNA, faster than conventional spectrophotometric titration.

#### Conclusions.

R-factor-eliminating drugs: ethidium, quinacrine, acridine orange, quinine, chlorpromazine, chloroquine and methylene blue are intercalated into DNA as was proved by viscometric titrations of closed circular DNA. At low ionic strength, the antimalarial drug, primaquine, is also intercalated. Triphenylmethane dyes form complexes with DNA and act as template poisons in the DNA polymerase I reaction; they also inhibit the hydrolysis by the same enzyme of its DNA polymerization



products. Electrical dichroism studies on complexes of DNA with distamycin A, quinacrine, chloroquine and methylene blue yielded precise determinations of the placement of these molecules relative to linear DNA. R-factor DNA was isolated by lysis of E. coli, containing a Pseudomonas R-factor, on top of a linear sucrose gradient, followed by centrifugation and collection of fractions. The R-factor R1 was transferred into, and maintained by, a mutant of E. coli which was deficient in DNA polymerase I.

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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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.							
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.							
25. (U) 75 07 - 76 06 The procedure for isolating mRNA and the cell-free translation of mRNA is perfected. An extremely sensitive method for characterization of immunoglobulin has been perfected using a defined hapten as antigen. Studies are underway to identify a suitable host-parasite system that will yield relatively reticulocyte-free preparations. Preliminary results indicate that the active T. rhodensei polysomes can be obtained to initiate these studies. Purification methods for S. dysenteriae toxin are being perfected and purification has been achieved. Cytotoxicity, neurotoxicity and enterotoxicity of the highly enriched toxin preparation is being determined and also evaluated for antigenicity in other laboratories within WRAIR. Purified toxins are being labeled radioactively for localization studies. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

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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

Investigators.

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The objective of this work unit is to utilize the knowledge gained through basic and molecular research in order to obtain the solution to the existing military medical problems. Studies are conducted on disease and injury-induced variations in cellular processes to define biochemically, the macromolecule synthetic mechanisms by employing physico-chemical and structural characteristics of macromolecules. The following studies were conducted:

1. DNA relatedness among enterobacteriaceae.
2. In vitro isolation and characterization of structural and functional genes.
3. Isolation, purification and characterization of S. dysenteriae I toxin.
4. Development of antibody and RIA for detection of variation in enzyme levels in disease states.
5. Biochemical studies on cellular injury and regeneration.

1. DNA relatedness among Enterobacteriaceae.

The family of organisms known as the Enterobacteriaceae contains pathogens that cause a variety of diseases including: diarrhea, enteric fever, dysentery, urinary tract infection, food poisoning, and bacteremia. These enteric organisms also include 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 assess the lines of evolutionary divergence in pathogenic bacteria.
- (2) To develop a molecular (genetic) definition of a bacterial species.
- (3) To develop a classification based on genotypic relatedness, instead of only a few phenotypic characteristics.
- (4) To accurately classify newly described organisms.
- (5) To identify atypical clinical isolates for purposes of treatment and epidemiology.

Previously obtained data 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. These organisms exhibit about 10-15% relatedness to members of all other genera of the Enterobacteriaceae.

Our research in the past year has concentrated on assessing relatedness within specific genera of medical importance, as well as intergenetic relatedness. Our studies have also involved the identification and classification of clinical atypical organisms.

Genotypic and phenotypic similarity in Hafnia alvei (Enterobacter hafniae). Previous studies of the family Enterobacteriaceae found that E. hafniae DNA was 20-25% related to DNA of other members of the enterics. Therefore it was of interest to further study this group of organisms to determine if they (E. hafniae) formed one or possibly more relatedness groups.

Using a hydroxyapatite assay, 34 clinical isolates of typical and biochemically atypical Hafnia isolates were tested for DNA relatedness. Most strains were in two DNA relatedness groups (75% or higher relatedness) that were 45-60% interrelated. Three strains formed a third relatedness group that was 25-30% related to Groups 1 and 2. One atypical strain (Group 4) was 48% related to Group 3 and 25% related to Groups 1 and 2.

All but one of the Group 1 and Group 2 strains were lysed by Guinea's Hafnia-specific bacteriophage, whereas Groups 3 and 4, had anti-biograms typical of H. alvei. Tests of value in the biochemical identification of these groups have been determined. On the basis of these data we recommend the establishment of additional species within the genus Hafnia.

Polynucleotide sequence relatedness among atypical clinical isolates of *E. coli*. The incidence of atypical clinical isolates appears to be increasing and most general hospitals have neither the facilities, manpower, nor funds to attempt identification of difficult clinical isolates. In most cases the identification is never made. In other instances, problem cultures are sent to a reference laboratory for identification. This procedure takes from two to five weeks and in cases of the most difficult organisms, an almost arbitrary designation is made between two or more possibilities. The average bacterium contains sufficient DNA to specify some 3,000 average size genes, yet most organisms are classified on the basis of 10-25 characters. Nucleic acid hybridization is the only technique that is not significantly affected by atypical biochemical reactions.

A collaborative study with Dr. Don J. Brenner, Center for Disease Control, Atlanta, Ga. has been initiated with approximately 90 clinically atypical isolates of *E. coli* being studied. The biochemical data, in addition to the DNA relatedness data will give the clinical personnel an idea of what to expect when these organisms are found. This data will be programmed in a computer for reference for characterization and epidemiological purposes as well as lead to an accurate and fast identification of atypical organisms.

These cultures have been classified atypical because biochemical tests, which are not normal for 90-95% of the *E. coli*'s tested have occurred. These include strains which are singularly or multiply  $H_2S$ +, urease +, KCN=, adonitol +, inositol +, lysine decarboxylase +, ornithine decarboxylase +, and arginine dihydrolase +. The possibility exists that cultures of this nature may be falsely identified as other members of the Enterobacteriaceae, although there may be only one or two genes different in the bacterial chromosome.

Our study with DNA relatedness have shown that 14 of 85 isolates identified as atypical *E. coli*'s are not *E. coli* as compared to a reference *E. coli* DNA. Some of those not identified as *E. coli* have tentatively been classified by biochemical tests as *Yersinia*, *Proteus*, *Citrobacter*, and *Enterobacter agglomerans*. We are now in the process of further studies with DNA relatedness to corroborate or refute these findings.

Additional work with Dr. Brenner on the characterization of *Yersinia enterocolitica* and *Y. pseudotuberculosis* has been completed.

DNA from *Y. pseudotuberculosis* strains were highly related (80% or higher) but *Y. pseudotuberculosis* was only 40-60% related to *Y. enterocolitica*. *Y. enterocolitica* strains formed three DNA relatedness



groups, and possibly a fourth relatedness group. All of the yersinia tested are distantly, but significantly related to other Enterobacteriaceae.

Also a study of DNA relatedness among species of Enterobacter and Serratia is completed. Strains of Enterobacter cloacae from two separate hybridization groups that correlate with the presence or absence of yellow pigment. These two groups are 40-50% interrelated. Serratia marcescens strains form one closely related group. S. liquefaciens strains formed a single, more disperse, relatedness group, as did isolates of S. rubidaea. Phenetic similarity and DNA sequence homology of root nodule bacteria from New Zealand native legumes and Rhizobium strains from agricultural plants. In collaboration with B.D.W. Jarvis, Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand.

A comparison was made between 65 strains of root nodule bacteria from indigenous New Zealand legumes and 45 reference strains including: Rhizobium trefolii, R. phaseoli, R. leguminosarum, R. meliloti, and both acid-producing and non-acid-producing strains from the Lotus-Lupinus-Ornithopus, cross-inoculation group. The strains were classified into 10 clusters on the basis of 37 morphological, cultural and physiological tests. Relationships disclosed among the reference strains were in accordance with current ideas concerning Rhizobium taxonomy. The indigenous strains were well separated from both the trefolii-phaseoli-leguminosarum complex and R. meliloti but acid-producing strains from the Lotus-Lupinus-Ornithopus cross-inoculation group segregated with the indigenous strains. The principal characters differentiating the clusters are discussed. The base composition of DNA from representative indigenous strains was determined and base sequence homology studied. It was shown that the relative reassociation at 65°C in 0.14 molar/phosphate buffer between DNA from R. trefolii TA 1 and DNA from 3 R. lupini strains and 9 indigenous strains averaged 18%. Reassociation between R. lupini strain 809a or cc811 and indigenous strains averaged 52% and 56% respectively. Reassociation between DNA from the indigenous strain 5105 and other indigenous strains averaged 60%. The melting profiles of reassociated duplexes formed between DNA from two strains of R. trefolii and between cc811, 809a, 5105 and indigenous strains were also studied. It was concluded that DNA from indigenous New Zealand rhizobia contained an average of 56 and 52% of base sequence in common with those from R. lupini cc811 and 809a respectively. DNA from strain 5105 had an average of 60% of its base sequences in common with DNA from other indigenous rhizobia and base sequences in DNA from two strains of R. trefolii were identical. Those relationships are discussed in relation to those reported for the genus Agrobacterium where the genetic composition of the population is much more clearly understood than is



the case for Rhizobium.

2. Isolation characterization of structural and functional genes:  
Studies on their transcriptional and translational products. Transfer  
RNA gene clusters in E. coli DNA.

The results of this investigation to-date establish that there is extensive clustering of the tRNA cistrons in the E. coli chromosome. The ratio RNA:DNA in the hybrids, determined by density and isotopic measurements, was found to be about 0.6 to 0.7 for hybrids constructed from tDNA fragments of 125,000 daltons. Thus, these fragments, which are 4 to 5 times as large as a mature tRNA molecule, contain, on the average, 3 to 4 tRNA cistrons.

Treatment of these hybrids with N. crassa endonuclease resulted in the production of monocistronic hybrids, which suggests that single-strand gaps occur between adjacent cistrons. If spacer sequences do occur between tRNA cistrons and the cistrons are distributed evenly along the tDNA, it is possible to estimate the size of the spacer regions from our results.

Of the 125,000-dalton tDNA fragment, - 30 to 35% is not complementary to native tRNA. This corresponds to 120 to 140 unpaired nucleotides. If each tDNA fragment contains, on the average, 3 to 4 tRNA cistrons, then the average spacer region is - 30 to 45 nucleotides in length. In view of the possibility that some tDNA fragments may also contain other cistrons, this estimate may be somewhat high. It is interesting, however, that the estimated number of bases that could serve as spacers corresponds to the number of "extra" nucleotides in a precursor to E. coli tyrosine tRNA. Perhaps most E. coli tRNA's are formed from larger precursors. This question and the one regarding the occurrence of polycistronic units of transcription for tRNA will receive much attention in the near future.

Conservation of tRNA and 5s RNA cistrons in Enterobacteriaceae.

Isolated, labeled, tDNA and 5s DNA genes from Escherichia coli were hybridized with bulk DNA from representative members of Enterobacteriaceae. Both tDNA and 5s DNA cistrons are highly conserved as compared to total DNA, but are not as conserved as ribosomal DNA cistrons.

Both tDNA and 5s DNA cistrons are highly conserved as compared to total DNA among enteric bacteria. This fact can be quite useful in assessing evolutionary divergence in distantly related bacteria and also in establishing a system of classification based on gene similarity.

There are now four levels at which DNA relatedness can be measured: ribosomal DNA, 5s or tDNA, total DNA, and DNA from genes that specify enzyme such as tryptophan synthetase. Ribosomal DNA is the DNA conserved to the highest degree. It can, therefore, be used to group organisms at the family or suprafamily level. tDNA or 5s DNA relatedness can be used to separate these organisms at the family or tribe level. Species separation is best obtained by determining total relatedness. Subspeciation may best be determined by testing relatedness within specific genes common to a given species. Strict quantitative rules are not yet available; however, bulk DNA relatedness is already in extensive use for speciation, and rDNA is frequently used to determine relatedness among distantly related bacteria.

We know of no proposed theory to explain the fact that in enteric bacteria tDNA and 5s DNA, though conserved, show more divergence than rDNA. As an integral part of the protein synthesizing system, it is not surprising that rDNA is highly conserved. 5s DNA is also intimately associated with the ribosomes, and tDNA is certainly essential in protein synthesis. Why then do these molecules exhibit greater divergence than rDNA?

We propose that, in fact, tDNA and 5s DNA are conserved to the same degree as rDNA, and that the higher extent of divergence is due to spacer regions between the tDNA and 5s DNA cistrons. It is well known from both genetic and hybridization studies that tDNA and 5s DNA cistrons are clustered in *E. coli* and presumably in all enteric bacteria. Fournier et al performed the following experiment to determine whether tDNA cistrons are contiguous or whether they are separated by spacer DNA. Purified  $^{32}\text{P}$ -tDNA (an average single-strand fragment size of 125,000 daltons), which is approximately 4.5 times as large as a tRNA molecule, was hybridized to  $^{35}\text{S}$ -tRNA. The hybrids were treated with a single-strand specific endodeoxyribonuclease. If the hybridized segments were contiguous, without spacer sequences, the resulting hybrids would contain three or four tRNA molecules. If the hybridized segments were separated by single-stranded gaps, the endonuclease treatment would generate hybrids containing only one tRNA molecule and its corresponding cistron. After endonuclease treatment, 30% of the DNA was digested and the ratio of RNA to DNA in the remaining hybrid was approximately 1.0. The molecular weight of these hybrids was approximately 60,000 daltons, that expected for a single tRNA and its corresponding cistron. These results are consistent with the presence of approximately 20% DNA between clustered tDNA cistrons.

While comparable experiments were not done for 5s DNA, it is assumed that there is spacer DNA present between 5s DNA cistron clusters. Since 5s RNA is approximately a 25% larger molecule than tRNA, one would expect somewhat less spacer DNA per 125,000 DNA fragments than

is present on the tDNA fragment. We hypothesize that tDNA and 5s DNA are conserved to almost the same extent of rDNA, but that the spacer DNA is species or group specific and diverges at a rate close to that to total DNA. Spacer DNA is probably present next to rDNA cistrons. Its effect is not seen, because rDNA cistrons are among the largest cistrons in bacteria; 500,000 daltons to 1,000,000 daltons, and therefore most 125,000 dalton rDNA fragments contain no spacer DNA.

Decoding of Met-tRNAs, Met-tRNA<sub>m</sub> and other AA-tRNAs in rabbit reticulocyte lysates (In collaboration with Dr. B. Hatfield et al NIH).

Incubation of <sup>35</sup>S-Met-tRNA<sub>f</sub> and <sup>35</sup>S-Met-tRNA<sub>m</sub> in lysates of rabbit reticulocytes and peptide analysis of globin products have shown: 1) approximately 5% of the methionine from mammalian Met-tRNA<sub>f</sub>, approximately 30% of the methionine from E. coli Met-tRNA<sub>f</sub> and none of the methionine from yeast Met-tRNA<sub>f</sub> are transferred into internal positions of globin while the remaining methionine from each of these isoacceptors is lost during the assay; 2) in the presence of cycloheximide or anisomycin the loss of methionine from eucaryotic Met-tRNA<sub>f</sub> is inhibited during the assay while that from E. coli is unaffected suggesting that the loss of methionine from eucaryotic Met-tRNA<sub>f</sub> is due to initiation of protein synthesis while that from E. coli is due to mechanisms other than initiation (e.g., Met-tRNA<sub>f</sub> deacylase, Met-tRNA synthetase) and 3) mammalian and E. coli Met-tRNA<sub>m</sub> are rapidly incorporated into internal positions of globin. Preliminary studies with other aa-tRNAs have shown that Leu-tRNA<sub>CUG</sub> and Arg-tRNA<sub>AGG</sub> are incorporated more rapidly into globin than other isoacceptors of Leu-tRNA and of Arg-tRNA.

The two preceding studies demonstrate the feasibility of the test systems and provide the bases for the determination of the control mechanisms responsible for regulation of toxin formation in various strains of pathogens. These leads will be applied to the studies of Shigella Toxin biosynthesis.

### 3. Isolation, purification and characterization of S. dysenteria 1 toxin.

The causative agent for Shigella mediated dysentery has not been clearly established. A toxigenic material has been demonstrated in media supernatants from broth cultures and alkaline extracts from S. dysenteria 1 which has cytopathic, neurotoxic and enterotoxic properties. These studies were undertaken to isolate and characterize the toxin activities and to determine the feasibility of producing a stable toxoid.

Small scale purification procedures were used to obtain a homogenous material as determined by polyacrylamide gel electrophoresis with a molecular weight of approximately 70,000 and isoelectric point of pH 7.3. This material demonstrated cytotoxicity in HeLa cell culture at  $10^{-9}$  microgram. Neurotoxicity was demonstrated in mice ( $LD_{50}$  approx. 0.1 microgram) and enterotoxicity in rabbit ilial loops (fluid secretion induced with approximately 0.25 microgram). The material used in characterization work varied from 40-100% homogeneous. The small yield of toxin from the purification procedure outlined below presented problems in storage and it was found convenient to store toxin at about 40% homogeneity to maintain stability. Purified toxin was utilized for studies including effects on HeLa cell morphology (in collaboration with Dr. A. Takeuchi) and on protein synthesis. Toxoid preparation for the preparation of specific antisera was attempted.

Toxin preparation: cell free extracts of strains 60R and 3818T, *S. dysenteriae* 1 were prepared by the method of van Heyningen in the Department of Applied Immunology, WRAIR. Throughout purification, cytotoxicity assays were performed to determine specific activities. The purification procedure consists of the following steps:

1. 28% - 70% ammonium sulfate precipitation
2. Resuspend pellet in 0.25 M NaCl, 20 mM Tris buffer, pH 7.8
3. Apply to DEAE sephadex A50 column
4. Concentrate with 0 - 70% ammonium sulfate
5. Resuspend pellet in 0.1 M NaCl, 20 mM Tris buffer, pH 7.8
6. Apply to biogel A .5 M gel filtration column
7. Pool and concentrate active fractions with polyethelene glycol
8. Apply to preperative isoelectric focus column
9. Pool active fractions and apply to preparative electrophoresis column
10. Store active fractions at  $-70^{\circ}\text{C}$

One kilogram (wet weight) of packed cells yields approximately one milligram of homogeneous toxin demonstrating all three toxicities. The same extraction procedure was used on *S. flexneri*, strain M4243, a

strain that demonstrates no toxin activity in crude extracts. This strain has been shown pathogenic in man and primates and is known to be invasive. Performing the extraction procedure presented above on this strain demonstrated for the first time the presence of all three toxin activities but at greatly reduced strengths compared to S. dysenteriae.

Initial attempts to produce a toxoid have demonstrated that low levels of toxin must be effectively cross linked (for example, with 2% glutaraldehyde) and injected with incomplete Freund's adjuvant to immobilize the toxin. Incomplete immobilization and/or crosslinkage prove lethal to rabbits, even at microgram levels of toxin. Foot pad injections were performed with 50-50 mixtures of incomplete adjuvant and glutaraldehyde-treated toxin monthly with toxins from M42443, 60R and 3818T. Antisera production was monitored with a neutralization assay for cytotoxicity. Results indicate that antisera produced in response to all three toxin preparations are capable of protecting against any of the three toxins in the HeLa cell cytotoxicity assay.

No concrete evidence has been obtained demonstrating a specific morphological change in HeLa cells at various times of incubation in the presence of toxin. Changes observed in the presence of toxin as observed with electron microscopic studies appear to parallel those observed in the absence of toxin.

The effect of highly purified toxin on cell free protein synthesis was studied from brain, liver and intestinal cells utilizing polyuridylic acid as a messenger to direct the synthesis of polyphenylalanine. Studies were also performed on a natural messenger (endogenous messenger RNA)-directed system synthesizing polypeptides. Specific steps in the protein synthesis were observed in the presence and absence of toxin. The following results were obtained:

The effect of S. dysenteriae 1 toxin (Shiga toxin) on aminoacylation of calf liver tRNA and yeast tRNA was studied using a rat liver supernatant enzyme preparation. Toxin concentrations to 60 microgram/assay failed to demonstrate an inhibitory effect on this reaction. This observation was independent of the concentration of tRNA and enzyme in the system. Hence, in the mammalian system we employed there is no inhibitory effect by Shiga toxin on in vitro aminoacylation.

Toxin was demonstrated to inhibit the transfer of  $^3\text{H}$  phenylalanine ( $^3\text{H}$ -phe) from  $^3\text{H}$ -phe-tRNA to ribosomes freed of endogenous messenger RNA in a poly U directed system. Similarly, the transfer of  $^3\text{H}$ -amino acids ( $^3\text{H}$ -AA) from previously formed  $^3\text{H}$ -AA-tRNA to nascent

polypeptide chains using endogenous messenger directed ribosomes (polysomes) was inhibited. These results demonstrated that this toxin affects the transfer reaction in protein synthesis.

In order to further establish that the toxin inhibited the transfer of amino acids from tRNA to ribosomes, the following experiments were carried out. First, the effect of various toxin concentrations on the poly-U directed polyphenylalanine synthesis was studied using purified yeast tRNA and  $\text{NH}_4\text{Cl}$ -washed ribosomes (to remove endogenous messenger). As can be seen in Table 1, the inhibition of polyphenylalanine synthesis is dependent upon toxin concentration in the incubation mixture. Similarly Table 2 presents the effect of toxin concentration on nascent polypeptide synthesis utilizing natural messenger containing polysomes. These findings demonstrate that Shiga toxin inhibits the transfer of amino acids from tRNA to nascent polypeptide chains in mammalian cell free protein synthetic systems.

It has been shown by Collier that diphtheria toxin fragment A inactivates the free form of elongation factor -2 (EF-2) by catalyzing the attachment of the ADP-ribose moiety of intracellular  $\text{NAD}^+$  to an amino acid residue on EF-2. The modified factor is inactive in promoting translocation on ribosomes. The effect of cholera, diphtheria and Shiga toxins in the presence and absence of  $\text{NAD}^+$  on nascent polypeptide chain synthesis using polysomes and amino acid mixture is shown in Table 3. These results indicate that Shiga toxin, unlike diphtheria toxin, does not require  $\text{NAD}^+$  to inhibit cell free protein synthesis.

These studies were based upon Shiga toxin from strain 3818T isolated from a recent epidemic of shigellosis in Guatemala. Toxin purified from another strain, 60R has also been purified in this lab and preliminary experiments indicate a similar effect on protein synthesis. Toxin partially purified from S. flexneri strain M4243 has not been purified sufficiently to study its effect on protein synthesis at this time. Preliminary kinetics indicate that inhibition involves a ribosome-toxin complex or a ribosome-elongation factor-toxin complex.

TABLE 1

## INHIBITION OF POLYPHENYLALANINE SYNTHESIS BY PURIFIED

S. DYSENTERIAE 1 TOXIN

Toxin Concentration ( $\mu$ g)	[ $^3$ H]-phe Polymerized (cpm)	Inhibition %
0	147,800 $\pm$ 18,500	0
2.2	59,850 $\pm$ 12,200	60
4.4	37,091 $\pm$ 9,800	75
11.0	29,022 $\pm$ 7,400	80
55.0	16,123 $\pm$ 3,500	89

The cpm represents an average of three samples.

TABLE 2

INHIBITION OF NASCENT POLYPEPTIDE CHAIN SYNTHESIS BY  
PURIFIED S. DYSENTERIAE 1 TOXIN

Toxin Concentration ( $\mu$ g)	Nascent Peptide Chain Synthesized (cpm)	Inhibition %
0.0	33,230 $\pm$ 110	0
9.9	26,750 $\pm$ 100	19
1.8	21,600 $\pm$ 160	35
2.2	19,590 $\pm$ 1,010	41
4.4	17,950 $\pm$ 1,100	46
7.7	13,320 $\pm$ 1,400	60

The cpm represent an average of three samples.



TABLE 3

## COMPARISON OF EFFECT OF CHOLERA, SHIGA AND DIPHTHERIA

TOXINS ON IN VITRO PROTEIN SYNTHESIS

Toxin	Nascent Polypeptide Synthesized - NAD <sup>+</sup> (cpm)	Nascent Polypeptide Synthesized + NAD <sup>+</sup> (cpm)
None	30,785 ± 1,000	26,770 ± 300
Cholera	27,732 ± 100	
Shiga	11,432 ± 1,100	10,900 ± 350
Diphtheria	25,730 ± 450	13,308 ± 450

The cpm represents an average of two samples. 6.6 µg toxin was used for each assay; the concentration of NAD<sup>+</sup> was 10 µg/ assay.

Effects of Shigella dysenteria enterotoxin on PHA stimulated human peripheral lymphocytes.

In order to understand the mechanisms of action of Shigella dysenteria enterotoxin various concentrations of a highly purified preparation were exposed to PHA stimulated peripheral blood lymphocytes at day 0 and day 4 of culture. The preliminary results indicate:

(1) There is an increase in DNA synthesis at the higher dilutions of  $10^{-7}$  and  $10^{-6}$  with a subsequent decrease at  $10^{-6}$  to  $10^{-3}$  when added to 4 day old cultures. These changes were not noted however when the enterotoxin was added at 0 day. (2) Accompanying the changes in DNA synthesis there was a decrease in protein synthesis when the enterotoxin was added at both 0 and 4 days of culture. These results correlate well with the inhibition of protein synthesis found in an in vitro polyribosome system reported by others in this department.

4. Development of antibody and RIA for detection of variation in enzyme levels in disease states.

Radioimmune assay procedures for the detection of prostatic acid phosphatase (PAP) have been developed as a model system for the establishment of methodology necessary for a larger project with the goals of determining cell death and/or multiplication during disease states by measuring either macromolecules or their metabolism in biological fluids. The relationship of PAP to prostatic carcinoma fulfills the requirements of such a project.

The progression of prostatic carcinoma is characterized by a spreading of cancerous cells in a stepwise manner from the prostatic capsule to the surrounding soft tissue, and finally, to the bone marrow, particularly that of the iliac crest. During the entire course of the disease PAP is an isozyme specific for prostatic tissue. Demonstration of PAP in serum and bone marrow is indicative of the disease and the extent of its progression. However, the phosphatase family consists of a large number of enzymes with broad substrate specificity. This perversity complicates colorimetric methods for the determination of PAP in clinical samples. Using an immunological approach we have developed a specific and sensitive assay for PAP.

Work on this project has concentrated on the purification and characterization of PAP from seminal fluid, on the production of antisera against PAP, and on the development of an RIA for PAP using the double antibody technique. During the last fiscal year our main emphasis has been on the development of techniques for the detection

of antigen-antibody interaction. However, refinements in the procedures for the purification of PAP and for the production of antisera have been made.

#### Purification of PAP

The purification scheme consists of two DEAE-Sephadex A-50 and one CM-cellulose ion exchange steps, each followed by concentration of the active fractions in polyethylene glycol (PEG) and then dialysis against the appropriate buffer for the next step. Overall, the purification results in a 15 fold enrichment of PAP with a recovery of approximately 50%. PAP elutes from the last column with constant specific activity and is homogeneous on SDS electrophoresis.

A 200 ml sample of pooled seminal fluid is dialyzed against 8 liters of 20 mM Tris, 100 mM NaCl, pH 8 for 24 hours and applied to a 9.5 X 25 cm column of DEAE Sephadex A-50 equilibrated with the same buffer. The column is washed with 2 liters of starting buffer and PAP activity eluted with a linear gradient consisting of 2 liters of starting buffer and 2 liters of 20 mM Tris, 400 mM NaCl, pH 8.0. Active fractions are pooled, concentrated overnight against PEG, and dialyzed against 1 liter of 20 mM Acetate, 25 mM NaCl, pH 4.2 for 16 hours.

The crude fraction from the previous step is then applied to a 1.5 X 25 cm column of CM Cellulose equilibrated with 20 mM Acetate, 25 mM NaCl, pH 4.2 and washed with 300 ml of the same buffer. PAP is eluted from the column with a linear gradient consisting of 750 ml of starting buffer and 750 ml of 20 mM Acetate, 225 mM NaCl, pH 4.2. Fractions containing activity are pooled, concentrated against PEG, and dialyzed against 1 liter of 20 mM Tris, 100 mM NaCl, pH 8.0.

Finally, the sample is applied to a 2.5 X 100 cm column of DEAE Sephadex A-50 equilibrated with 20 mM Tris, 100 mM NaCl, pH 8.0 and washed with 1 liter of the same buffer. PAP is eluted with a linear gradient consisting of 1 liter of starting buffer and 1 liter of 20 mM Tris, 300 mM NaCl, pH 8.0. Fractions containing greater than 400 units of PAP per a <sup>280</sup> are pooled, concentrated against PEG overnight, and dialyzed against 1 liter of 20 mM Tris, 100 mM NaCl, pH 8.0.

#### Production of antisera

New Zealand white rabbits are injected epidurally in 20 sites along the back with 0.5 mg of PAP in complete Freund's adjuvant. At regular intervals the animals receive booster injections in the foot pads with 0.5 mg of PAP in incomplete Freund's adjuvant. The antisera titers rise after the third injection and remain elevated for a period of several months.

Initially the antisera titer of the animals was established by classical passive hemagglutination procedures using tannic acid. However, because of the many drawbacks associated with the use of fresh RBC an improved procedure was developed in this laboratory using stabilized and activated RBC. This procedure uses two bi-functional cross linking reagents toluene-2,4-diisocyanate and glutaraldehyde to stabilize and activate the erythrocyte membrane.

An emulsion is prepared by adding 0.15 ml of TDI to 30 ml of saline and sonicating at an attenuation of 4 or 5 for two minutes using a Branson model S 75 sonifier with a pencil size probe. The emulsion is added to 200 ml of a 10% suspension of RBC, stirred, and then immediately mixed with 7.2 ml of 8% glutaraldehyde for a final concentration of 0.25%. The cell suspension is hand stirred for 5-10 minutes and then centrifuged at 1000g for 15 minutes. The supernate is discarded and the packed cells are washed twice with 200 ml of PBS of saline. The cells should pack tightly on centrifugation and the supernate following each centrifugation should be clear.

PAP is covalently coupled to the RBC's by adding 0.1 ml of PAP, 4 mg/ml to 2 ml of a 10% suspension of stabilized and activated RBC's and incubating at 37°C in a water bath. After 45 minutes centrifuge at 1000g for 10 minutes and resuspend the packed cells in 2 ml of PBS-1% NRS. Cells prepared in this manner are stable indefinitely as long as bacterial growth can be prevented. For titration of antisera the cells are added to dilutions of antiserum in the wells of a microtiter plate. The resulting agglutination patterns determine the titer of the antiserum.

Alternately, a solid phase system was developed to determine the titer of antisera. In this procedure PAP is allowed to bind non-specifically to the polyvinyl chloride wells of a microtiter plate. The plate is aspirated and carrier protein added to each well to saturate any non-specific binding sites remaining. The carrier protein is aspirated following a 4 hour incubation and then dilutions of antisera are added. After 24 hours the plate is washed extensively and  $^{125}\text{I}$  labeled goat antirabbit globulin is added to each well. The radioactivity fixed to the wells of the plate after washing is indicative of the amount of antibody bound to the wells and thus the titer of the antisera.

#### RIA for PAP

The various radioimmune assay methods differ in the means of separation of free from bound or complexed antigen. Several

techniques were investigated for use with the PAP RIA including two solid phase systems. The first solid phase system examined was based on PAP covalently bound to Sepharose-4B. The second relied on the non-specific binding of antibody to the polyvinyl chloride wells of microtiter plates. However, the only system examined to date with the required sensitivity has been the double antibody method.

Separation of free and complexed PAP in this procedure is based on the precipitation of the gamma globulin fraction of the primary antisera by a second antibody directed against the gamma globulin species of the first antisera. Thus the gamma globulin components and any PAP-antibody complex associated with it are effectively removed from the reaction by the addition of the species specific second antibody. Briefly, the procedure consists of allowing labeled and unlabeled antigen to compete for limiting amounts of rabbit antiserum, adding normal rabbit serum as carrier and goat antirabbit globulin (second antibody), and determining the radioactivity associated with the resulting precipitate and/or proportional to the concentration of unlabeled PAP in the initial reaction.

PAP labeled with carrier free  $^{125}\text{I}$  is prepared either by the solid state lactoperoxidase or chloramine T procedure. The labeled PAP routinely used contains 2-10  $\mu\text{Ci}$  of  $^{125}\text{I}$  per  $\mu\text{g}$  of protein.

We have examined some 20 bone marrow aspirates and over 100 serum samples by RIA for PAP content. The bone marrow samples were obtained from male patients with local or metastatic prostatic carcinoma by aspiration of the iliac crest. These samples have also been assayed by a colorimetric test, the General Diagnostic's Phosphatrate Acid Kit used by WRAMC clinics to evaluate serum for total acid phosphatase content.

Of the 20 bone marrow samples assayed by RIA 19 have been classified as normal with less than 40 ng of PAP per ml of serum and indicate local disease only. The remaining sample contained nearly 1000 ng of PAP per ml. Metastatic disease in this case has been confirmed by pathological examination of the cellular content of the aspirate, colorimetric enzyme analysis, and X-ray. Erythrocytes are lysed during the sampling procedure to varying degrees from sample to sample and red cell acid phosphatase contributes to the total acid phosphatase content of the serum. This makes the colorimetric evaluation of normal and abnormal bone marrow extremely difficult. However, erythrocyte acid phosphatase does not interfere with the RIA for PAP. We have been unable to find any cross reacting substance to interfere with the RIA for PAP.

Of the 80 normal male serum samples assayed for PAP by RIA we have

established an average of 3.1 ng of PAP per ml of serum with a range of 1.5 to 7.6 ng of PAP per ml of serum. The corresponding values of the colorimetric test average 1.1 IU and range from 0.2 to 2.5 IU. Assuming that the maximum specific activity of PAP is 400 units per mg of protein, then the value of 3.1 ng of PAP per ml of serum is equivalent to 1.2 IU, a value well within the average. The values for the content of PAP in abnormal serum samples varies with the extent and activity of disease. However, the colorimetric and RIA results correlate well.

##### 5. Biochemical studies on cellular injury and regeneration.

Development of a highly reproducible graft vs host system.

The graft-versus-host reaction (GVHR) in this system is induced by the injection of lymphoid cells from either allogeneic parent strain into its F<sub>1</sub> hybrid. The B10A/SgSn mouse was utilized as the source of donor spleen lymphocytes. These were injected into a two day old F<sub>1</sub> (B10A/SgSn ♀ X B10D<sub>2</sub>/n Sn ♂). Nine days later the F<sub>1</sub> was sacrificed and whole body and spleen weight were determined. The spleen was constantly frozen by dry ice as rapidly as possible following its removal and then weighed. They were stored at -70°C for cyclic nucleotide assay. The degree of splenomegaly was expressed as the ratio.

$$\frac{\text{Weight of spleen}}{\text{Weight of mouse}} \quad (\text{Experimental})$$

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$$\frac{\text{Weight of spleen}}{\text{Weight of mouse}} \quad (\text{Control})$$

Early attempts to obtain reproducible results quickly pointed out a need to develop proper animal husbandry techniques for better survival. Individual housing of mothers, quiet and dark breeding boxes, restricted contact with humans, special nesting material and a selection for proven good litter producing mothers were found to be indispensable factors for increased litter survival as well as the production of litter sizes suitable for experimentation.

The work of intraperitoneal injection of the donor cells was also found to be of great importance as injection through the abdominal wall or through the muscles of a rear leg either produced loss of inoculum by leakage through the injection site or a danger of injury to the mouse which led to its death. We perfected intraperitoneal injections by placing the needle through the triceps, and latissimus-doris muscles, subcutaneous over the right ribs anterior to the liver and into the peritoneal cavity as the best injection method for inoculum retention. Experiments indicated the highest GVHR values could be obtained utilizing 1-to  $5 \times 10^{-7}$  donor lymphocytes in

a 0.05 ml injection volume. With the development of these techniques we obtained GVHR ratios of 2.25 to 2.75. A ratio of greater than or equal to 1.3 IS considered a positive GVHR.

#### Macrophage sensitization.

In order to more fully understand the GVHR model we extended it to a series of experiments in which donor cells were pre-sensitized by allogeneic macrophage contact in vitro prior to injecting them into the newborn mice. The techniques were developed which enabled the macrophages to be attached to the surface of the culture plate so the donor lymphocytes could be exposed to them and be removed after a suitable incubation period without becoming contaminated with the macrophages. This procedure involved the sequential pre-treatment of the plastic culture tray with acetone (to make the surface irregular), sulfonation with concentrated  $H_2SO_4$  (to produce a negative charge) followed with poly-L-lysine (to make a positive charge which attracts the negative cells) following incubation with the macrophage monolayer the lymphocytes undergoing the same treatment were pooled, washed and resuspended to a cell concentration of  $2 \times 10^8$  cells/ml. Litters were divided so that half the individuals received the experimentally treated cells and the other half served as controls.

GVHR values of 1.97 higher than unsensitized controls were obtained by allogeneic macrophage in vitro pre-sensitization.

#### Cell free antibody synthesis.

As part of a project to develop a cell free antibody synthesizing system, methodology was developed for the identification and isolation of lymphocytes that are producing antibody specific for a simple hapten. These cells will be used as a source of the instructional molecules necessary for cell free protein synthesis. A hemolysis in gel procedure has been adapted to determine the time of maximal cell response to the immunogen and a photographic method of evaluating large numbers of plaques formed by antibody producing cells was developed.

#### Cyclic nucleotides in cellular regeneration.

Knowledge of basic cellular mechanisms will enable the better understanding of wound healing under ideal conditions as well as those of starvation, infection, chemical and radiation damage. Two models have been employed to help understand the mechanisms of the triggering step of cellular growth: the mixed lymphocyte culture and the graft vs host system. Cellular growth in each is triggered by a cell which carrier histocompatibility antigens different from its own.



The biochemical parameters which are set into motion are many. Two general areas of investigation however which seem to be quite important are: (1) The cyclic nucleotides cAMP, cGMP and their associated enzymes adenylate cyclase and guanylate cyclase, (2) the polyamines, putrescine, spermidine and spermine. Cyclic AMP and cGMP have been shown to be important in cellular control of DNA, RNA and protein synthesis. The polyamines are ubiquitous cellular components being involved in most systems of rapidly proliferating as well as degenerating cells.

Investigations and personnel in the section on cellular injury and regeneration have been divided into two main groups, biological and chemical. The biological section was responsible for the development and setting up of a reliable highly reproducible graft vs host system and mixed lymphocyte culture capabilities. This entailed the definition of the problem, proposed solutions and methodology, and organizing a functional highly efficient laboratory with tissue culture capabilities. The chemical section was assigned the task of evaluating existing methods (modifying where needed) and the development of new technology to measure cyclic nucleotides and associated enzymes in small quantities of cells. In addition, the extraction and purification as well as the analysis of polyamines was carried out by this section.

#### Chemical section

##### Protein binding assay

This was redeveloped by extracting and greatly purifying cAMP dependent protein kinase from rabbit skeletal muscle. This protein had a very high specific activity and gave an assay with a sensitivity of 0.05 - 0.1 picomoles of cAMP.

##### Adenylate cyclase

Method A - The measurement of adenylate cyclase was perfected utilizing an enzyme preparation extracted from cells. This enzyme then utilized  $^{32}\text{P}$ -ATP to form  $^{32}\text{P}$ -cAMP in a reaction volume of 50  $\mu\text{l}$ . The resultant cAMP was then purified by passage over sequential Dowex 50 X4 and neutral alumina columns. Methodology was developed to purify multiple samples quickly and efficiently utilizing small lightweight plastic racks which stacked one on the other. By this method, loss of cAMP was prevented.

Method B - This method was developed in this laboratory to allow the simultaneous measurement of guanylate cyclase and adenylate cyclase.



$^{32}\text{P}$ i was incubated with intact cells, thus labeling endogenous ATP. Following the cellular reaction (described under the biology section) the TCA extract was separated into cAMP and cGMP by sequential Dowex 50 X4 and PEI columns. Cyclic GMP was purified by washing Dowex 50 X4 with 0.05 N HCl and collecting a relatively pure fraction. The resultant fraction was further purified of contaminating  $^{32}\text{P}$ -compounds and HCl by the second passage over a Dowex 50 X4 and washing with water. The resultant cGMP peak was then passed over a PEI column and all remaining  $^{32}\text{P}$  contaminants were removed by washing with water. The cGMP was removed in the form of ethyl alcohol-ammonium acetate buffer and counted in scintillation vials. The cAMP which remained on the first Dowex 50 X4 column was removed with water. The resultant fraction was then purified by PEI column chromatography and the eluate of  $^{32}\text{P}$  cAMP was counted by a liquid scintillation spectrometer. This is the first method developed for the rapid reliable simultaneous assay of guanylate and adenylate cyclase without combining lyophilization and thin layer chromatography.

#### Polyamine analysis

Urines with suspected elevated levels of polyamines were treated in the following manner:

Nine ml of urine were filtered through a millipore filter 0.45  $\mu$  and added to an equal volume of 9 ml of concentrated HCl. This was hydrolysed for 16-18 hours at 110°C. The specimens were then filtered through a 0.45  $\mu$  millipore filter and evaporated to dryness. This was reconstituted into 3 ml of NaCl Na citrate buffer. The sample was then eluted from a strong cationic exchange column using a 3 step gradient buffer system. The effluent was mixed with ninhydrin, reacted at 95°C for 12 minutes and detected by absorbance at 570 nm. This is a fully automated system using a program tape. The analysis time is 16 samples/24 hours.

The graft vs host system has yielded approximately 150 spleens ready for cyclic nucleotide analysis. Further experiments will be designed depending upon results. Several experiments have yielded GVH spleens in which adenylate cyclase ( $^{32}\text{P}$ -ATP method) was measured. Preliminary results demonstrate increased adenylate cyclase activity in GVH (+) spleens greater than GVH (-) spleens. Measurement of cAMP in the MLC by the competitive protein binding assay has revealed that cyclic AMP rises in the syngeneic mixture (that mixture which will not proliferate) and falls in the allogeneic mixture (that mixture which undergoes blast transformation) during the first 10 minutes of incubation. Those experiments are preliminary but have been given added weight by the preliminary results of measuring adenylate cyclase in the MLC by the  $^{32}\text{P}$ i method. While neither results

are conclusive, their parallel results suggest that the proliferation signal may in part be associated with a fall in cellular cyclic AMP levels.

Amine analysis of urines of patients with testicular carcinoma (as a model for rapid cellular proliferation) has revealed that not only are the polyamines, putrescine, spermine and spermidine elevated. But also that other diamines such as 1,3 diaminopropane and cadaverine are elevated. They are demonstrated to be more elevated in those patients who have developed (clinically) a higher tumor burden. These diamine compounds might later become useful as chemicals to monitor the success or failure of treatment of cancer. In a model of rapidly dying cells (a patient with a transplanted kidney undergoing a rejection crisis) we have demonstrated a rise of diamine compounds prior to and coincident with the rejection of the kidney. The diamines may become useful markers of the success or failure of organ transplantation.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 075 Metabolic problems and biomedical variations  
associated with disease and injury

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
					DA OB 6448	76 07 01	DD-DR&E(AR)636
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCY	6. WORK SECURITY	7. REGRADING	8A. ORIGIN INSTN	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
75 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			
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22. KEY WORDS (Precede EACH with Security Classification Code)							
(U) Pharmacology; (U) Medicinals; (U) Drugs; (U) Shock; (U) Toxicity							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish 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 as well.							
25. (U) 75 07 - 76 06 WR 149,024 protected all dogs tested from a lethal dose of E. coli endotoxin when the drug was administered 45 minutes after the toxin. Further investigations on the mechanism of initial hypotension and bradycardia induced by intravenous WR 2823 in anesthetized cats strengthen the evidence that the effect may be mediated by an action on central vasomotor centers. Work unit number 192, project 3A161101A91C will be consolidated into this unit and project with a unified report. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							
Support in the amount of \$10,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

\*Available to contractors upon originator's approval.

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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 149,024 on lethal endotoxemia in the dog.

a. Background:

Gram-negative sepsis is still a major cause of shock despite advances in antibiotic therapy. Septic shock is characterized by inadequate tissue perfusion resulting from increased peripheral vascular resistance, pooling of blood in the microcirculation, diminished cardiac output and tissue anoxia. The vasoactive phenomena leading to this state of circulation failure have been attributed to the release into the circulation of endotoxin, the lipopolysaccharide moiety of the gram-negative bacterial cell wall. Endotoxin is thought to exert its major effect in arterioles with alpha-adrenergic receptor innervation (Petersdorf, 1974). In experimental studies, this effect is observed as a prolonged and deleterious vasoconstriction accompanying a massive sympathetic discharge (Spink et al., 1966).

No single therapeutic agent has met with consistent success in the treatment of endotoxin shock. Since the shock state is

accompanied by excessive stimulation of the alpha-adrenergic receptors, it is obvious that the sympathomimetic pressor agents so often used in other hypotensive states would be contraindicated since they would further reduce perfusion of vital organs (Petersdorf, 1974). Several pharmacological agents (isoproterenol, dopamine and corticosteroids) have had limited success in the treatment of septic shock. This success is most often associated with their prophylactic use or administration early in the course of the shock state. However, WR 149,024 (1,18-diamino-6,13-diaza-9,10-dithiaoctadecane tetrahydrochloride) has been shown to be effective in preventing mortality and modifying cardiovascular responses in a canine endotoxin shock model. This protective effect of WR 149,024 may be related to its reported production of vascular alpha-receptor blockade and enhancement of ascending aortic blood flow (Caldwell et al., 1972).

The studies with WR 149,024 (and most shock studies) involved treatment of the animal with the protective agent prior to the endotoxin insult. The present study was designed to determine the effectiveness of WR 149,024 when administered 45 min after the endotoxin insult. This regimen was selected to more closely approximate conditions observed clinically with a patient already in septic shock. The study has been divided into two parts. The first part was designed to determine whether WR 149,024, administered 45 min after endotoxin, would improve the 72 hour survival when compared to control. The second part involves an analysis of the hemodynamic alterations which might explain the enhanced survival associated with WR 149,024 administration, when given 45 min following endotoxin. Preliminary data on the 72 hour survival study have been included in this report.

#### b. Methods:

Ten adult mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg, iv). The right femoral artery and vein were catheterized for monitoring of arterial blood pressure and administration of drugs, respectively. The Lead II electrocardiogram was also monitored. After stabilization of blood pressure and heart rate following the surgical cutdown, endotoxin was administered as a slow iv infusion. Two doses of endotoxin were employed. Endotoxin (1 mg/kg iv) was infused over a 3 min period in 3 dogs. E. coli endotoxin (1.5 mg/kg iv) was infused over a 5 min period in the remaining 7 dogs. Forty-five min after the start of the endotoxin infusion, the dogs were given either WR 149,024 (10 mg/kg of the salt iv) dissolved in 5 ml of 0.9% saline, or 5 ml of the saline vehicle without drug. The dogs were monitored for four hours after the administration of WR 149,024 or saline. The catheters were then removed, vessels ligated and wounds sutured. The dogs were placed



in the intensive care quarters where they received food and water ad libitum. Survivors were defined as those animals alive 72 hours after endotoxin administration.

c. Results:

Preliminary results of the survival studies are given in Table 1. Control animals that were given the saline vehicle 45 min after endotoxin administration had a 72 hour survival rate of 20% (1 dog of 5). Those animals that received WR 149,024 (10 mg/kg iv) 45 min after endotoxin administration had a 72 hour survival rate of 100% (5 dogs of 5). These preliminary results indicate that post-endotoxin treatment with WR 149,024 is capable of modifying the shock sequelae, increasing the possibility of WR 149,024 being useful against septic shock clinically.

3. 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 cardiodepressor 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 pre-amplifier. 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.

Following a 30 minute equilibration period, 50 mg/kg (7 cats) or 25 mg/kg (3 cats), dissolved in 10 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.

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 animal preparations were allowed to stabilize for 30 min prior to the administration of WR 2823 (50 mg/kg).

The drugs utilized in the present experiments, the dosage (calculated as the free base) and the equilibrium period before WR 2823 (50 mg/kg) 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); lysergic 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.

Control observations of the various physiological parameters were made just before either surgical or pharmacological pretreatment and again before administration of WR 2823 (50 mg/kg). 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 electrocardiogram 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  $\mu$ g 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, 25 or 50 mg/kg intravenously, induced immediate hypotension and bradycardia in all 10 intact control animals. These results, together with the effect of WR 2823 in the surgical pre-treatment cats are summarized in Table 2. With 50 mg/kg WR 2823 changes were noted to begin during the infusion and reached maximum decreases of 32% to 48% in mean arterial pressure and 15% to 23% in the 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 magnitude of the hypotension and bradycardia seen after the 25 mg/kg dose was similar to that seen after 50 mg/kg of WR 2823. In contrast, recovery was more rapid at the lower dose, with both blood pressure and heart rate back to control levels within 15 to 30 min.

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 2). 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-blockade 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 3 through 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 blockage 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 dithiothreitol 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 25 or 50 mg/kg of WR 2823 was infused over a 4 min period. The cardiodepressant effects appeared before the injection was completed. Although not attempted in the present experiments, 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 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 α-methyldopa (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  
Effect of Post-Endotoxin Treatment with WR 149,024 on 72 Hour Survival Time in the Dog.

<u>Dog</u>	<u>Dose of <i>E. coli</i> Endotoxin (mg/kg)</u>	<u>Post-Endotoxin Dose of WR 149,024 (mg/kg)</u>	<u>72 Hour Survival</u>
1	1.0	10	yes
2	1.0	0	no
3	1.0	10	yes
4	1.5	0	no
5	1.5	10	yes
6	1.5	10	yes
7	1.5	0	no
8	1.5	0	no
9	1.5	10	yes
10	1.5	0	yes

Table 2

Comparison of the Blood Pressure and Heart Rate Changes After a Four Minute Intravenous Infusion of 50 or 25 mg/kg WR 2823 and the Effect of Surgical Pretreatment on Initial Cardiovascular Responses Following Intravenous Infusion of WR 2823 in the Anesthetized Cat

		% of Predosing (Control) Values					
		Mean Arterial Pressure <sup>a</sup>			Mean Heart Rate <sup>a</sup>		
Time Postdose (min)	No Operation <sup>b</sup> (25 mg/kg)	No Operation <sup>b</sup> (50 mg/kg)	Decerebrate <sup>b</sup> (50 mg/kg)	Spinal <sup>b</sup> (50 mg/kg)	No Operation <sup>b</sup> (25 mg/kg)	No Operation <sup>b</sup> (50 mg/kg)	Spinal <sup>b</sup> (50 mg/kg)
1	51±2	67±6	52±6	132±17	85±1	87±10	71±8
5	64±4	66±4	54±2	86±9	83±1	81±7	68±8
15	89±9	59±5	57±4	75±5	104±15	77±7	64±7
30	114±15	68±5	67±1	75±5	111±11	88±7	71±11
60	117±16	72±4	73±3	78±5	118±14	105±7	83±10
Control <sup>c</sup>	97±12	128±12	104±23	70±11	159±9	176±9	204±12
							135±15

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

<sup>b</sup>Number of experiments are three for 25 mg/kg WR 2823 "No Operation", seven for 50 mg/kg WR 2823 "No Operation", three for "decerebrate" and five for spinal."

<sup>c</sup>Control values are in mm Hg for blood pressure and bpm for heart rate.



Table 3

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 Control Values <sup>a,b</sup>				
	No Pretreatment	Mecamylamine 2.5 mg/kg	Reserpine 0.1 mg/kg x 2	Reserpine 0.1 mg/kg x 2 Mecamylamine 2.5 mg/kg
After Pretreatment		67±2	-	95±9
After WR 2823 (min)				
1	67±6	143±19	77±8	77±6
5	66±4	117±10	96±3	116±11
15	59±5	107±9	93±3	104±10
30	68±5	110±9	96±5	105±8
60	72±4	109±6	101±6	122±13
Predosing Values (mmHg)	128±12	106±7	79±9	64±3
Number of Experiments	7	5	3	4

<sup>a</sup>Control values obtained prior to WR 2823 administration.

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

Table 4

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 Control Values <sup>a,b</sup>						
	No Pretreatment	Hexamethonium 10.0 mg/kg	Phenoxybenzamine 5.0 mg/kg	dl-Propranolol 0.5 mg/kg	Atropine 1.0 mg/kg	LSD 0.05 mg/kg Diphenhydramine 10.0 mg/kg
After Pretreatment		86+4	83+5	98+3	96+3	109+5
After WR 2823 (min)						
1	67+6	96+4	56+1	62+6	52+6	54+10
5	66+4	79+5	64+5	68+9	54+2	67+7
15	59+5	79+5	68+5	60+7	57+4	72+4
30	68+5	79+5	78+9	66+7	66+7	87+1
60	72+4	91+8	93+4	68+10	68+10	89+3
Predosing Values (mmHg)	128+12	88+6	118+16	111+16	111+8	119+19
Number of Experiments	7	3	3	3	4	3

<sup>a</sup>Control values obtained prior to WR 2823 administration.

<sup>b</sup>Values are mean  $\pm$  S.E.M. and represent at least three cats per group.

Table 5

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 Control Values <sup>a,b</sup>				
	No Pretreatment	Mecamylamine 2.5 mg/kg	Reserpine 0.1 mg/kg x 2	Reserpine 0.1 mg/kg x 2 Mecamylamine 2.5 mg/kg
After Pretreatment		74±7	-	101±5
After WR 2823 (min)				
1	87±10	139±6	94±1	106±6
5	81±7	113±7	90±3	91±2
15	77±7	104±8	91±7	95±3
30	88±7	102±3	97±12	92±2
60	105±1	100±1	92±9	99±5
Predosing Values (bpm)	176±9	208±19	141±13	142±20
Number of Experiments	7	5	3	4

<sup>a</sup>Control values obtained prior to WR 2823 administration.

<sup>b</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 Control Values <sup>a,b</sup>						
No Pretreat- ment	Hexameth- onium 10.0 mg/kg	Phenoxy- benzamine 5.0 mg/kg	dl-Propan- olol 0.5 mg/kg	Atropine 1.0 mg/kg	LSD .05 mg/kg	Diphenhy- dramine 10.0 mg/kg
After Pretreat- ment	82+6	106+3	90+3	102+1	99+1	105+8
After WR 2823 (min)						
1	87+10					
5	81+7	144+9	97+4	100+2	82+9	77+2
15	77+7	125+7	91+7	98+2	80+6	73+1
30	88+7	98+10	83+6	100+3	76+6	73+1
60	105+1	95+8	84+11	101+3	86+13	83+6
		99+11	106+5	116+7	104+6	116+11
Predosing Values	176+9	152+19	173+15	163+13	179+20	180+4
(bpm)						
Number of Experiments	7	3	3	3	4	3

<sup>a</sup>Control values obtained prior to WR 2823 administration.

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

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>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL 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. DISEASE INSTN <sup>a</sup>	8B. SPECIFIC DATA CONTRACTOR ACCESS <sup>a</sup>	9. LEVEL OF SUM <sup>a</sup>
75 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	3A161102B71Q	00	165			
B. CONTRIBUTING							
C. XXXXXXXX	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Parasitic Diseases of Military Importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002600 Biology							
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				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER <sup>a</sup>				FISCAL YEAR		388	
C. TYPE				CURRENCY		239	
D. KIND OF AWARD:				77		6	
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 CD&I			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DEAN if U.S. Academic Institution)			
NAME: JOY, R.J.T., COL				NAME: BURKE, James C., COL			
TELEPHONE: (202) 576-3551				TELEPHONE: (202) 576-2273			
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) Parasite; (U) Schistosomiasis; (U) Pathology; (U) Primate; (U) Chemotherapy; (U) Immunology; (U) Trypanosomiasis; (U) Filariasis				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) 75 07 - 76 06 Surface proteins in the cell membranes of Trypanosoma rhodesiense were endogenously labelled with 3H glucosamine and a 14 C-amino acid mixture. These antigenic proteins were extracted with 3M-KCl in the cold for 16-20 hours. After centrifugation the supernatant was dialyzed and further purified by G-200 Sephadex column chromatography and sucrose electrophoresis. One major glycoprotein peak containing 3H and 14 C radioactivity was obtained. The molecular weight was 65,000-70,000 daltons as determined by SDS-polyacrylamide gel electrophoresis. This glycoprotein inhibited E-rosette formation of sheep erythrocytes and thymus-derived lymphocytes from chimpanzees. These antigens demonstrated varying levels of antibody sensitivity in sera obtained from infected chimpanzees. In the soluble antigen fluorescent antibody test the sera from chimpanzees produced higher titers against the homologous antigen than either heterologous antigens or sonicated crude antigen. Mice immunized with 3M-KCl extract glycoproteins had produced strain specific immunity when challenged with T. rhodesiense. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$110,000 from FY 77 funds is programmed for the period 1 Jul - 30 Sep 76.			

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PII Redacted



Task 00 Communicable Diseases and Immunology

Work Unit 165 Parasitic diseases of military importance

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1. Isolation, purification and characterization of surface glycoprotein antigens of *Trypanosoma rhodesiense*.

Antigenic variation in African trypanosomes appears to be the key to the survival of the parasites in the presence of high titers of circulating antibodies. Antigens are variant specific in that trypanosomal neutralization only occurs in the presence of homologous serum antibodies. Antigen-antibody complexes are formed along the cell membrane of *Trypanosoma brucei* which suggests that the glycocalyx or surface glycoproteins are the antigenic determinants that induce immune responses in the vertebrate host. Recently, Cross showed that surface glycoproteins isolated from cloned *Trypanosoma brucei* induced specific immunity to the cloned parasites. The present studies analyze some of the properties of surface glycoproteins of *Trypanosoma rhodesiense* by attempting to endogenously label the surface glycoproteins with radioactive glucosamine and amino acid mixtures and to isolate, purify and determine some of the biochemical and immunological characteristics of these antigens.

The Wellcome strain and EATRO 1886 strain of *Trypanosoma rhodesiense* were used. Either adult Swiss albino mice or adult albino rats were inoculated intraperitoneally with  $10^4$  parasites. Three to five days later, the animals were bled out and the parasites were separated from the formed blood elements by column chromatography. The parasites were washed three times at 1800g at 4 C for 20 minutes in glucose and amino acid deficient Medium 199.

Endogenous Labelling. After washing, the parasites were adjusted to a concentration of  $10^7$ /ml in Medium 199 deficient in glucose and amino acids containing 3% fetal bovine serum. After 20-30 minutes at room temperature,  $^3\text{H}$  glucosamine (10  $\mu\text{Ci}/\text{ml}$ ) and  $^{14}\text{C}$  amino acid mixture (1  $\mu\text{Ci}/\text{ml}$ ) were added to the parasites and the suspension was incubated for two hours at 37 C followed by 16 hours at 4 C. The parasites were then incubated at 37 C for an additional two hours. They were washed a minimum of six times with Hank's Balanced Salt Solution until only background counts of the supernatant were obtained using a Packard Tri-Carb

Scintillation counter.

Extraction of Soluble Antigens. The washed parasites were centrifuged at 1000g at 4 C for 10 minutes and the packed cell volume (PCV) was determined. The Packed cells were resuspended in 10 volumes of Phosphate Buffered Saline (PBS), pH 7.4, and KCL was slowly added with constant stirring to a final concentration of 3M. The 3M KCL suspension of parasites was stirred for 16-20 hours at 4 C and then centrifuged at 100,000g for two hours. The supernatant was removed and immediately diluted 15-fold with distilled water containing 0.02% sodium azide. This was concentrated by negative pressure dialysis back to the original volume and then dialyzed in two changes of normal saline at a 4 C and clarified at 100,000g for three hours. Antigen preparations were sterilized by filtering through a 0.45 um millipore filter and stored in one ml aliquotes at -20C until used.

Gel Filtration Chromatography. Radioactively labelled extracts of parasite antigens were fractionated on a G-200 Sephadex column under the following conditions: A five ml aliquot of antigen was placed on a 2.5 by 96 cm column of G-200 Sephadex equilibrated with 0.05M Tris-HCL, 0.15M NaCl, pH 7.4 buffer and 4 ml fractions were collected at a flow rate of 16 ml/hr.

Sucrose Electrophoresis. Although the radioactive peak from the G-200 Sephadex column appeared symmetrical, it was sufficiently broad to warrant further purification. The principle of density gradient electrophoresis is that hydrodynamic stability is obtained in a vertical column of free liquid by preparing the liquid in such a way that the density steadily increases from top to bottom. Thus, the column is characterized by a density gradient, which effectively opposes the currents tendency to develop thermal convection and electro-osmotic streaming. The migration of molecules down the column is due exclusively to the net electrical charge on the surface of the molecules.

Rate-zonal electrophoresis was essentially the same as reported elsewhere. In this experiments column of 9mm x 150mm were used. The lower end of the column was closed with a dialysis membrane. A sucrose gradient of 10-25 (w/v)% in 0.05 M Tris-HCL, pH 8.2, was generated within the column to the height of 140mm. A sample of 0.5 ml from the peak fraction of the G-200 Sephadex column eluate was layered on the gradient. To stabilize the sample, another sucrose solution of 3% was added above the sample. A current of 10 milliamperes/tube (cathode in the upper, and anode in the lower reservoir) was applied for three hours. Bromphenol blue (BFB) was used as a forward marker and human hemoglobin as a trailing marker.

Polyacrylamide Gel Electrophoresis. Protein(s) were disassociated by mixing the peak fraction from Sucrose electrophoresis above with sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2ME), each to form a concentration of one per cent, and boiling at 100 C for 10 minutes. Co-electrophoresis was conducted with Bovin serum albumin (BSA) and

purified egg albumin. Procedures employed in the preparation of polyacrylamide gels electrophoresis and counting of radioactive samples, have been described previously.

Protein and Carbohydrate Determinations. Protein determinations were performed according to the method of Lowery using Bovine serum albumin as the standard. Carbohydrates (neutral and basic sugars) were determined by modifying the methods of Kirby and Davidson, and Krystal and Graham to give a larger volume of working reagents and the increased sensitivity at low concentrations of carbohydrates. The mean optical density unit of 100  $\mu\text{g}/\%$  solution of glucose, galactose, and acetyl glucosamine and acetyl galactosamine were used as standards in this study. Acid hydrolysis was accomplished in 2N HCl at 100 C for 7 hours. The hydrolysate was dehydrated under streaming nitrogen gas and reconstituted in 0.5 ml of 70% methanol and applied to a column to remove amino acid from the neutral and basic sugars. A sample dilution of 100  $\mu\text{l}$  was placed in a 13 x 100mm glass culture tube containing 1 ml of dilute ferricyanide reagent and boiled at 100 C for 15 minutes, then cooled in 0 water bath. To the cooled sugar and reagent was added 1.4 ml of distilled water. Aliquots were then read at 437nm in a Zeiss spectrophotometer using reagent blank to adjust to zero.

Lymphocyte and Sheep Erythrocyte Preparation. Venous blood was obtained from adult chimpanzees. Four of which were infected with *T. rhodesiense* EATRO 1886. A one ml suspension of  $2 \times 10^4$  parasites in HBSS was inoculated intravenously. Three non-infected chimpanzees served as normal controls. Serum was collected periodically from these animals and stored at -40 C until used. Lymphocytes were isolated from defibrinated blood by dextran (one part dextran to nine parts blood) sedimentation of erythrocytes. After sedimenting for one hour, the lymphocytes rich supernatant was recovered and centrifuged at 400g for 10 minutes at room temperature. The resulting pellet was further purified by resuspending the cells in HBSS and floating the suspension on a Ficoll-Hypaque gradient and centrifuged at 700g for 35 minutes. The lymphocytes suspended at the interface of the gradient were removed and washed in HBSS three times by centrifugation at 200 x g for 10 minutes. They were finally suspended in medium 199 containing 20% FBS at a concentration of  $4-8 \times 10^6$  cell/ml.

Sheep erythrocytes preserved in Alsever's solution were obtained regularly from the Department of Immunology at Walter Reed Army Institute of Research. The sheep red blood cells (SRBC) were washed 4 times in HBSS and brought to a final concentration of 2%.

Rosette Formation. Rosette testing was performed by placing 0.2 ml of  $4-8 \times 10^6$  cell/ml lymphocyte suspension in a 12 x 75 mm glass culture tube and adding 0.2 ml of 2% SRBC. The mixture was centrifuged at 150g for five minutes and placed in the cold for 16 hours. Following incubation at 4 C the cell buttons were gently resuspended using a Pasteur pipette and placed on a Hemacytometer chamber. A total of 200 lymphocytes

were counted scoring all cells with 3 or more adherent SRBC's. For testing these antigens a given concentration, 6.1  $\mu\text{g}$  of glycoprotein was added to the standard mixture.

Serum Antibody Determinations. Serum antibody titer were documented by the soluble antigen fluorescent test essentially as described elsewhere using as antigens 3M KCl extracts of *T. rhodesiense* strains EATRO 1886 containing 450  $\mu\text{g}$  of protein per ml and Wellcome containing 852  $\mu\text{g}$  of protein per ml and sonicated crude homogenate of stock strain Wellcome trypanosomes containing 1200  $\mu\text{g}$  of protein/ml. The results obtained using selected sera of non-infected and infected chimpanzees were compared. The tests were performed as follows briefly. Milipore disc of 0.45  $\mu\text{m}$  HAWG was saturated with each antigen solution and allowed to dry. Smaller discs of 0.5 cm diameter were placed in varying dilution of serum diluted in .05M Tris-HCl-1.5M NaCl pH 8 Buffer (TBS) containing 2% Tween 80 and rotated slowly on a rotation for 45 minutes. The discs were then washed in three changes of TBS for 5 minutes each. After the third wash 0.1 ml of the predetermined dilution of species fluorescent conjugated anti-globulin for 30 minutes and washed as above then placed on black electrical tape. The fluorescence was measured in a fluorometer using the antigen disc of preinfected or normal sera to set the instrument to zero.

TABLE I

<u>Lot of KCl</u> <u>Extract</u>	<u>Protein</u> <u><math>\mu\text{g}/\text{ml}</math></u>	<u>Carbohydrate*</u> <u><math>\mu\text{g}/\text{ml}</math></u>	<u>Carbohydrate</u> <u><math>\mu\text{g}/100\mu\text{g}</math> protein</u>
111976W	152	ND <sup>+</sup>	ND
41576W	852	37.5	4.4
42976E	452	17.5	3.8

<sup>+</sup>Not Done.

Immunogenicity of Glycoprotein Antigens. The ability of the glycoprotein to induce immunity in mice was tested by injection of 0.1 ml of glycoprotein solution (31.4  $\mu\text{g}/\text{ml}$ ) intramuscularly on day 1 and intradermally on day 7 and 14. Control mice received injections of buffered saline solution only. Mice were challenged by intraperitoneal injections of approximately 1000 trypanosomes 7, 14, 21, 28, or 42 days following the last antigen injection. After day four of the challenge, tail blood of mice was examined daily for trypanosomes and the day of death was recorded.

TABLE II

Chimpanzee No.	No. of Rosettes Formed		% Rosettes Formed	
	Without 3M KCl-X	With 3M KCl-X	With 3M KCl-X	Inhibited with 3M KCl-X
Non-infected				
P544	32	23	72	28
M760	18	18	100	0
Mean	25	21	86	14
Infected				
488	27	18	67	33
759	56	8	14	86
681	25	4	16	84
109	59	12	20	80
Mean	42	11	29	71

Effect of 3M KCl-X on rosettes formed by sheep erythrocytes and lymphocytes from infected and uninfected chimpanzees.

TABLE 111

Fluorescent antibody titers in sera from chimpanzees infected with *Trypanosoma rhodesiense* EATRO 1886 using homologous and Wellcome 3M KCl extracted glycoproteins and Wellcome crude antigens.

Animal No.	Day Post Infection	Serum antibody titers obtained using antigens of <i>T. rhodesiense</i>		Crude Sonicated Wellcome
		Glycoprotein		
		188 Strain	Wellcome	
Non-infected				
P544	233	0	0	0
P546	0	0	0	0
	177	0	0	0
	233	0	0	0
M760	177	0	0	0
Infected				
759	12	128	0	32
	19	2048	8	128
	24	2048	8	128
	38	2048	8	128
	41	2048	32	128
	99	2048	32	128
	198	2048	128	2048
681	12	2048	8	32
	99	2048	32	512
	149	2048	32	128
	233	2048	128	2048
478	21	2048	8	512
	44	2048	32	512
	149	2048	32	512
	177	2048	32	512
	198	2048	32	512
	P109	149	2048	32

#### Labelling and Purification of *T. rhodesiense* Antigens. A typical

elution profile of 3M KCl extract on G-200 Sephadex column chromatology is shown in Figure 1. Only on protein peak containing both  $^3\text{H}$  (tritium) and  $^{14}\text{C}$  (carbon) radioactive counts was eluted from the column at the position corresponding to that of human hemaglobulin (65,000 daltons). Approximately 90% of the radioactivity applied to the column was recovered.

The total protein and carbohydrates (neutral and basic sugars) are shown in Table I. Although there is a 50% difference in protein concentrations per ml of extract the concentration of carbohydrates 3.8 - 4.4 mg/100  $\mu\text{g}$  is constant in the glycoprotein extracts.

The glycoprotein peak from G-200 Sephadex column chromatology was further purified by electrophoresis in a sucrose density gradient. Figure 2 shows that again a single radioactive peak was obtained contained both  $^3\text{H}$ -glucosamine and  $^{14}\text{C}$  amino acid mixture.

The peak from sucrose electrophoresis was used for protein molecular weight determination on SDS acrylamide gel electrophoresis (Figure 3). Co-electrophoresis with standard markers Bovine serum albumin (MW 67,000 daltons) and egg albumin (45,000 daltons) show the molecular weight of the extracted protein is approximately 65-70,000 daltons (Figure 4).

E-Rosette Inhibition Studies. The biological characteristic of thymus-derived lymphocytes to form rosettes (E-rosette) with sheep erythrocytes was used to evaluate the biologic activity of the 3M-KCl extracts (glycoprotein antigens). As shown in Table II a mean of 25 rosette forming lymphocytes from the peripheral blood of non-infected chimpanzees under normal E-rosette test conditions of the 25 rosette formed 14% failed to form rosettes in the presence of glycoprotein antigens. Whereas of the 42 rosetting cells the peripheral blood of the infected chimpanzees, 71% failed to form rosettes in the presence of the glycoprotein antigens.

Soluble Antigen Fluorescent Antibody Testing. When sera from chimpanzees infected with *T. rhodesiense* EATRO 1886 were tested, there were notable differences in the sensitivity of the antigens used in the SAFA test. Antibodies were detected by the SAFA test using homologous 1886 3M KCl-X, heterologous Wellcome 3M KCl-X and heterologous Wellcome sonicated crude antigens. However, as shown in Table III higher titers were consistently demonstrated when homologous 1886 3M KCl extracted antigens were used and the results compared to the results obtained using the heterologous Wellcome 3M KCl extracted, or sonicated crude antigens in aliquots of the same serum dilutions. Antibodies to trypanosome were not detected in the sera of non-infect chimpanzees with any of the antigenic preparations.

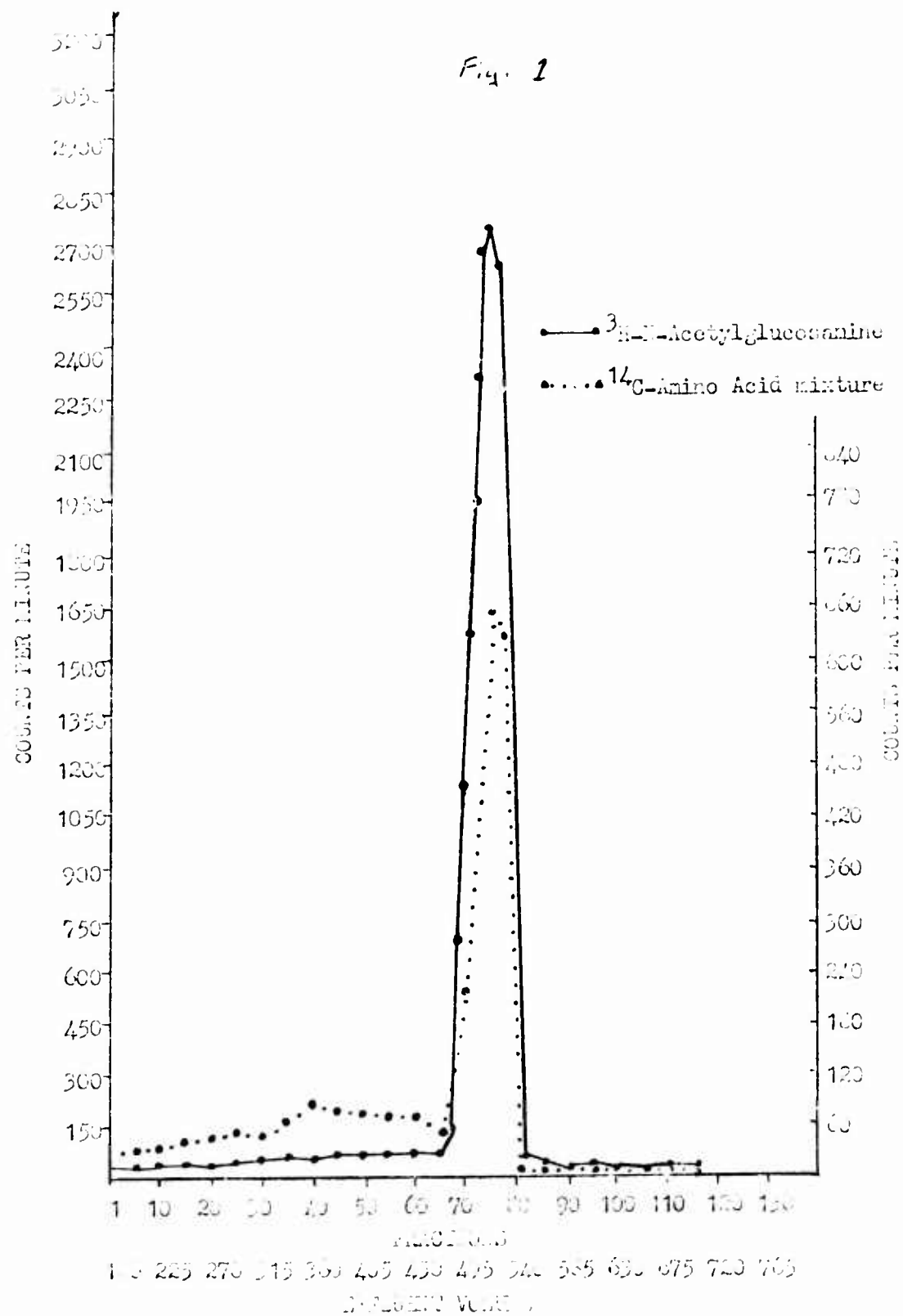
Fig. 1. G-200 column chromatography elution profile of radioactivity labeled Trypanosoma rhodesiense glycoproteins extract with 3M KCl.

Fig. 2. Sucrose electrophoresis fraction of 3M KCl extracts of Trypanosoma rhodesiense fraction #75 from G-200 Sephadex chromatography.

Fig. 3. Polyacrylamide gel electrophoresis of 3M KCl extract of Trypanosoma rhodesiense from each fraction of sucrose electrophoresis.



Fig. 1



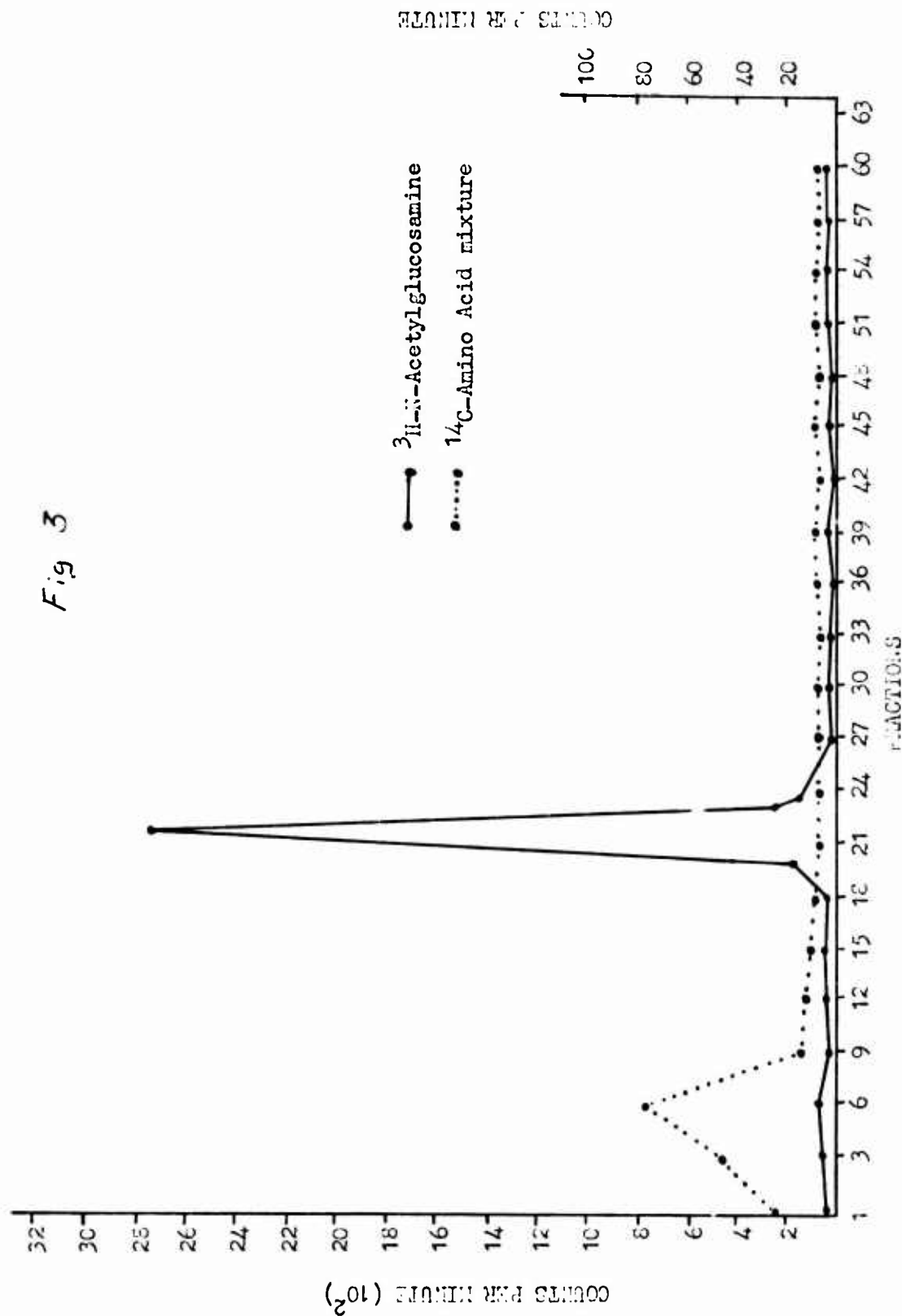
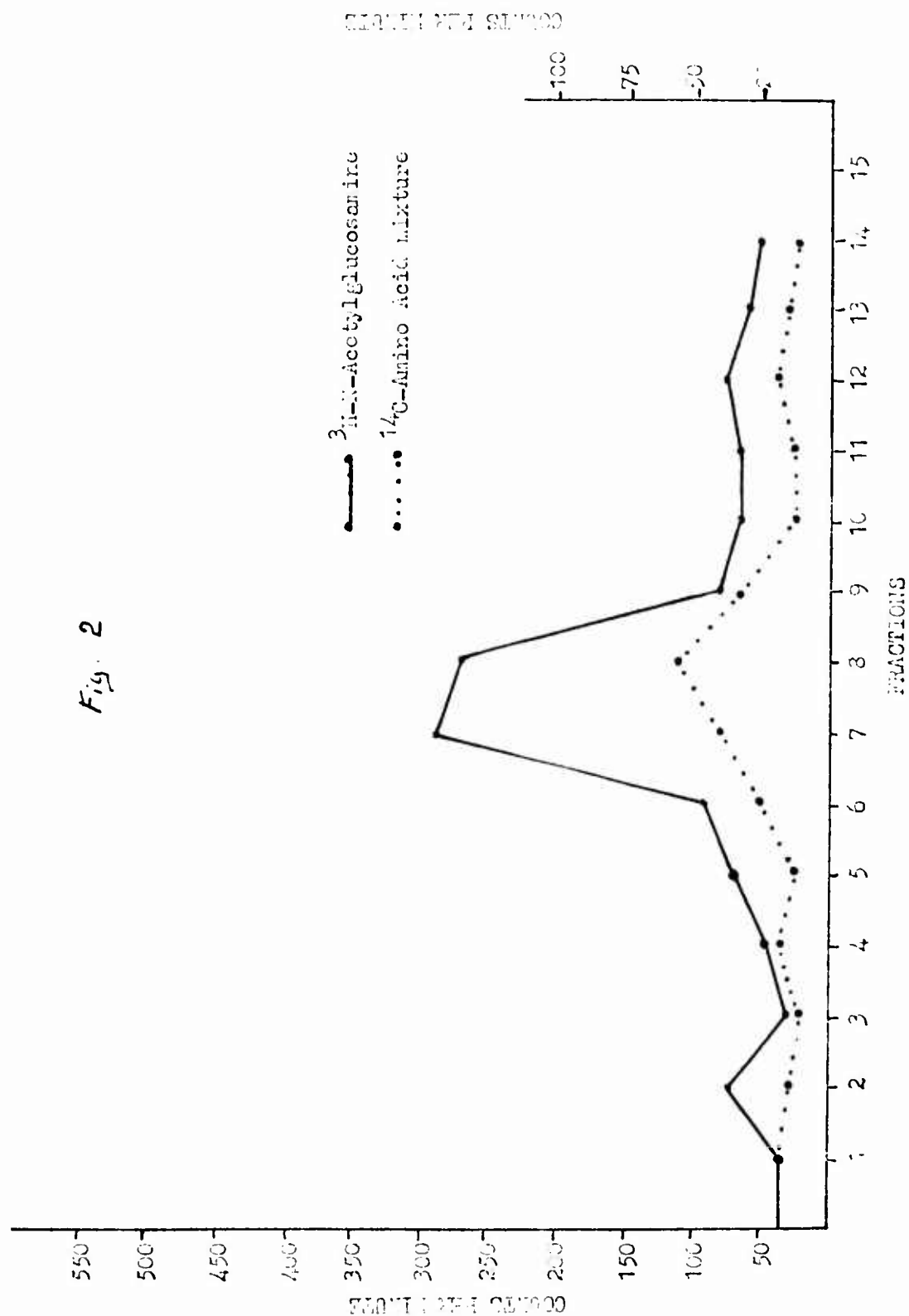


Fig 3



Immunogenicity of Soluble Glycoprotein Extracts. By immunizing mice intramuscular with 0.1 ml (3.1 mg of protein) of glycoprotein on day one and intradermally on day 7 and day 14 an immune state was induced in mice which appeared to be related to the day of challenge following the last immunizing injection. Table IV the mean per cent of protection was 73% with extremes of 40%-100% protection. The lowest per cent (40%) of protection was accomplished as early as day 7 following the last immunizing dose. The greater per cent of mice protected (90%-100%) was achieved when the animals were challenged on day 28 and 42 post immunization. None of the non-immunized (control) mice survived the challenge with T. rhodensiense EATRO 1886 when injected with approximately 1000 trypanosomes intraperitoneally.

The endogenous labelling of the glycoprotein of surface membranes of T. rhodensiense with  $^3\text{H}$  glucosamine and  $^{14}\text{C}$  amino acid mixture and subsequent extraction and purification is an important step in the study of antigenic variations and immunization studies. Extraction with 3M KCl and subsequent purification on G-200 Sephadex was facilitated by being able to locate the radioactivity labelled glycopeptide molecules in the column eluate. This purified glycoprotein is similar to those of cloned T. brucei. Molecular weight is about the same (65,000-70,000 daltons) there are carbohydrate moieties on a peptide backbone which were cleaved off by acid hydrolysis under nitrogen. It could be used to detect strain specific antibodies and in a protocol of immunization induce complete protection in mice.

This antigen may remove one of three major obstacles in the introduction of a practical and an effective program of immunizing man and/or animals. It is possible to rationally determine the antigenic types endemic in geographic locations and prepare multivalent vaccines. Blood forms of trypanosomes can be obtained by quick passages 2-3 days about 4 times. They would then be 3M KCl extracted purified and pooled and sterilized by filtration and used as immunogens. Spontaneous formation of rosettes of SRBC and thymus-derived lymphocytes, a non-immunological event, has been shown to occur with lymphocytes obtained from mice, monkey, chimpanzee and man. This rosetting can be inhibited or blocked chemically by sodium iodoacetate which interrupts the glycolytic pathway of the lymphocytes and by competitive inhibition of SRBC with various glycoprotein, i.e., fetuin glycopeptide I, human erythrocyte glycopeptide I and human transferrin glycopeptide. It is suggested by the ability of 3M KCl extracted glycoprotein of T. rhodensiense to inhibit E-rosette formation that these glycopeptide molecules share a common structural feature at least of the carbohydrate moiety of the molecule. Galactose mannose and N-acetylglucosamine have been shown to be present in the glycoproteins listed above and of cell membranes of T. brucei and T. equiperdum. Since it was concluded by Boldt and Anderson that glycopeptide showing the basic structure Sialic acid  $\longrightarrow$  galactose  $\longrightarrow$  Nacetylglucosamine  $\longrightarrow$  (mannose)<sub>N</sub> are effective inhibitors of rosette formation and all of these moieties are in the structure of cell membrane of sheep red blood cells and T. rhodensiense there is a more than casual relationship between SRBC and trypanosome cell membranes.

TABLE IV

Day Post	No. of Mice Challenged	Number of Mice Dead by Day							Percent Survivors
		5	6	7	8	9	10	15	
7	Immunized 5	2	3					3	40%
	Controls 5	4	5					5	0
14	Immunized 5		2					2	60%
	Controls 5	2	5					5	0
21	Immunized 20	1	3					3	85%
	Controls 10	7	10					10	0
28	Immunized 10			1				1	90%
	Controls 10	5	10					10	0
42	Immunized 5							0	100%
	Control 5	4	5	1				5	0

Effect of delay in challenge on mortality of mice immunized with 9  $\mu$ g of Wellcome 3 M KCl extracted glycoproteins.



Fig. 4. Co-electrophoresis values with 3M KCl extract of Trypanosoma rhodesiense (A) and with bovine serum albumin and egg albumin as markers (B).

When selected serum of infected and noninfected chimpanzees were tested antibodies were preferentially demonstrable dependent upon the antigen preparation used. When using the heterologous 3M KCl extracted glycoproteins the antibodies were consistently lower than the 3M KCl extracted EATRO 1886. The difference in antigenicity is probably due to key changes of amino acids or amino acid sequence as shown by Cross in glycoproteins of 6 cloned T. brucei variants. The available amount of protein in EATRO 1866 glycoproteins at 450  $\mu\text{g/ml}$  is approximately 50% less than that present in Wellcome glycoproteins. Sonicated Wellcome crude antigen detected antibodies which are probably due to the stable antigens that are of somatic origin, as opposed to the variant or strain antigens found in the cell membranes which are detected by specific glycoprotein extracts.

2. The cynomolgus monkey (*Macaca fascicularis*) as an experimental primate host for visceral leishmaniasis.

Cynomolgus monkeys inoculated intravenously with 40-100 million amastigotes of *Leishmania donovani*/kg host body weight invariably developed a clinical illness similar to that observed in visceral leishmaniasis of man. The initial clinical symptom usually was splenomegaly in 3 to 7 weeks post-exposure followed shortly by the onset of pancytopenia and significant weight loss. Typical changes included a 6 to 12-fold increase in spleen weight, a drop in platelet count from 5-600,000 to less than 100,000/ $\text{mm}^3$ , a decrease in packed cell volume from 32-40% to 15% or less, a drop in total white blood count from 5-10,000 to 1-3,000/ $\text{mm}^3$ , and weight loss approximating 10-35%. Animals usually died within 6 to 13 weeks post-exposure. Parasites were seen microscopically in liver, spleen, bone marrow, and numerous other organs at necropsy. Promastigotes were easily cultured from infected bone marrow using Tobie's diphasic, Tanabe's, or Mansour's medium. Histopathologic lesions included severe diffuse granulomatous inflammation of spleen, liver, and the abdominal lymph nodes. Non-suppurative interstitial pneumonia and epididymitis also were evident. Sub-meningeal hemorrhages occasionally were observed. These results suggest that this monkey model may be suitable for testing of anti-leishmanial compounds and also in studies relating to the biology and pathogenesis of the disease.

3. A filarial parasite in greyhounds in Southern Ireland.

There appears to be no previous evidence of filarial infections in dogs in Great Britain and Ireland. A search of the literature fails to show any reported cases. Furthermore, personal conversations and correspondence with several veterinarians and parasitologists in Great Britain and Ireland have not revealed any knowledge of such infections.

This is a report of the discovery of microfilariae in the peripheral blood of seven dogs from County Limerick and County Cork in Southern Ireland. Positive identification of the species has not yet been made since there has thus far been no opportunity to perform necropsies to

obtain adult worms. However, the microfilariae appear to be a species of Dipetalonema. This report is being published in the hope that it will stimulate additional investigations regarding the prevalence, geographical distribution, and biology of the parasite in Southern Ireland and perhaps elsewhere in Ireland and Great Britain.

Dogs 1 to 4--In May 1973, four greyhounds from Palm Beach County in south Florida, United States, were referred to one of us (RFJ) for heartworm treatment. Examination of their bloods revealed microfilariae which were different from those of either Dirofilaria immitis or Dipetalonema reconditum seen in native dogs in the United States. Those are the only two filariae commonly found in dogs in the USA. The newly found microfilariae appear to resemble D. reconditum in the following respects: they exhibit progressive motility in a fresh blood smear and the anterior end is blunt as seen in a modified Knott preparation. The new microfilariae differ from D. reconditum in that they are shorter and do not possess button-hook tails as usually seen in D. reconditum in native dogs in the United States.

Greyhounds 1 to 4 were born and raised in Ireland and shipped to the USA when they were about one year of age, six months before our examination. While in south Florida they had been housed in a kennel in close proximity to numerous other greyhounds. No similar microfilariae were found, however, in any of the other dogs in the kennel. Dogs 1 to 4 were moved to Putnam County in north Florida after our examination and were placed in a kennel with approximately 100 other greyhounds. Since this area is endemic for heartworm disease and the dogs were not housed in screened pens, all were given the recommended daily dose of diethylcarbamazine as a heartworm preventive. All dogs in this kennel were examined periodically for microfilariae during the entire two year period that dogs 1 to 4 remained there. No other dogs were found with the new microfilariae. The microfilarial count in dogs 1 to 4 gradually decreased until the last time they were examined they had only approximately 1 microfilaria per 10 ml of blood.

Dogs 5 to 7--On April 28, 1975, blood was examined from a greyhound (dog 5) shipped to south Florida from Ireland four days previously. Microfilariae similar to those found in dogs 1 to 4 were found. On May 22, 1975, one of us (RFJ) travelled to Ireland and examined blood from eight dogs on the premises in County Cork from which dogs 1 to 4 had originated. No microfilariae were found. Blood from one year old dogs on the premises in County Limerick from which dog 5 originated contained microfilariae in two (dogs 6 to 7) of the five dogs examined. Dog 6 was a litter mate of dog 5. Another litter mate of dogs 5 and 6 was found to be negative.

These newly found microfilariae in County Limerick in Southern Ireland were fixed in 2 per cent formalin (modified Knott preparation; 1 ml of blood in 10 ml of 2 per cent formalin) and mailed to the United States. The sediment was air dried on slides, fixed in equal parts of ether and 95 per cent ethyl alcohol, and stained with Delafield's hema-



toxylin.

Microfilariae were observed to be essentially straight with no evidence of button-hook tails. The anterior end was bluntly rounded often with a slight swelling apparent at the level of the cephalic space. The two anterior-most body nuclei were distinct in that they were typically elongate, side by side, and more lightly stained than the remaining oval nuclei. The posterior nuclei characteristically ended in a single row of five or six oval to elongate nuclei which did not extend to the tail tip leaving a caudal space. The last and third to last nuclei were often the most elongate with the latter often taking on the lightest stain. Measurements of 15 of these microfilariae yielded the following parameters: body length-- $249\mu$  (237 to  $259\mu$ ); maximum body width-- $4\mu$  (3 to  $5\mu$ ); length of cephalic space-- $11\mu$  (10 to  $13\mu$ ); cephalic ratio (width of cephalic space/length--0.34 (0.23 to 0.40). The percentage distance of various landmarks from the anterior end are: cephalic space--4.4 (4.0 to 5.1); nerve ring--21.2 (20.6 to 22.0); excretory pore--30.0 (28.5 to 31.4); anal pore--78.2 (76.0 to 79.2); and last nucleus--92.1 (90.7 to 94.0).

These specimens differ from the microfilariae of what is presently termed D. reconditum from native dogs in the United States. The latter average 15 to  $20\mu$  longer, commonly have a button-hook tail, and have a shorter cephalic space. Furthermore, the anterior and posterior nuclei differ in shape and configuration.

Further information including description of the adults will be required for identification of this newly found parasite.

#### 4. Brugia pahangi: Effects upon the flight capability of Aedes aegypti

The effects of developing filarial worms upon their mosquito hosts have been studied extensively by numerous investigators. Rosen demonstrated that Wuchereria bancrofti in Aedes polynesiensis caused excessive mortalities during the later stages of parasite development in heavily infected mosquitoes. Wharton showed similar higher death rates in Brugia malayi-infected Mansonia longipalpis. Townson further demonstrated that among Aedes aegypti infected with Brugia pahangi that survived throughout the entire development of larval worms, there was a significantly greater number of nonflying mosquitoes than among uninfected controls. He also reported a significantly higher infection rate among infected nonflying than in infected flying mosquitoes. He demonstrated, however, by using a wind tunnel, that increased flight duration in noninfected versus infected mosquitoes was only "barely significant" ( $P = 0.065$ ).

This paper reports the results of experiments with Brugia pahangi-infected Aedes aegypti using flight mills that automatically measure distance, speed, and duration of tethered flight. We have compared flight characteristics and other parameters of A. aegypti at various times after exposure with those of noninfected controls.

Female A. aegypti (strain 2, Macdonald and Sheppard relevant genotype (+fm/+fm)) were used. Standard rearing procedures were followed. Female pupae were picked on a single day, and approximately 75 were placed in each of a number of pint cartons covered with fine mesh screen. After adults had emerged, water was drained from the cartons, and the mosquitoes maintained at 25 C and 80% relative humidity and fed on 10% sucrose solution.

All infected mosquitoes were 12 days old when flown. This was accomplished by feeding 3-, 6-, and 9-day-old mosquitoes on B. pahangi-infected cats and flying them 9, 6, and 3 days later, respectively. Each experimental group was paired with a control group fed simultaneously on a noninfected cat. Microfilaremiias in cats ranged from 19 to 24/mm<sup>3</sup>. 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 Resources, National Academy of Sciences-National Research Council.

At 3, 6, and 9 days postexposure (PE), the number of dead mosquitoes in each group was recorded; also, the number of living mosquitoes incapable of flight when stimulated by touch was determined.

For each of Days 3, 5, and 9 a  $\chi^2$  test ( $P = 0.05$ ) was made for comparing the infected vs control groups (each day) on the basis of (1) dead vs surviving and (2) nonflying survivors vs flying survivors.

A random sample was taken of those mosquitoes apparently capable of flight according to Townson's (1970) procedure. These mosquitoes were flown on flight mills patterned after those described by Rowley et al. (1968). The mills consisted of a lightweight metal arm that pivoted on a central vertical axle. A short hook made of thin nichrome wire was suspended below the tapered end of the metal arm. A small drop of low-melting-point wax was used to attach a lightly anesthetized mosquito to the wire hook. The mosquito was affixed by the dorsum of its thorax and oriented at a right angle to the pivot arm.

Each revolution flown by the mosquito equaled 1 m. This was recorded by a digital counter and a continuous-event recorder. Each flight mill was enclosed in a transparent Plexiglas box to eliminate wind currents. All mosquitoes were flown for 24 hrs. in artificial light at 23 C and 53% relative humidity. Individual flight distances and number and duration of flights were recorded for each mosquito after 24 hrs. A  $t$  test ( $P = 0.05$ ) was made to determine whether the infected mosquitoes differed from the controls at 3, 6, and 9 days PE on the basis of (1) distance flown and (2) total time flown.

All flown mosquitoes along with representative mosquitoes that were incapable of flight were then dissected and the number of parasites per mosquito determined. A  $t$  test ( $P = 0.05$ ) was made for comparing infection

Results of this study indicate that the development of *B. pahangi* in *A. aegypti* has a detrimental effect upon survival and flight capability of the vector. A significantly higher percentage of infected mosquitoes was killed or rendered incapable of flight than of controls throughout the experiment. These differences represent those vectors that in nature would be incapable of transmitting their infection to a definitive host. Dissection of mosquitoes to determine parasite burdens suggest that heavier infections tend to cause greater mortalities and nonflying rates.

TABLE V

*Death Rates and Flight Capabilities of Brugia pahangi-Infected Versus Control Aedes aegypti at 3, 6, and 9 Days Postexposure*

Days post-exposure	No. of mosquitoes		% Dead		% Nonflying		% Flying	
	Infected	Control	Infected	Control	Infected	Control	Infected	Control
3	365	601	20	18	25 <sup>a</sup>	2	53 <sup>a</sup>	80
6	966	217	18 <sup>a</sup>	6	15 <sup>a</sup>	1	67 <sup>a</sup>	93
9	674	295	16 <sup>a</sup>	6	27 <sup>a</sup>	6	57 <sup>a</sup>	88

<sup>a</sup> Significant [ $\chi^2$  contingency test ( $P = 0.05$ )].

Significant pathologic findings involving flight muscles of mosquitoes as a result of developing filarial worms have been reported by several investigators: Townson, Beckett, and Kan and Ho. The histopathologic findings in the present study are consistent with those previously reported. Both minor and severe damage as described by Beckett was observed. These observations seem sufficient to explain the marked morbidity and mortality seen in the present study.

The explanation for the ability of control mosquitoes to fly further and longer with increasing time after the blood meal is unclear. In nature, engorged females remain relatively sessile after engorging for 2-3 days during the period of egg development. Subsequently, they disperse in search of sites to oviposit. Perhaps these behavioral patterns also are being manifested under the conditions of this experiment.

Decreased flight capabilities in the infected mosquitoes may be due to the parasites depriving the vectors of energy by direct usage and/or damaging the flight muscles so that they are incapable of proper functioning. The latter aspect could be purely mechanical or due to the muscles' inability to utilize or convert energy for flight activity.

intensities in flying versus nonflying mosquitoes at 3, 6, and 9 days PE.

Mosquitoes with 1- to 10-day-old infections were examined histologically. They were fixed in Carnoy's, embedded in paraffin, serially sectioned at 6  $\mu$ m, and stained with hematoxylin and eosin.

Infected mosquitoes flew significantly shorter total distances and exhibited a significant reduction in total time flown throughout the experiment. The total distance flown by infected mosquitoes was reduced 24, 43, and 50% (Fig. 5) and total flight time reduced 26, 37, and 48% (Fig. 6) at 3, 6, and 9 days PE, respectively. The total flight range and duration of time flown by infected mosquitoes remained relatively constant throughout the infection process, while control mosquitoes flew further and longer with increasing time after their blood meal.

No significant differences were detected between infected and control mosquitoes in length of initial flight or in the number or duration of individual flights during the 24-hr flight periods.

A significantly greater percentage of infected mosquitoes were dead at 6 and 9 days PE compared to the controls (12 and 10%, respectively) (Table V). Furthermore, a greater percentage of infected mosquitoes (23, 14, and 21%, respectively) was not capable of flying at 3, 6, and 9 days PE than of the controls (Table V). A significantly reduced percentage of infected mosquitoes was found alive and able to maintain sustained flight at 3, 6, and 9 days PE (25, 26, and 31%, respectively) (Table V).

All infected mosquitoes flown at 3, 6, and 9 days PE, along with a representative number of nonflying mosquitoes from the same samples, were dissected and the mean intensities of infection compared (Table VI). Nonflying mosquitoes with 6-day-old infections harbored a mean of 25% more worms than did the 6-day flying mosquitoes. Over twice the number of worms on the average were found in each nonflying mosquito at 9 days PE as in the 9-day flyers. Mosquito dissections of 3-day-old infections showed no significant differences in intensity of infection between the two groups.

Examination of histologic sections of infected mosquito flight muscle using light microscopy indicated that microfilariae entered the flight muscle within the first observed time period (15 min postfeeding). Little damage was apparent at 24 hr PE except for occasionally enlarged or hyperchromic muscle fiber nuclei. Eosinophilic material began to accumulate anterior and posterior the worm 2-3 days after infection and was apparently being ingested by Day 5. By this time, hyperchromic and enlarged nuclei were more prevalent and observed in both parasitized and nonparasitized fibers. In some specimens, pyknosis and disintegration of the nuclei was observed to the point that eventually the entire flight mass was anucleate. This was followed by degeneration of the myofibrils to form an amorphous mass.

TABLE VI

*Infection Intensities of Brugia pahangi in Flying Versus Nonflying Aedes aegypti at 3, 6, and 9 Days Postexposure*

Days post-exposure	No. of mosquitoes		Mean no. of worms recovered		Standard error		
	Flying	Nonflying	Flying	Nonflying	Flying	Nonflying	
3	47	49	6.3	7.0	0.94	0.83	0.5
6	61	49	9.3	12.2	0.84	1.12	2.1*
9	42	50	8.9	21.0	0.87	1.94	5.7*

\* Significant ( $P = 0.05$ ).

Whatever the mechanism, it is clear that under laboratory conditions, development of filarial larvae within this mosquito reduces its ability to survive and to transmit infection by reducing its flight capabilities. It is important, however, to note that only *A. aegypti* mosquitoes homozygous for the gene *fm* are fully susceptible to this filarial parasite. Therefore, conclusions from this study relate only to one particular genotype and not the vector species *A. aegypti*.

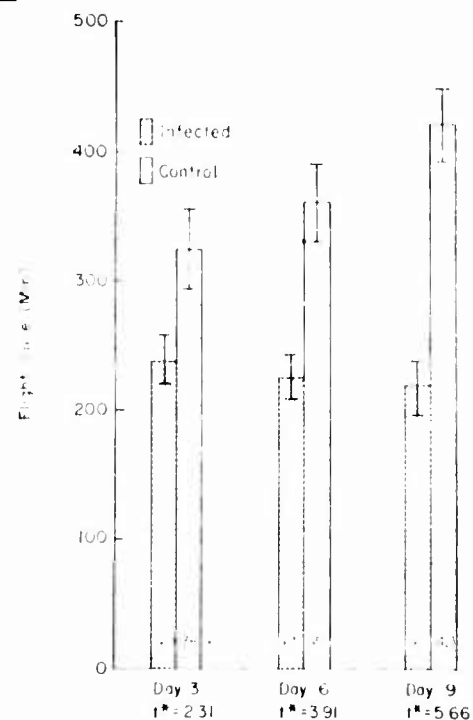


FIG. 5.—Total time flown in a 24-hr period by *Brugia pahangi*-infected versus control *Aedes aegypti* at 3, 6, and 9 days postexposure. The vertical brackets indicate standard error of the mean. \* Significant ( $P = 0.05$ ). † Number of mosquitoes per group.

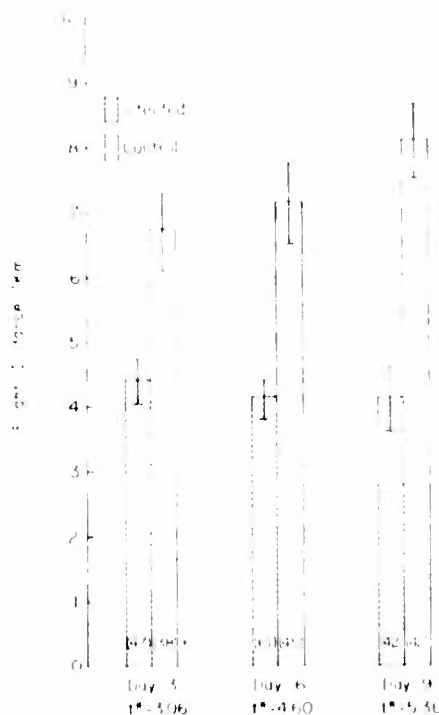


FIG. 6. Distances flown in a 24-hr period by *Brugia pahangi*-infected versus control *Aedes aegypti* at 3, 6, and 9 days postexposure. The vertical brackets indicate standard error of the mean. \* Significant ( $P = 0.05$ ). † Number of mosquitoes per group.

5. A method for estimating blood meal volume in *Aedes aegypti* using a radioisotope.

Numerous investigators have attempted to estimate blood meal volume of *Aedes aegypti* by weighing the mosquitoes, individually or en masse, before and after feeding. The commonly accepted average blood meal volume for this mosquito species using this method is ca. 2.5  $\mu$ l. Boorman reported that gravimetric methods significantly underestimated blood volume because no allowance was made for the excretion of clear fluids by the mosquitoes during and shortly after engorgement. In his studies, he determined that the average blood volume ingested by *A. aegypti* was ca. 4.0 - 4.5  $\mu$ l and that the volume due to excretion of clear fluids was ca. 1.5  $\mu$ l. He concluded that the latter figure represented the error inherent in previously reported gravimetric estimates. Boorman's experiments lacked tight control, however, because he employed a different lot of mosquitoes in each of his 3 critical experiments, i.e. quantitation of fluid loss, radioisotope estimation of blood meal volume, and gravimetric estimation of blood uptake. The present study is an attempt to reassess Boorman's conclusions and critically evaluate this technique as a tool for estimating blood meal

volume in A. aegypti.

Experimental mosquitoes. Female A. aegypti strain 2, Macdonald and Sheppard, relevant genotype (+fm/+fm) were used. Standard insectary rearing procedures were followed. Emerged female mosquitoes were placed in screened pint cartons and maintained at  $26 \pm 2^{\circ}\text{C}$  and  $80 \pm 5\%$  relative humidity and fed on 10% sucrose solution ad lib. from cotton pads. A 12 hr daylight cycle was maintained. Unless otherwise described, mosquitoes were 7 days old and starved for 1 day when utilized. All experimental procedures were performed outside the rearing insectary but mosquitoes were either kept under a bell jar containing a vessel of warm water or if possible returned to the insectary between manipulations.

Excretion of clear fluid. Five cartons of 75 mosquitoes each were exposed in turn to the shaved flank of a dog. Four cartons contained 7-day-old females; 1 group each was starved for 1, 2, or 3 days; the fourth group was not starved. The fifth carton contained 2 day-old females never given food. The proportion of mosquitoes which fed in each carton was noted and a few engorged individuals from each were placed into screened glass tubes and observed with a dissecting scope to characterize fluid excretion.

Excretion of radioisotope. Mosquitoes were lightly anesthetized with ether and individually placed into small glass feeding tubes 27 mm long and 11 mm in diameter. The bottom of each tube contained a piece of filter paper and the open end was covered with a fine mesh screen. After a 2-hr recovery period, mosquitoes were allowed to engorge on blood through a membrane feeding apparatus patterned after Rutledge which used a natural lamb skin membrane and contained a mixture of 8 ml of heparinized rhesus monkey blood and 160  $\mu\text{l}$  ( $\mu\text{Ci}$ ) of pH neutralized  $^{144}\text{Ce}$ . A magnetic stirrer was used to keep the blood thoroughly mixed. Each mosquito was left undisturbed in its tube for 3 hr after feeding, then it was killed by freezing and transferred to a clean glass tube. Each feeding tube was then individually counted for 5 min in a well-type gamma counter. Each mosquito was counted in its clean tube in a similar manner. Counts exceeding 150 per 5 min were considered to be above the baseline level. All glass tubes utilized in this experiment were counted in place before use and only those with less than 135 background counts per 5 min were accepted.

Radioisotope and gravimetric blood meal volume estimates. Each mosquito was lightly anesthetized and weighed using a single pan substitution balance having an accuracy of  $\pm 0.01$  mg. Each was then placed into a feeding tube and after a 2-hr recovery period fed on  $^{144}\text{Ce}$ -labeled rhesus monkey blood from a membrane feeder as described above. Immediately after feeding, each mosquito was again anesthetized, weighed, and placed back into its screened feeding tube. At 3 hr postfeeding (PF), mosquitoes were killed by freezing, individually weighed, and each placed into a clean tube. Gamma counting of the feeding tubes and mosquitoes was done as described above. The specific gravity of 1.027 was used to convert

Table VII

Gamma emission count rates from engorged Aedes aegypti fed on  $^{144}\text{Ce}$ -labeled blood and count rates from clear excreta expelled during the interval between onset of feeding until 3 hr post-feeding.

Mosquito No.	Gamma Counts Per 5 Min. (3 hr Post-Feeding)	
	Engorged Mosquito	Mosquito Excreta
1	3,167	154
2	1,919	124
3	5,687	132
4	3,613	136
5	4,935	132
6	3,266	127
7	2,636	121
8	2,264	112
9	2,191	133
10	4,889	119
11	2,130	136
12	2,566	106
13	5,630	116
14	2,617	135
15	868	100
16	3,756	125
Mean	3,258	126
Range	868-5,687	100-154



ingested blood weight vs volume.

Both a rank test and t-test were utilized to analyze differences between blood meal volume estimates by the radioisotope versus the gravimetric method. Comparison of the variances between the 2 methods was done using the F-test. The r statistic was used to correlate blood meal volume with fluid volume loss within 3 hr PF.

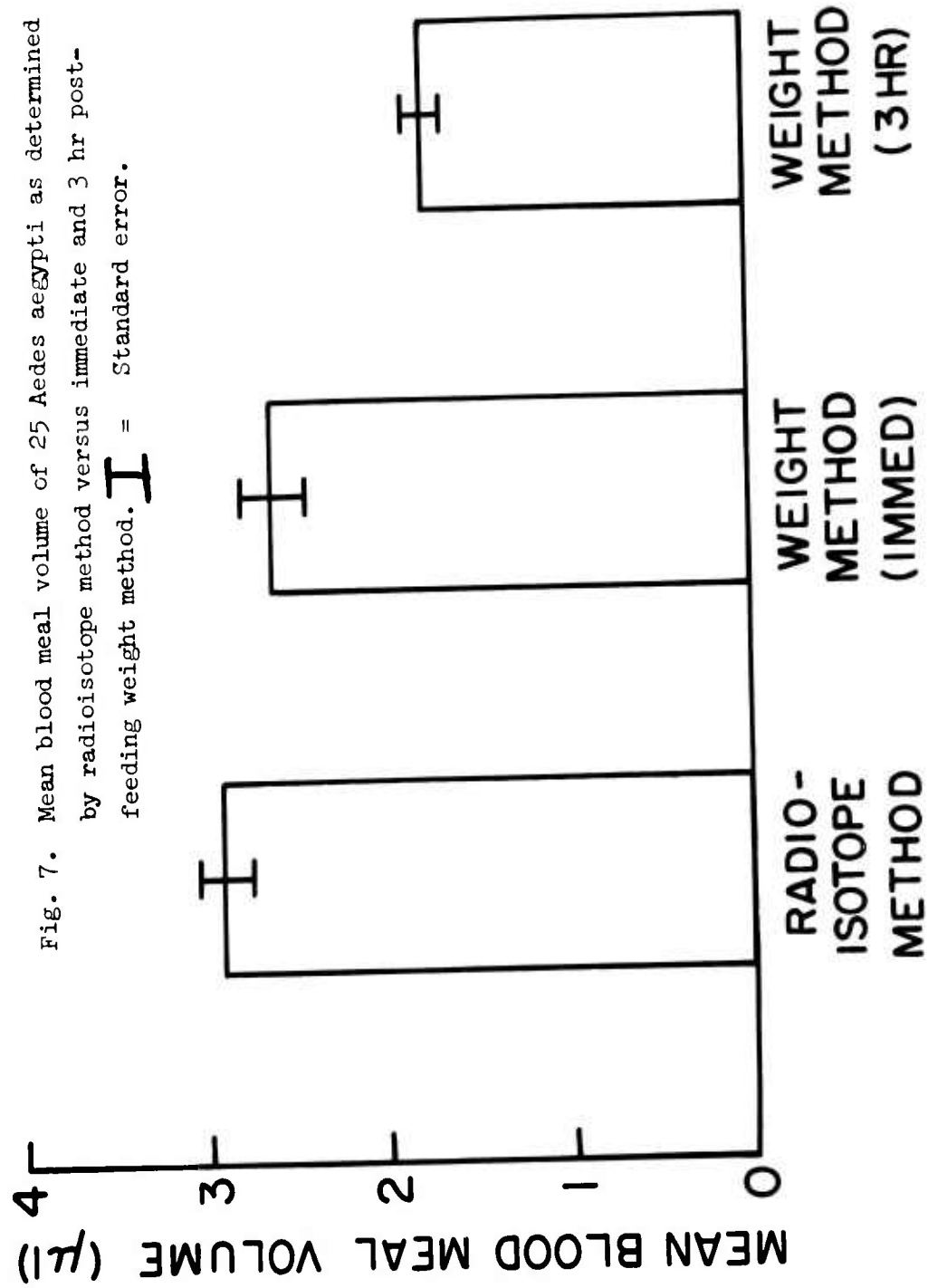
Radioisotope elimination by mosquito. Twelve females were membrane fed on  $^{144}\text{Ce}$ -labeled rhesus monkey blood as described above and then maintained individually in screened glass tubes with filter paper liners for 96 hr PF in the insectary. Ten per cent sucrose solution was provided ad lib. At 12 hr PF, the mosquitoes were lightly anesthetized with ether and transferred to clean tubes. The original tubes were monitored in the gamma counter for excreta radioactivity levels. This procedure was repeated at 24, 36, 48, 72, and 96 hr PF. Also at 96 hr, radioactivity levels were determined for each mosquito.

Radioisotope tagging characteristics in blood. One ml of heparinized rhesus monkey blood was labeled with 20  $\mu\text{l}$  (1 $\mu\text{Ci}$ ) of neutralized  $^{144}\text{Ce}$  with constant mixing. After 1 hr at room temperature, four 200  $\mu\text{l}$  aliquots of whole blood were centrifuged at 800 x G for 10 min, the plasma separated from the cells, and both phases counted from each sample. The cells from 2 of the samples were then each washed twice in equal volumes of normal saline, centrifuged, and the supernatant and cells counted separately.

When *A. aegypti* were allowed to feed on a dog, it was noted that they began to defecate numerous droplets of a clear fluid which continued for up to 3 hr PF. An initial experiment was conducted to determine if age or the ingestion of sucrose influenced the excretion of clear fluids by the mosquito. About 75% of the 7-day-old mosquitoes which were never starved, or which were starved for 1 day, fed well and excreted large amounts of clear fluid. Of those which were starved for 2 days, only ca. 25% fed; fluid excretion was only ca. half that of the first 2 groups. Less than 5% of those starved for 3 days survived and all these remaining failed to feed. Thirty per cent of the 2-day-old starved mosquitoes fed and excreted a significant amount of fluid comparable to that of the 7-day-old females which had been starved for 2 days.

When 16 seven-day-old mosquitoes which were starved for 1 day ingested  $^{144}\text{Ce}$ -labeled monkey blood, no significant amounts of radioisotope were passed in their excreta, either during feeding or up to 3 hr PF (Table VII). The excreta of mosquito #1 emitted 154 counts per 5 min which slightly exceeded the established baseline, but this was not considered to be of significance.

Blood meal volume in 25 individually fed mosquitoes was then estimated by the radioisotope versus the gravimetric blood (Fig. 7). Immediate and 3 hr PF weighings indicated mean blood volumes to be 2.63  $\mu\text{l}$  (90.4%),



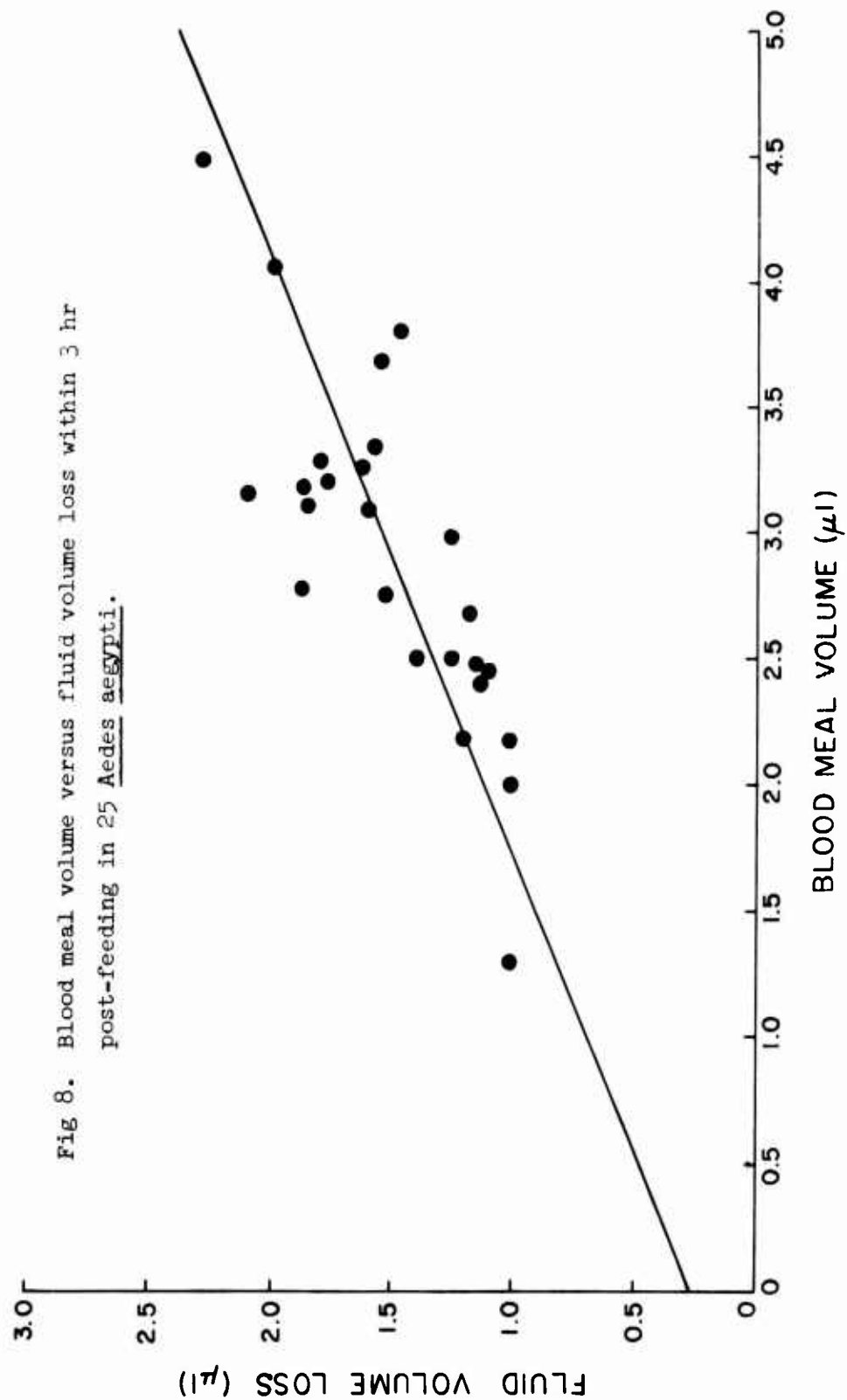
and 1.78  $\mu$ l (61.2%), respectively, compared to the mean estimate of 2.91  $\mu$ l as determined by the radioisotope technique. These results indicate an average fluid loss of 0.28  $\mu$ l immediately PF and a 1.13  $\mu$ l loss by 3 hr PF. Comparisons of blood meal volume estimates employing the rank test indicated significant differences between the 2 methods, both immediately and at 3 hr PF ( $P = 0.05$ ). However, analysis of the same data by the t-test indicated that the differences between the 2 methods were significant only at 3 hr PF ( $P = 0.05$ ). Comparisons of the variances between radioisotope estimates versus both immediate and 3 hr PF weight estimates indicated that in neither case was there a significant difference (F-test;  $P = 0.05$ ).

Blood meal volumes of the above 25 mosquitoes as determined by the radioisotope method were then correlated with fluid volume loss determined gravimetrically within 3 hr PF. Figure 8 indicates a close direct relationship ( $r = 0.804$ ).

The results of the experiment in which 12 females were individually fed on  $^{144}\text{Ce}$ -labeled monkey blood and their excreta monitored for radioactivity for 96 hr are shown in Figure 9. No radioisotope was passed prior to 36 hr PF. Between 36 and 48 hr PF, an average of 4.1% (range = 0.2 - 17.1%) of the  $^{144}\text{Ce}$  ingested was passed. The major portion of  $^{144}\text{Ce}$  was passed by 72 hr PF (95%; range = 83.5 - 99.2%); by 96 hr PF, 96.6% (range = 87.0 - 99.2%) of the radioisotope could be accounted for in the excreta. When the mosquitoes were monitored separately in the gamma counter at 96 hr PF, 8 of them contained no detectable  $^{144}\text{Ce}$  while the remaining 4 contained from 0.2 - 0.7% of the total amount of radioisotope ingested.

When 4 aliquots of  $^{144}\text{Ce}$ -labeled rhesus monkey blood were centrifuged and the radioactivity levels of the cells and plasma determined separately, an average of ca. 17% of the radioisotope remained with the cell fractions. The average radioactivity levels from 2 of these cell fractions dropped to 3% and then to 1.5% of the total after 2 saline washings.

The need for accurate determination of blood meal volume in mosquitoes has been clearly established. Several authors have related the amount of blood ingested to nutrition and fecundity; these subjects are thoroughly reviewed by Clements. The factor of blood meal volume as it relates to the epidemiology of various mosquito-transmitted disease agents also has been emphasized by numerous authors. Hovanitz, who worked with an *A. aegypti* - *Plasmodium gallinaceum* model, noted great variability in the amount of blood ingested by mosquitoes and concluded that this factor was responsible for a portion of the variations in infectivity of individual mosquitoes. Jeffrey maintained that accurate blood meal estimates were necessary to critically compare relative susceptibility among species of *Anopheles* to various strains of *Plasmodium*. He also felt that these data would aid in determining minimum infective gametocyte densities in the blood consumed. Kershaw et al. used blood meal volumes to compare the actual versus the expected



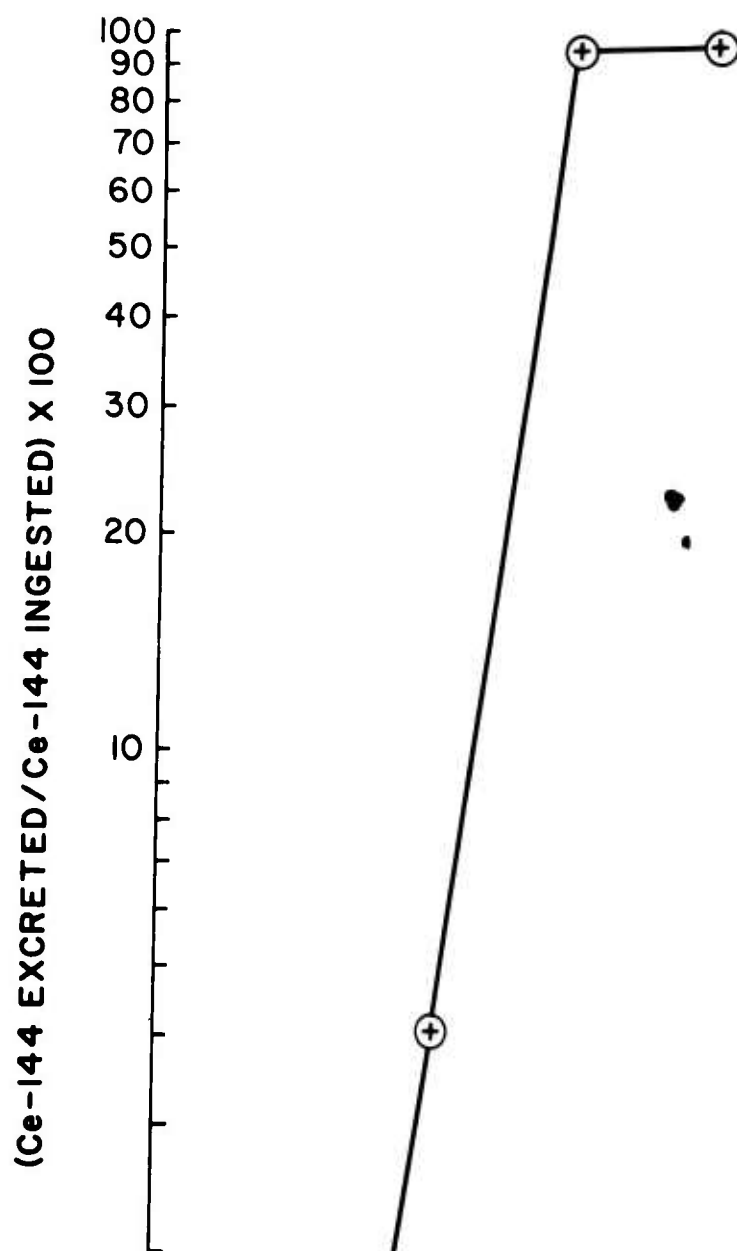
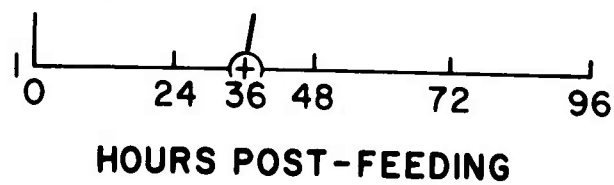


Fig. 9. Average cumulative excretion rate of  $^{144}\text{Ce}$  from 12 Aedes aegypti following membrane feeding on radioisotope-labeled blood.



uptake of microfilariae of Dirofilaria immitis by A. aegypti. Wharton performed similar vector efficiency experiments utilizing blood meal volumes in his study of Mansonia longipalpis infected with Brugia (Wuchereria) malayi. Smithburn and Haddow used blood meal volume in A. aegypti to evaluate the significance of low levels of yellow fever virus in the circulating blood of animals or man by determining the minimum virus titer likely to infect a biting mosquito. Yuill and Thompson, studying Cache Valley virus in 2 species of Aedes, concluded that variation in virus titers of individual mosquitoes or, in some cases, failure to infect, may have been due in part to differences in blood meal volumes.

Most of the studies to determine blood meal volume in Aedes aegypti by the gravimetric method do not mention the observation of clear fluid excretion related to feeding. Other workers mention the fact but either minimize its importance or offer no resolution to the problem.

The present study confirms the work of Boorman by demonstrating that blood meal volume of A. aegypti is underestimated by gravimetric means because the significant amount of clear excreta expelled both during and after feeding is not taken into account by weighing. Expulsion of excreta does not appear to be related to the age or prior nutritional conditioning of the mosquito. Because no significant amount of  $^{144}\text{Ce}$  is passed in the clear excreta but remains with the blood meal, the radioisotope method is considered to be more accurate than weighing. Furthermore, the longer the PF weighing is delayed, the more inaccurate the gravimetric estimate becomes. This study clearly demonstrates that mosquitoes which take in larger blood meals also excrete larger quantities of clear fluid. Thus, the magnitude of error by the weighing method is increased in these mosquitoes.

A significant difference exists between the average blood meal volume as calculated by Boorman using  $^{144}\text{Ce}$  compared to our average estimate, 4.21  $\mu\text{l}$  and 2.91  $\mu\text{l}$ , respectively. A difference in average mosquito size may be partially responsible but Boorman provides no data on unfed mosquito weights in his radioisotope experiment; thus, direct comparison with similar weights in the present study is not possible. A second factor to be considered is that Boorman fed his mosquitoes on a  $^{144}\text{Ce}$ -inoculated mouse with mosquitoes having unrestricted access to this animal for 30 min. Under these conditions, mosquitoes probably tended to engorge more fully than in the present study in which mosquitoes were individually placed in small glass tubes and fed from a membrane feeder. We have now exposed A. aegypti en masse to  $^{144}\text{Ce}$ -labeled blood using the membrane feeder and have found that individuals engorged an average of 3.24  $\mu\text{l}$  of blood (unpublished data). A third element to be taken into account concerns a flaw in the design of Boorman's uptake experiment. We have inoculated  $^{144}\text{Ce}$  intravenously into cats and have discovered that this radioisotope does not remain in the blood vascular system at a constant level as evidenced by decreasing amounts of radioactivity seen in venous blood samples taken sequentially during the

first hour after  $^{144}\text{Ce}$  inoculation (unpublished data). Boorman exposed a mouse to mosquitoes for 30 min beginning 10 min after radioisotope inoculation. He then took aliquots of heart blood for baseline gamma emission counts after the feeding period was completed. Thus, Boorman overestimated blood meal volumes since the engorged blood no doubt was more radioactive than the heart blood which he used as the standard. We have exposed mosquitoes en masse to a  $^{144}\text{Ce}$ -inoculated cat under rigidly controlled condition and have found that engorged individuals contained an average of 3.25  $\gamma\text{l}$  of  $^{144}\text{Ce}$ -labeled blood. Detailed results of our in vitro and in vivo feeding experiments will be discussed in a subsequent report.

The eventual excretion of  $^{144}\text{Ce}$  by A. aegypti appears to be closely associated with the color and presumably content of the mosquito excreta. The radioisotope is not present in the relatively light-colored excreta up to 36 hr PF, but only after this time when dark-colored protohaematin first appears. By 96 hr PF when mosquitoes exhibit little or no radioactivity, neither their abdomens nor their excreta appear to contain any haematin pigment.

The radioisotope estimation of blood meal volume is not only more accurate but also is more convenient than that of weighing. The laborious task of individually separating and then weighing mosquitoes before and after feeding is eliminated. Females can be fed en masse and then individually monitored for radioactivity levels anytime up to 36 hr PF with no loss in accuracy. Furthermore, once mosquito abdomens appear cleared of blood at approximately 4 days PF, radioisotope handling precautions are no longer necessary and mosquitoes may be managed in a normal manner.

Estimation of blood meal volume in a mosquito species using  $^{59}\text{Fe}$  also has been reported. Using a Culex pipiens fatigans model, these workers also found that radioisotope estimation of blood meal volume was a simpler and more accurate method than that of weighing. A number of significant differences exist between  $^{144}\text{Ce}$  and  $^{59}\text{Fe}$ : 1) the highest energy peak of  $^{144}\text{Ce}$  is only about one-tenth that of  $^{59}\text{Fe}$ ; 2)  $^{144}\text{Ce}$  does not tag red blood cells as does  $^{59}\text{Fe}$ ; and 3)  $^{144}\text{Ce}$  is entirely eliminated by the mosquito with the blood meal excreta while  $^{59}\text{Fe}$  is incorporated into the body of the mosquito making it permanently radioactive.

Regardless of the radioisotope chosen, this technique offers the investigator a more accurate and simple method than weighing for the determination of blood meal volume in A. aegypti and presumably in other mosquito species having similar excretion behavior.

#### 6. Mesocestoides in the baboon and its development in laboratory animals.

Three of 35 African baboons (Papio sp.) which had been imported for research purposes at this Institute were found to be harboring infections of tetrathyridia larvae of the cestoe genus Mesocestoides. Although such

primate infections are not unknown, the definitive experimental development to adults from larvae obtained from baboons has not been successfully undertaken. In view of the extensive use of wild-captured primates as biomedical research models, additional information is needed on the biology and effects of naturally acquired infections on the laboratory hosts. We have investigated the adult development of Mesocestoides in laboratory animal feeding experiments.

The subject baboons (all young adult males) had been wild-captured and imported from East Africa (Tanzania/Kenya border area) at different times but by the same supplier. Baboon No. 1 had been in captivity for several months and appeared to be in good health. During a routine surgical laparotomy numerous tetrathyridia were found distributed free throughout the abdominal cavity and encapsulated on internal organs. Some of these were referred to one of us for diagnosis. Before identification was completed, however, the incision was closed and the animal was returned to his cage. We diagnosed the specimens as cestode larvae and referred them to Dr. R. E. Kuntz, parasitologist, Southwest Foundation for Research and Education, who further confirmed our identification as Mesocestoides tetrathyridia. The baboon was maintained in our facilities until it was sacrificed 3 months later. It was not used for any experimental protocols during that time, partially because of the uncertainty of the effects that the cestode infection might have had upon experimental results.

Baboon No. 2 had been only recently imported almost a year after Baboon No. 1. Routine screening for tuberculosis indicated positive skin tests and the animal was euthanized and autopsied. Baboon No. 3 exhibited tetrathyridia during a routine autopsy, but none were collected for feeding experiments.

Tetrathyridia were recovered during autopsy from the baboon hosts, placed into physiological saline and counted. At least 20% of the individual specimens were fixed in hot AFA (alcohol-formalin-acetic acid), and preserved in 70% ethanol for histological and whole mount preparations. The remaining specimens were maintained in saline preparatory to immediate feeding to laboratory hosts. Baboon tissues were collected in formalin for histopathologic examination.

Feeding experiments similar to those of Witenberg were carried out on laboratory reared dogs, cats, rats and mice. Preliminary fecal examinations were done on the dogs and cats to detect pre-existing parasite infections. All of the animals appeared to be healthy. Dogs and cats (except Dog No. 3) were starved for 24 hours prior to baboon autopsy and were fed larvae immediately thereafter. Feeding was accomplished by depositing a counted number of larvae in the center of a portion of canned dog food and presenting this to the dog or cat. Each animal was observed to insure complete ingestion. Rats and mice were exposed by manually forcing a counted number of larvae into the esophagus.



Table VIII. Feeding experiments of Mesocestoides sp. tetrathyridia to laboratory hosts

Tetrathyridia host	Laboratory feeding host	Number of tetrathyridia administered	Number of adult cestodes recovered <sup>1</sup>	Age of infection (days)
Baboon 1 (>500 larvae)	Dog 1	50	17 (34%)	72
	Dog 2	50	27 (54%)	90
	Cat 1	50	9 (18%)	72
	Cat 2 <sup>2</sup>	50	10 (20%)	90
	Rat 1	50	None	89
	Rats 2-10	20 each	None	3-89
Baboon 2 (103 larvae)	Dog 3	80	65 (81%)	15
Cat 2 <sup>2</sup> (5 larvae)	Mouse 1	1	None	42
	Mouse 2	4	None	42

<sup>1</sup>Minimum possible worm recovery as determined by number of scoleces removed.

<sup>2</sup>One encapsulated and 4 unencapsulated larvae obtained from Cat 2.

Two dogs and 2 cats were each fed 50 larvae from Baboon No. 1. Weekly fecal exams were conducted beginning 21 days post-exposure. When no proglottids were detected by direct examination of normally deposited feces, we obtained fecal material from dogs by rectal probe. Dog No. 1 and cat No. 1 were sacrificed and autopsied on day 66 post-exposure. Dog No. 2 and cat No. 2 were sacrificed on day 90. Individuals from the rat group were sacrificed on days 3, 7, 14, 21, 29, 35, and 42, 49, and 89 respectively. The single dog (dog No. 2) fed 80 larvae from Baboon No. 2 was sacrificed on day 31, after presenting proglottid-positive feces on day 14.

Dogs and cats were euthanized by an intravenous injection of sodium pentobarbitol and were immediately autopsied. The internal organs were examined for the presence of tetrathyridia, followed by removal and opening of the intestine. Attempts to remove adult cestodes immediately resulted in excessive breakage and the best worm recoveries were accomplished by relaxing the worms overnight in cold (4°C) saline. The relaxed cestodes were removed, counted, measured, fixed in hot AFA and preserved in 70% ethanol. Worm recovery was determined by the numbers of scoleces obtained.

Host tissues were subjected to routine histopathologic procedures and stained with H&E. Parasite materials (larvae and mature worms) were processed and stained as whole mounts (Semichon's aceto-carmin) or as serial sections (H&E). Parasite material from dogs and cats were fixed in cacodylate buffered paraformaldehyde (pH 7.37) for 2 1/2 hours and forwarded to Dr. J. E. Ubelaker for ultrastructural level examination.

Parasite burdens and feeding protocols are summarized in Table VIII. Tissue sections of the larvae revealed the unarmed scolex bearing 4 well developed suckers typical of Mesocestoides tetrathyridia (Figs. 10, 11, 12). The majority of the tetrathyridia found in the baboons and in one cat were free and apparently causing no pathological changes. There was a significant concentration of organisms in the pelvic cavities of the baboons, with some located within the scrotum. A few tetrathyridia were encapsulated in the mesentery and the connective tissues separating the lobules of the seminal vesicles of the baboons (Fig. 13). A single encapsulated organism was observed in the diaphragm of Baboon No. 1. These forms were enclosed within a connective tissue capsule with a minimum of inflammatory cell accumulation.

Feeding experiments produced variable results depending upon the experimental host used. All rodent hosts were negative for larval or adult cestodes 89 days post-exposure. The 3 dogs served as excellent hosts for adult cestode development and only mature worms were recovered from each animal. All worms were attached within a 60 cm length of the small intestine approximately 60 cm from the stomach. Gravid proglottids were distributed throughout the colon and this indication of patent infection was evidenced as early as 14 days post-exposure. Adult tapeworms were well tolerated by the hosts and no gross lesions were observed. A very mild focal inflammatory reaction was present in the area of the scolex

Table IX. Measurements of adult Mesocestoides sp. from experimentally infected dogs

	Specimen treatment	Number of measurements	Measurement	
			Range	Mean
Worm length	Living, relaxed in cold saline	40	25-85	48.6
Scolex diameter	AFA fixed	9	280-390	332
Sucker diameter	AFA fixed	30	150-230	182
Mature proglottid	AFA fixed	30	1.4-2.1 x 1.9-2.3	1.8 x 2.1
Testes number	AFA fixed	58	41-71	52
Cirrus pouch	AFA fixed	100	90-210	153
Gravid proglottid	AFA fixed	23	2.7-32 x 1.0-1.4	3.0 x 1.2
Paruterine organ	AFA fixed	30	600-720 x 408-528	691 x 487
Oncosphere	AFA fixed	75	16-30 x 10-16	22 x 18
Oncosphere	Living, saline	50	24-30 x 18-28	27 x 21

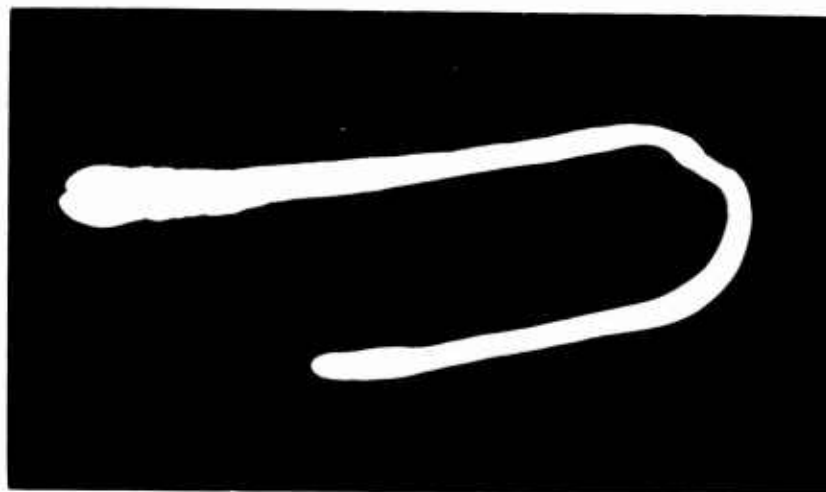


Fig. 10. Tetrathyridium (AFA-fixed) from the abdominal cavity of Baboon No. 1. X 6.



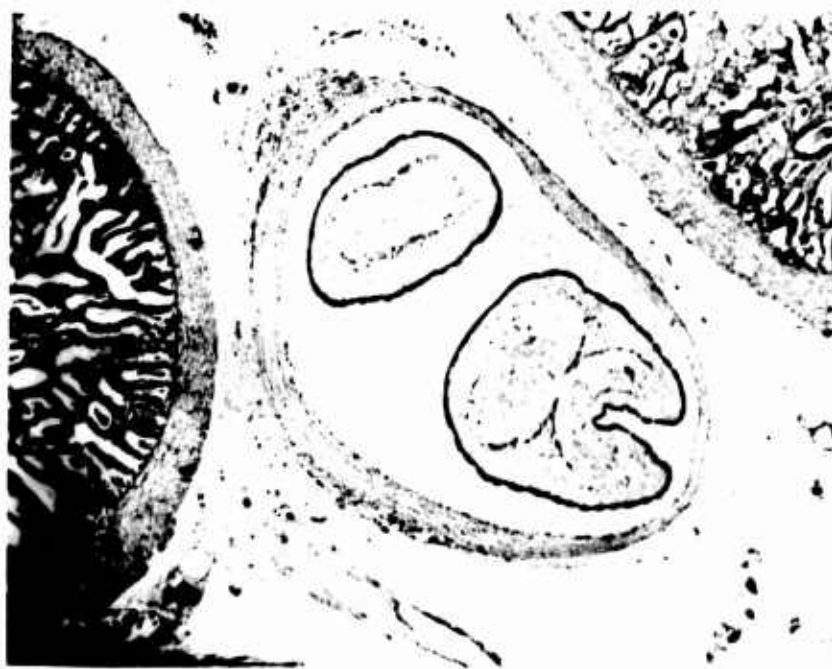
Fig. 11. Section of a Tetrathyridium showing invaginated scolex (arrow). H&E. X 52.

Legend for Figures

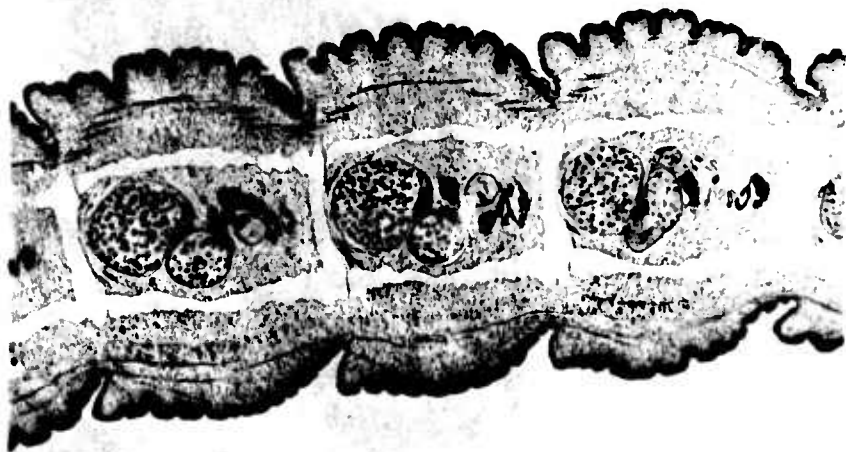
- Fig. 12. Enlargement of the scolex in Fig. 11. H&E. X 325.
- Fig. 13. Encapsulated tetrathyridium in the connective tissues separating the lobules of the seminal vesicle of Baboon No. 2. H&E. X 375.
- Fig. 14. Sections of the mature proglottids of Mesocestoides sp. recovered from Dog No. 1. H&E. x 44.
- Fig. 15. Gravid proglottid of Mesocestoides sp. Semichon's aceto-carmin. X 38.



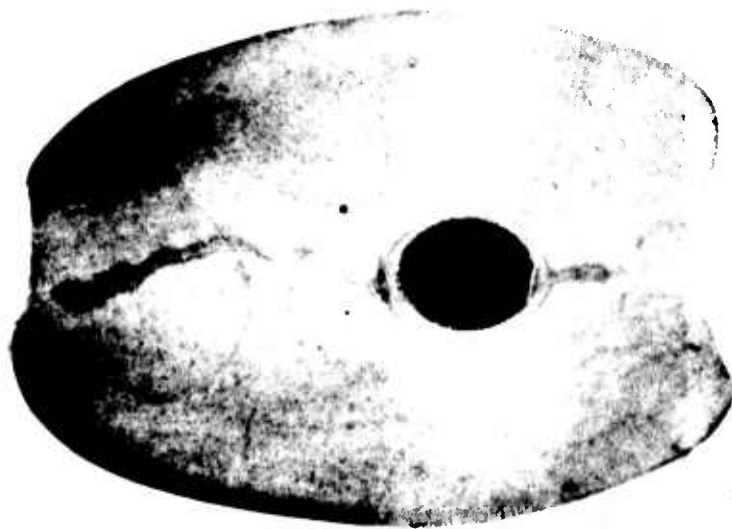
*Fig. 12*



*Fig. 13*



*Fig. 14*



*Fig. 15*

attachment to the intestinal epithelium.

Cats were relatively poor hosts as evidenced by the low numbers and poor conditions of recovered worms. These cestodes were considerably smaller than those recovered from dogs. They tended to fragment easily following overnight refrigerated incubation and manual disturbance. Only 3 intact worms were eventually recovered from the 2 cats. Gravid proglottids were produced, however, and active oncospheres were obtainable from disrupted paruterine organs. Cat No. 2 also harbored 5 tetrathyridia in the abdominal cavity.

The morphology of adult worms (Figs. 14 and 15) was consistent with the described criteria for the genus Mesocestoides. Species identification was complicated, however, by overlapping characteristics of M. lineatus and M. caestus. Table IX summarizes the pertinent measurements of our specimens from dogs. Measurements from cat specimens were not noticeably different from those of dogs except that the three intact worms were only 11 cm, 15 cm and 18 cm long respectively. There appear to be no morphologic differences on an ultrastructural level between parasites from dogs and cats. Serious questions have been raised concerning the validity of species within the European and African groups of the genus (see below) and we prefer to designate our material simply as Mesocestoides sp.

The cestode genus Mesocestoides has long been recognized as a ubiquitous group occurring in larval or adult forms in a large variety of vertebrate hosts. This fact contributes to the present confusion in taxonomy of the group and is compounded by the lack of complete biological life cycle evidence, leading to dependence upon often vague morphological criteria in the establishment of new taxa.

Our experimental results are in agreement with those of Witenberg and emphasize the problems of species identification of worms derived from the same larval populations which vary in development in different definitive host species. Witenberg's tetrathyridia collections from Palestine were from lizards, cats, hedgehogs, a jackal and a fox. It is interesting to note that, despite the variety of larval hosts, adult worm development followed essentially the same pattern within the same experimental host species. In all cases, as in our experiments, the dog was extremely susceptible to developing a patent infection and the cat was only partially susceptible.

Myers and Kuntz list only 2 cases of tetrathyridia in Papio spp. and in neither case had the adult form been characterized by feeding experiments. The occurrence of these larvae in other primates has been reported and reviewed, as has the pathology of the infection in monkeys. Concurrently, wild and domestic canines have been reported frequently as harboring naturally acquired adult worm infections. One might speculate, therefore, that the definitive hosts for our baboon parasites could reasonably be a wild canine carnivore common to the natural habitat of the baboon, such as the hyena or the wild hunting dog



of Africa. The occurrence of this infection in 3 different baboons trapped on 3 different occasions supports our belief that the infections were naturally acquired rather than developed in captivity and that the incidence of natural infection in baboons may be higher than previously implied. The possibility that wild captured primates might possess such infections should be carefully considered before using them for biomedical research. Additionally, our experience with Baboon No. 2, in which tetrathyridia were found only in the lower pelvic cavity and the scrotum, should alert the worker to the highly localized nature of some infections and to the possibility that a diffuse, easily discernible distribution of parasites will not always occur.

7. The cellular and humoral immune response to *Schistosoma mansoni* infections in inbred rats

Previous studies have shown that augmented resistance to schistosomiasis *mansoni* can be specifically induced through exposure to parasites or parasite antigens, but research has been hampered by a difficulty in developing suitable models for experimentation. In many ways the inbred rat represents an ideal animal for experimentation as it is "naturally" resistant to the development of a *Schistosoma mansoni* infection. In addition, the biology of schistosomiasis has been extensively studied in rats. Present studies describe the response of Fischer rats to initial exposure to *S. mansoni* and define the optimal conditions for reproducible infections, including the effects of age susceptibility and magnitude of exposure on subsequent worm burden. These biologic parameters are then related to the kinetic development of host cellular and humoral immune mechanisms of defense.

Unless otherwise noted 4.5- to 5-wk-old (40-50 g) male Charles River Fischer inbred rats<sup>5</sup> (CDF) were used in this study. Animals tolerated an exposure of up to  $10 \times 10^3$  *S. mansoni* cercariae without any demonstrable ill effects. A total of 3070 animals were utilized.

The Puerto Rican strain of *S. mansoni* was maintained as previously described. Life cycle support hosts were albino strain *Biomphalaria glabrata* snails and Walter Reed strain albino mice. Cercariae were shed from infected snails by exposure to light and rats were exposed by tail immersion and penetration by these cercariae within 90 minutes of photic release. Cercarial viability was judged by motility and penetration was judged by counting residual tailless forms following exposure. Both criteria were consistently met with a frequency of greater than 95%.

Animals were killed by the IP injection of 600 mg of sodium barbital and 300 units of sodium heparin/kg of body weight. This procedure afforded both the required anticoagulation and the narcosis which facilitated the release of the schistosomes from the endothelium. The mesenteric and hepatic vessels were then perfused with 0.85% saline via aortic cannulation. The worms were collected on a filter paper disk via a suction apparatus and were counted after staining with Lugol's iodine.

Cell collections. Animals were killed by intracardiac exsanguination. The spleens, lymph nodes, and thymuses were carefully excised free of fatty tissue, decapsulated, and minced. The cells were pressed through a no. 60 mesh stainless steel screen and prepared as previously described. Media consisted of RPMI-1640 plus 1% syngeneic normal rat serum, and 0.005 M "Hepes" buffer. After settling, the cells were washed twice in preparation for injection or subpopulation fractionation. Bone marrow cells were prepared by perfusion of femori and humori, similarly sedimented, passed through a no. 26 gauge needle and washed in media. Peritoneal exudate cells were harvested by peritoneal lavage with iced, heparinized, L-15 Leibovitz media 3 days following an intraperitoneal injection of 25 ml light mineral oil. The cells were washed twice in media in preparation for fractionation.

Adherence fractionation. Nonadherent and adherent cell populations were prepared via incubation on and elution from glass bead columns as previously described.

Antibody fractionation. The nonadherent lymphoid cells ( $5 \times 10^7/\text{ml}$ ) were incubated in neat rabbit anti-rat gamma globulin anti-serum or rat anti-rat thymic-cell sera (kindly provided by Dr. David Luberoft, University of Iowa) at  $4^\circ\text{C}$  for 30 minutes and then at room temperature for 15 minutes. Cells were diluted to 40 times the original volume, centrifuged, and resuspended in fresh guinea pig serum (previously adsorbed with agarose and rat thymocytes). They were then diluted 1 to 3 and incubated an additional 45 minutes at room temperature in the presence of  $2 \mu\text{g}$  DNase/ml. The cells were washed and injected intravenously into rats 4 hours before exposure of these animals to schistosome cercariae. Direct cytotoxicity was determined by specific  $^{51}\text{Cr}$  release, using aliquots of cells labeled with  $^{51}\text{Na}$  chromate, as previously described.

Serum collections. Animals were exsanguinated by cardiac puncture. Blood was allowed to clot at  $37^\circ\text{C}$  for 30 minutes and retract at  $4^\circ\text{C}$  for 4 hours and centrifuged at 15,000 rpm for 20 minutes. The sera were stored at  $-70^\circ\text{C}$  until use. Immediately before intravenous injection into recipient animals all sera were again centrifuged and sterilized by filtration ( $0.22 \mu\text{m}$  Millipore).

Serum fractionation. 1. Ammonium sulfate precipitation: Serum was sequentially precipitated with ammonium sulfate, using 50, 40, 35% saturations (30). The final precipitate was resuspended in and dialyzed against PBS (pH 7.2).

1. Gel filtration. Three milliliter aliquots of rat serum were applied to a Sephadex G-200 (Pharmacia) column ( $90 \times 2.5 \text{ cm}$ ). The eluting buffer was 0.02 M Tris HCl-NaCl (pH 8.3) and the flow rate was 4 ml/hr. Three milliliter samples were collected and their absorbance at 280 nm determined. Each serum yielded three individual protein peaks which were, respectively, pooled, equilibrated by dialysis against PBS (pH 7.2), and concentrated by vacuum filtration.

2. QAE chromatography: QAE-Sephadex A-50 (Pharmacia) was swollen and equilibrated with ethylenediamine acetic acid (ionic strength 0.1, pH 7.0) according to the method of Joustra. Six milliliters of rat serum, previously equilibrated with the same buffer, were applied to a QAE-Sephadex column (11 x 1.5 cm), with an elution rate of 8 ml/hr. A single protein peak was obtained which by immunoelectrophoretic criteria was pure IgG.

3. Immunoabsorbent chromatography: Sieved G-200 Sephadex (Pharmacia) was swollen and equilibrated with 0.2 N NaOH (pH 10.3) in the presence of cyanogen bromide (1 mg/10 mg dry Sephadex) for 10 minutes, followed by extensive washing in Borate Buffered Saline (BBS pH 8.3). Coupling of ammonium sulfate precipitated goat-anti-rat IgG (10 mg/ml) in 0.54 M NaCl bicarbonate buffer) was accomplished over a 4-hours period and terminated by washing with BBS and the addition of ethanolamine (1 M). Six milliliters of rat serum were passed sequentially over BBS washed columns (11 x 1.5 cm) with an elution rate of 8 ml/hr until the eluate, when concentrated to 6 ml by vacuum ultrafiltration, was free of IgG by immunoelectrophoretic criteria.

All samples were concentrated to their respective original whole serum volume and stored at -70°C. They were filtered immediately before intravenous injection.

Indirect hemagglutination. Sheep red blood cells initially were fixed in HBS and 1% glutaraldehyde. After washing in PBS, graded amounts of schistosome antigen (34) were added with coupling accomplished at 37°C for 15 minutes in the presence of tannic acid. All anti-sera were adsorbed in equal volumes of 30% washed fresh sheep red cell suspensions before dilution, and heat inactivated at 56°C for 30 minutes before testing. Isotonic PBS contained 1% normal rabbit serum which was previously heat inactivated and adsorbed with sheep red blood cells. Fifty microliters of the cell-antigen mixture were added to 50 µl of serum in the NRS-PBS. The plates were incubated at room temperature for 4 hours, subsequently in the cold for 12 hours and then observed for hemagglutination.

Complement fixation. Complement fixing activity was determined as previously described. All tests included antigen block titrations and used triethanolamine buffered salt solutions containing optimal concentrations of magnesium and calcium. Complement was reconstituted from lyophilized guinea pig serum and was standardized immediately before use.

Fluorescence microscopy. Adult worms from rats or mice were washed three times, incubated at room temperature for 30 minutes in rat antiserum in varying dilutions, washed in PBS, and incubated with fluorescein-conjugated rabbit anti-rat IgG. After three washings in PBS, binding was determined using a Zeiss dark field fluorescence microscope. Specificity was established through inhibition of specific binding by serum preadsorption with schistosome antigen and the use of fluoresceinated anti-rat C3, albumin and fibrinogen.

## A. Kinetic studies on *Schistosoma mansoni* infection in the inbred rat

### 1. Effect of age of animal at exposure upon susceptibility to schistosome infection.

Animals of varying ages (1-18 weeks of age) were exposed to 1000 *S. mansoni* cercariae and the number of recoverable worms were determined as function of time (Fig. 16). Worms could be recovered by perfusion as early as 6 days after initial exposure with peak worm burdens occurring 3-4 weeks after exposure. Thereafter, there occurred a rapid decrease in worm burden to very low levels by 6-8 weeks; however, the worms were not completely eliminated. Fifteen of 16 animals when perfused 6 months after exposure and 13 of 14 animals when perfused 12 months after exposure still contained active residual worms ( $11 \pm 3.1$  and  $5.2 \pm 2.6$ , respectively). As can also be seen, the maximum numbers of worms were recovered earlier

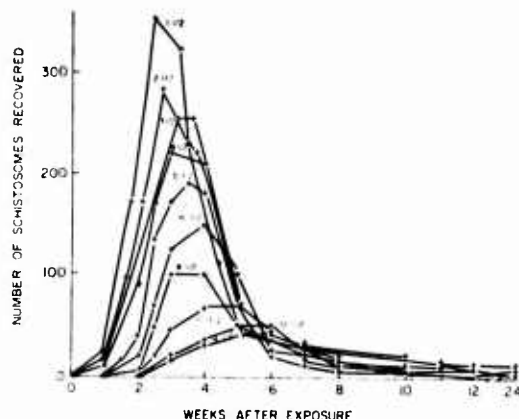


FIG. 16 Effect of animal age at exposure on subsequent schistosome worm burden: Fischer rats varying from 1.5 to 14.5 wk of age were exposed to 1000 *S. mansoni* cercariae. Following exposure, animals were sacrificed and perfused at intervals from 1 to 24 wk to determine the number of surviving schistosomes. Worms were recovered within 1 wk of exposure. Maximum recovery occurred 2-6 wk after exposure, depending upon the age of the animals at the time of exposure. Each point represents the mean of determinations on a minimum of six individual animal determinations.

from younger animals. In addition, the total number of worms recovered and the rate of subsequent elimination of worm burden was greater in the younger animals. This age dependence is further delineated when the number of worms recovered at the time of peak burden is plotted against the age of the animal at exposure (Fig. 17).

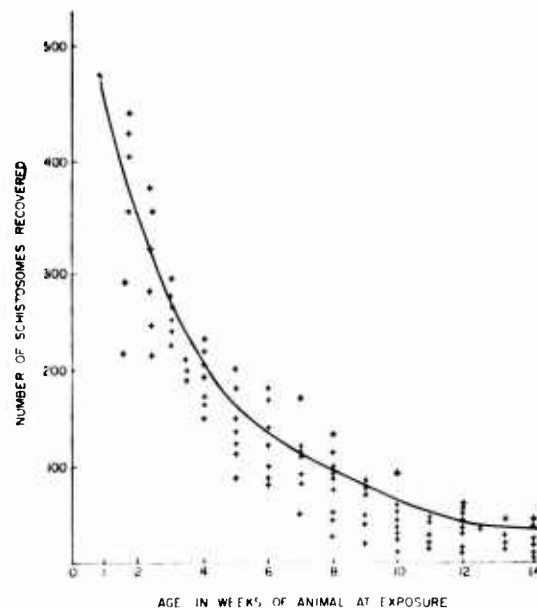


FIG. 17. Effect of animal age at exposure upon subsequent schistosome worm burden: Fischer rats varying from 1.5 to 14.5 wk of age were exposed to 1000 *S. mansoni* cercariae. The number of schistosomes found at the time of maximum recovery was plotted against the age of the animal at exposure.

## 2. Effect of the magnitude of exposure upon subsequent worm burden.

An analysis of the percentages of recoverable worms 6 wk after initial rat exposure (well after peak worm burden) relative to varying numbers of cercariae indicated that the greater the number of cercariae, the less the relative number of surviving worms found (Fig. 18). This dose effect was not reflected in the time or rate of initial appearance of peak worm burden.

As a result of these initial observations, all quantitative assays were performed using 4.5-week-old (50 g) rats exposed to 1000 cercariae and perfused 2.5 weeks after exposure. Under these conditions cumulative recoverable worm burdens were increasing, being approximately 70% of the projected maximum burden. This model afforded maximum discrimination between experimental groups with the parasite burden changing rapidly with time in a manner analogous to the response obtained at the midpoint of a sigmoid shaped titration curve. Additional studies analyzed the number of parasites at peak burden or the time of peak burden as well as "total parasite burden," determined by integration of the plot of "worm burden" vs "time after exposure" curves. No change was observed in the trend of the results using these latter methods of analysis.

B. Immunologic studies of *Schistosoma mansoni* infection in the inbred rat.

1. Studies of cellular immunity.

Animals exposed to *S. mansoni* cercariae at weekly intervals were killed, and their worm burdens were determined. Various cell populations, obtained from these animals, were injected into syngeneic recipients, followed by exposure of these recipient animals to cercariae. Figure 19 summarizes the results of three experiments utilizing  $2 \times 10^8$  viable splenic mononuclear cells per recipient. Protection was observed when cells were obtained from animals exposed to cercariae 3-4 weeks earlier. Figure 20 illustrates the effect of spleen, lymph node, peritoneal exudate, and thymus cells upon the number of recovered parasites. Only peritoneal exudate and spleen cells were protective in the experiment shown; preliminary studies have also demonstrated protection using lymph node cells specifically obtained from regional nodes draining the tail. Protection was directly related to the number of cells transferred. (Figure 21).

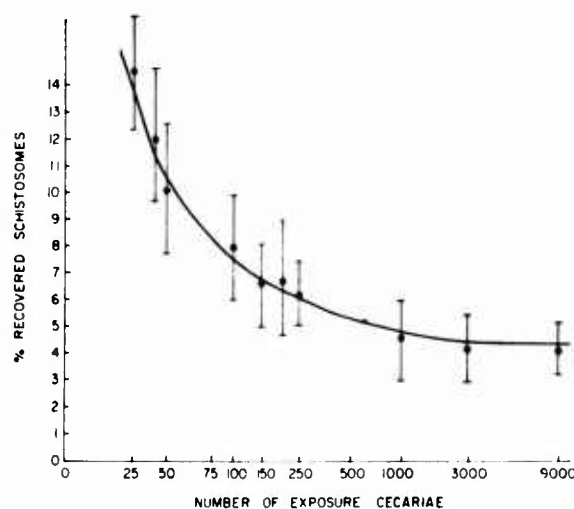


FIG. 18. Effect of exposure magnitude upon subsequent schistosome worm burden: 4.5-wk-old Fischer rats were exposed to varying numbers of *S. mansoni* cercariae. Six weeks after exposure, the animals were sacrificed and worm burden determined. Results are expressed as the percent of worms recovered. Vertical isobars represent 95% confidence interval for each group mean ( $N \geq 8$ ).

Further studies were performed to determine the properties of those cells responsible for protection in the cell transfer experiments. Table X demonstrates the protection afforded by adherent and nonadherent cell fractions of the peritoneal exudate. The majority of the protection was found with the nonadherent population, which was consistently found to be greater than 95% lymphocytes; the adherent population was less than 5% lymphocytic in morphology. A portion of the protection may be attributable to the adherent population, but appears at least partially

nonspecific since a slight protective effect was also noted with the adherent peritoneal exudate cell from unsensitized animals. Although this protective effect was not significant in the experiment shown, the trend was confirmed in four additional experiments, two of which demonstrates statistically significant ( $P < 0.05$ ) differences.

The nonadherent population provided specific protection which evolved with time in a manner analogous to the timing demonstrated by the unfractionated cell components (Table XI). Adherent cells showed a fixed nonspecific protective effect, causing a mean reduction of worms of 19%, regardless of exposure history of the host. This adherent population provided additional protection 2-3 weeks after exposure of cell donors to *S. mansoni*, indicating a specific protective effect as well. Additional evidence for a possible nonspecific component of protection was provided by the injection of 0.1 ml of complete Freund's adjuvant or PBS into each footpad and six additional subcutaneous sites. Animals were exposed to cercariae simultaneously and perfused 3.5 weeks later. Table XII demonstrates that animals exposed to adjuvant had a significantly lower

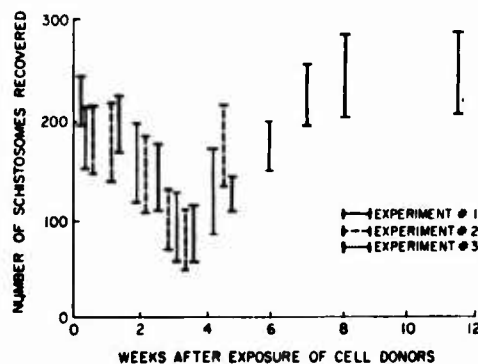


FIG. 19 Effect of spleen cell transfer on subsequent schistosome worm burden: 4.5-wk-old Fischer rats were exposed to 1000 *S. mansoni* cercariae and sacrificed at weekly intervals as cell donors.  $2 \times 10^6$  spleen cells were injected intravenously into 4.5-wk-old Fischer rats 4 hr before their subsequent exposure to 1000 *S. mansoni* cercariae. The worm burdens of these cell transfer recipients were determined 17-days after exposure. Three separate experiments are shown.





FIG 2. Effect of cell transfer upon subsequent schistosome worm burden; 4.5-wk-old Fischer rats were exposed to 1000 *S. mansoni* cercariae; 3.5 wk after exposure, they were sacrificed as cell donors. Peritoneal exudates were induced by the injection of 25 ml of light mineral oil 3 days before sacrifice. These animals were not used as sources of spleen, lymph node, or thymus cells.  $2 \times 10^6$  cells were injected intravenously into 4.5-wk-old Fischer rats 4 hr before their subsequent exposure to 1000 *S. mansoni* cercariae. The worm burdens of these cell transfer recipients were determined 17 days after exposure. Each point represents the mean of the value obtained from a minimum of eight individual animal determinations.

However, peritoneal exudate cells obtained from animals exposed to CFA were not significantly more effective at transferring immunity when compared to cells obtained from animals injected with PBS, suggesting that CFA was able to increase resistance, perhaps through nonimmunologic means. However, when cells obtained from animals who received both CFA and initial exposure to worms were used, an augmented protection was observed. These results suggest a specific adjuvant effect, probably mediated through immunologic mechanisms.

Further studies on the nature of the protecting cells are demonstrated in Table XIII. The specific protection was mediated by a population of cells which was highly susceptible to exposure to anti-thymocyte serum and complement. Conversely, exposure of these cells to anti- $\gamma$ -globulin anti-serum had little effect on their ability to transfer resistance. Also shown in this table is the relative susceptibility of the cells to lysis as assessed by chromium release. These latter studies, performed in parallel on aliquots of similarly treated cells, indicate that cell populations appear to be nearly mutually exclusive as demonstrated by the nearly additive cytotoxicity of the two antibodies when used sequentially on the same cell population; however, only that subpopulation which was susceptible to anti-thymocyte serum appeared to be specifically implicated in the protective assay.



TABLE X

## ADHERENCE CHARACTERISTICS OF ADOPTIVELY TRANSFERRED PERITONEAL CELLS

Cell population	No. worms recovered $\pm$ SEM <sup>d</sup>	$\Delta^c$	P <sup>f</sup>
Exposed <sup>a</sup>			
Unfractionated	62 $\pm$ 14	-115	<0.005
Nonadherent	83 $\pm$ 19	-93	<0.01
Adherent	117 $\pm$ 21	-60	<0.05
Unexposed <sup>b</sup>			
Unfractionated	141 $\pm$ 29	-36	>0.05
Nonadherent	169 $\pm$ 32	-8	>0.05
Adherent	139 $\pm$ 38	-38	>0.05
None <sup>c</sup>	177 $\pm$ 28	—	—

<sup>a</sup> Cells obtained from animals exposed to 1000 *Schistosoma mansoni* cercariae 3.5 week before sacrifice.  $2 \times 10^6$  cells were injected iv 4 hr before recipient exposure to schistosome cercaria.

<sup>b</sup> Cells obtained from animals not exposed to *Schistosoma mansoni* cercariae. Cell injections as above.

<sup>c</sup> Animals injected with media alone.

<sup>d</sup> SEM = Standard error of the mean.

<sup>e</sup> Change in number of recovered worms vs control (c).

<sup>f</sup> P value experimental group vs control (c) (Student's *t* test).

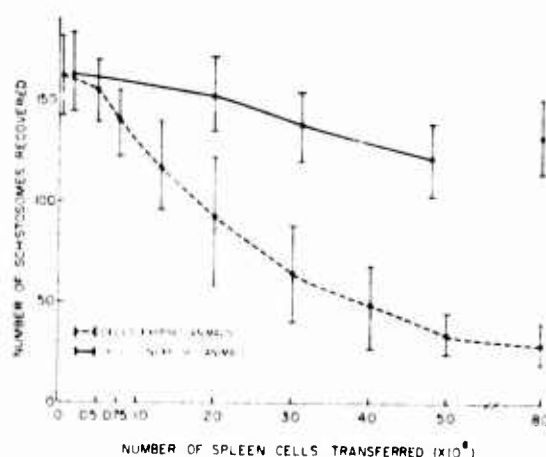


FIG. 21 Effect of cell transfer upon subsequent schistosome worm burden: 4.5-wk-old Fischer rats were exposed to 1000 *S. mansoni* cercariae. These animals were sacrificed 3.5 wk after exposure and served as spleen cell donors.  $0.5 \times 10^6$ – $8 \times 10^6$  cells were injected intravenously into 4.5-wk-old syngeneic Fischer rats 4 hr before their exposure to 1000 *S. mansoni* cercariae. The worm burdens of these cell transfer recipients were determined 17 days after exposure.

## 2. Studies of humoral immunity.

Serum injected into appropriate recipients also affected the subsequent worm burden. The maximum protective effect, however, was not noted until the sixth to seventh week following initial infection of the serum donors (Fig. 22), at which time the major parasite burden had already markedly diminished. The effect of serum upon protection was, however, quite complex and highly dependent upon the amount and source of serum used (Fig. 23). When serum from an animal that had been exposed to *S. mansoni* cercariae 3-4 weeks previously was used, moderate amounts (approximately the calculated serum volume of the recipient per injection) actually led to an increase in or enhancement of the number of recoverable parasites, whereas larger amounts led to a small degree of host protection. Significant enhancing activity was observed in four of eight experiments with a mean increase in the number of recovered worms of 57%. In addition, when this serum was administered simultaneously with cells (Table XIV) it apparently ablated the protective effect afforded by the nonadherent lymphoid cell population. However, serum from an animal infected for 7 weeks gave greater protection and the parasite enhancing phenomenon was not observed (Fig. 23).

TABLE XI

EFFECT OF TIMING OF EXPOSURE ON PROTECTION BY ADOPTIVELY TRANSFERRED ADHERENT AND NONADHERENT PERITONEAL CELLS

Cell population	Weeks after exposure				
	0	1	2	3	4
Exposed <sup>a</sup>					
Unfractionated	-11 <sup>c</sup>	-19	-61**	-70*	-31
Nonadherent	-3	-2	-46*	-59*	-22*
Adherent	-16	-18	-42*	-53*	-23
Unexposed <sup>b</sup>					
Unfractionated	-13	ND <sup>d</sup>	-18	-20	-11
Nonadherent	+3	ND	-4	+4	+6
Adherent	-15	ND	-21	-13	-19

<sup>a</sup> Cells obtained from animals exposed to *S. mansoni* cercariae at varying intervals before sacrifice. Cell injections as above.  $2 \times 10^6$  cells were injected iv 4 hr before recipient exposure to cercariae.

<sup>b</sup> Cells obtained from animals not exposed to *S. mansoni* cercariae but matched in age to respective exposed animal group. Cell injections as in *a*.

<sup>c</sup> % change in mean number of recovered schistosomes compared to age matched control animals receiving no cell transfer. ( $N \geq 6$ )

<sup>d</sup> Not determined.

<sup>e</sup> *P* value, experimental group vs respective week 0 value,  $<0.05$  (Student's *t* test).

TABLE XII

EFFECT OF FREUND'S ADJUVANT UPON WORM BURDEN AND ADOPTIVE TRANSFER CAPABILITY

Animal treatment	Direct worm burden $\pm$ SEM <sup>a</sup>	Transfer worm burden <sup>c</sup> (preexposed)	Transfer worm burden <sup>b</sup> (unexposed)
CFA <sup>d</sup>	86 $\pm$ 29	74 $\pm$ 31	153 $\pm$ 29
PBS <sup>e</sup>	162 $\pm$ 38	109 $\pm$ 25	182 $\pm$ 36
	$P^f < 0.05$	$P^f < 0.05$	$P^g > 0.05$

<sup>a</sup> Animals were injected with 1.0 ml of CFA or PBS simultaneously with exposure to 1000 *S. mansoni* cercariae. They were perfused for collection of worms 2.5 wk later. SEM = Standard error of the mean.

<sup>b</sup> Animals were injected with 1.0 ml of CFA or PBS. They were lavaged 3.5 wk after exposure and  $2 \times 10^6$  unfractionated peritoneal exudate cells were injected iv into syngeneic 4.5 wk old recipients. These animals were then exposed to 1000 *S. mansoni* cercariae and perfused 2.5 wk later.

<sup>c</sup> Animals were injected with 1.0 ml of CFA or PBS simultaneously with exposure to 1000 *S. mansoni* cercariae. Lavage and transfer as in b. The number of recovered worms from both of the experimental groups above differed significantly ( $P < 0.05$ ) from those recovered from recipients of cells from unexposed animals.

<sup>d</sup> Complete Freund's adjuvant.

<sup>e</sup> Phosphate buffered saline.

<sup>f</sup>  $P$  value CFA injected group vs PBS injected group (Student's  $t$  test).

<sup>g</sup>  $P$  value recipients of cells from donors injected with CFA vs donors injected with PBS (Student's  $t$  test).

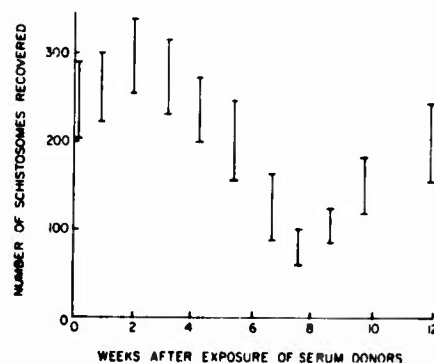


FIG. 22 Effect of serum transfer upon subsequent schistosome worm burden: 4.5-wk-old Fischer rats were exposed to 1000 *S. mansoni* cercariae. These animals were exsanguinated at weekly intervals after exposure. 2 ml of serum were injected intravenously into 4.5-wk-old syngeneic Fischer rats 4 hr before, and 7 and 14 days after their exposure to 1000 *S. mansoni* cercariae. The worm burdens of these recipient animals were determined 17 days after exposure.

The mechanism of this protective effect was further studied by varying the interval between serum injection and cercarial exposure (Table XV). Serum obtained from animals exposed to *S. mansoni* cercariae 7 weeks before perfusion was most effective when given simultaneously with the cercariae. When the serum transfer was delayed until 3 days after exposure, protection was markedly diminished and when it was delayed for 7 days, virtually no protection was observed.

Further studies (Table XVI) on the physical characteristics of the mediator of protective activity demonstrated it to be in the IgG QAE column chromatographic fraction and with a sedimentation coefficient of 7S estimated by Sephadex G-200 gel filtration. In addition, it could be removed with the use of anti-rat immunoglobulin G (immunoabsorbent column). Activity could also be removed by 33% ammonium sulfate treatment and recovered in the precipitate. Protective activity was not observed in the 4S + 19S Sephadex G-200 fractions.

Specific antischistosomal antibody activity was observed within 1 week of initial exposure to *S. mansoni* cercariae (Table XVII). Three different forms of antibody activity were determined: complement fixation; indirect hemagglutination; and fluorescence binding. These activities increased slightly with time and were maximum at the time when serum was protective in transfer experiments. Indirect hemagglutination titers were also lower in younger animals. All of these antibody activities were detected for many weeks after infection during times when serum seemed to have no effect on resistance.

TABLE XIII  
SUSCEPTIBILITY OF ADOPTIVELY TRANSFERRED PERITONEAL  
LYMPHOCYTES TO ANTIBODY TREATMENT

Cell treatment	Complement	% Release <sup>c</sup>	No. worms recovered ± SEM	$\Delta^d$	$P^e$
0	0	—	73 ± 14		
0	+	7	77 ± 19	+4	>0.05
Anti-thymocyte <sup>a</sup>	0	4	ND <sup>f</sup>		
Anti-thymocyte	+	38	181 ± 32	+108	<0.02
Anti-IgG <sup>b</sup>	0	6	80 ± 22	+7	>0.05
Anti-IgG	+	43	101 ± 27	+28	>0.05
Anti-IgG + Anti-thymocyte	+	74	188 ± 22	+115	<0.02

<sup>a</sup> Cells exposed to rat anti-rat thymocyte serum.

<sup>b</sup> Cells exposed to rabbit anti-rat  $\gamma$ G.

<sup>c</sup> Percent specific Chromium release:  $\frac{\text{cpm Experiment} - \text{Cpm Control}}{\text{cpm Freeze thaw} - \text{cpm Control}} \times 100$ .

<sup>d</sup> Mean increase in recovered worms.

<sup>e</sup>  $P$  value vs untreated cell population control (Student's  $t$  test).

<sup>f</sup> Not determined.

No. of worms recovered without cell transfer 217 ± 41.

These studies clearly demonstrate that the Fischer inbred rat is an appropriate host for the assessment of defense mechanisms to schistosomiasis infection. Moreover, they strongly implicate the animal's immune system as being germane to the generation of specific host defenses over and above those which may naturally exist. The degree of this natural resistance is inversely related to the age of the animal although the mechanisms of this dependency are not clear. It is of interest that, if age susceptibility were to be extrapolated backward in time, 100% recovery of worms would be predicted at near the time of conception of the host animal. A similar observation has been described in *Ascaridia galli* infections in chickens. The implication is that the rat "natural" defense mechanisms mature continuously from the time of conception. Specific factors such as immune maturation, animal size, skin penetration, etc., are presently under evaluation.

The size of the initial worm challenge, perhaps analogous to total antigenic load, affects the subsequent immune response of the animal to infection. When larger numbers of worms are used it may be that the animal's immune system is more efficiently stimulated by the greater amount of antigen and thereby manifests a more efficient elimination of the subsequent parasite burden. These findings are compatible with those

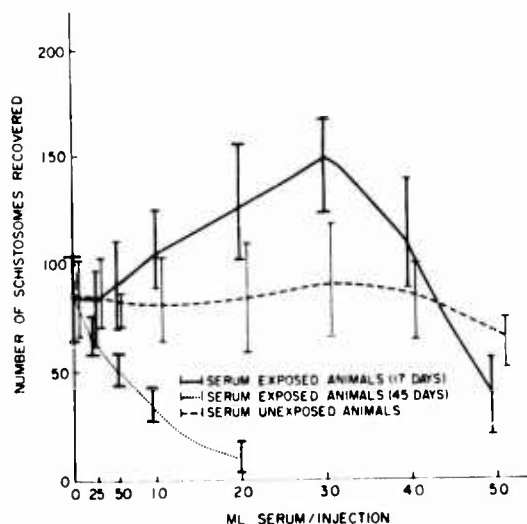


Fig. 23 Effect of serum transfer upon subsequent schistosome worm burden: 4.5-wk-old Fischer rats were exposed to 1000 *S. mansoni* cercariae. These animals were exsanguinated either 17 days (—) or 42 (---) days after exposure. Varying amounts of serum (0-5.0 ml) were injected intravenously into 4.5-wk-old syngeneic Fischer rats 4 hr before, and 7 and 14 days after their exposure to 1000 *S. mansoni* cercariae: The worm burdens of these recipient animals were determined 17 days after exposure.

Stirewalt and Ritchie who found a decreasing percentage of surviving schistosomes with increasing cercarial density at time of exposure. The kinetics of worm recovery agree quite closely with those reported by Madison, Clegg, and Ritchie *et al.* An exception to this general agreement was the failure of Ritchie to observe the age dependent resistance described by our studies. The studies of Fadl, however, did demonstrate this increased resistance with age. In addition, by emphasizing the rather striking differences in susceptibility to infection shown by various strains of rats, he may have provided an explanation for the apparent contradiction.

Previous studies have implicated the importance of the immune response in modulating host responses to schistosomiasis. Most recently the work of Sher and Maddison have clearly shown that mice develop acquired immunity as a result of prior exposure to *S. mansoni* cercariae. Additional observations have implicated cellular mechanisms both *in vivo* and *in vitro*. Cellular transfer experiments have not previously met with great success, perhaps due to these animals' relatively weak endogenous resistance to schistosomiasis. The failure by Maddison to reduce resistance by thoracic duct drainage and antilymphocyte serum and her minimal success in transferring resistance with serum and her minimal success in transferring resistance with serum and cells may similarly be in part methodologic. Successful transfer of resistance using cells has been accomplished in other parasitic diseases including malaria, trichinosis, and trichostrongylosis.

TABLE XIV

EFFECT OF SIMULTANEOUS TRANSFER OF CELLS AND EARLY  
SERUM UPON SUBSEQUENT WORM BURDEN

Agent	No. of worms recovered ± SEM	$\Delta^c$	$P^d$
Cells <sup>a</sup>	98 ± 19	-78	<0.02
Serum <sup>b</sup>	192 ± 32	+16	>0.05
Both	184 ± 29	-8	>0.05
Neither	176 ± 24	—	—

<sup>a</sup>  $2 \times 10^8$  cells obtained from animals exposed to *S. mansoni* cercariae 3.5 wk before sacrifice were injected iv into recipients 4 hr before their exposure to cercariae.

<sup>b</sup> 3 ml of serum obtained from animals exposed to *S. mansoni* 3.5 wk before sacrifice was injected iv into recipients 4 hr before their exposure to cercariae.

<sup>c</sup> Change in number of recovered worms vs uninjected animals.

<sup>d</sup>  $P$  value vs uninjected animals (Student's  $t$  test).

It appears that the initial immune response of rats to schistosomiasis is mediated through the generation of specifically sensitized thymus-dependent lymphocytes. Evidence for this conclusion is provided by discrete cell transfer experiments in which the cell population demonstrating protection has been shown to be nonadherent, lymphoid in morphology, susceptible to antithymocyte serum treatment, and insusceptible to anti-IgG antibody. These findings are compatible with those of Dennert and Cioli who observed that the number of parasites reaching maturity in rats quadrupled following thymectomy. The recent use of transfer factor to convey delayed hypersensitivity against *S. mansoni* antigens is also compatible with the importance of these lymphocytes. The findings are also consistent with those of Colley who observed increased susceptibility to the consequences of *S. mansoni* infection following depletion of thymic dependent lymphocytes. This latter work, along with that of Warren and Boros strongly implicates thymic-derived lymphocytes in the pathologic reaction of host against egg antigens deposited in the liver.

TABLE XV

EFFECT OF TIMING OF LATE SERUM INJECTION UPON PASSIVE PROTECTION

Interval <sup>a</sup>	No. worms recovered $\pm$ SEM	$\Delta^b$	$P^c$
Exposed			
0	55 $\pm$ 21	-130	<0.01
3	103 $\pm$ 32	-79	<0.05
7	152 $\pm$ 34	-30	>0.05
14	174 $\pm$ 40	-8	>0.05
Unexposed <sup>d</sup> (0, 3, 7, 14)	182 $\pm$ 37	—	—

<sup>a</sup> Interval in days between time of serum injection and cercarial exposure to *S. mansoni* cercariae. 3.0 ml of serum from animals exposed 7 wk prior to sacrifice was injected intravenously into four male recipients rats.

<sup>b</sup> Decrease in number of recovered worms compared to animals injected with serum from donors not previously exposed (d) to *S. mansoni* cercariae.

<sup>c</sup>  $P$  value experimental group vs respective control (d) (Student's  $t$  test).

<sup>d</sup> Animals injected 0, 3, 7, or 14 days after exposure to cercariae with serum from donors unexposed to *S. mansoni* cercariae:  $N = 24$ . No difference in the number of recovered worms was observed between these four groups.

Table XVI

## EFFECT OF SERUM FRACTIONATION UPON PASSIVE TRANSFER PROTECTION

Serum preparation	No. worms recovered $\pm$ SEM	$\Delta^f$	$P^g$
Exposed			
Whole <sup>a</sup>	58 $\pm$ 18	-136	<0.02
IgG (QAE) <sup>b</sup>	89 $\pm$ 28	-105	<0.05
7S (G-200) <sup>c</sup>	77 $\pm$ 31	-117	<0.03
IgG <sub>2</sub> (GAR IgG) <sup>d</sup>	166 $\pm$ 47	-38	>0.05
Unexposed <sup>e</sup>	194 $\pm$ 53	—	—

<sup>a</sup> Whole serum (2.0 ml) obtained from animals exposed to *S. mansoni* cercariae 7.5 wk previously.

<sup>b</sup> IgG containing fraction obtained from QAE-Sephadex fractionation, 2 ml equivalent V:V.

<sup>c</sup> 7S fraction obtained from G-200 column chromatography; 4S + 19S fractions did not demonstrate significant protective effect.

<sup>d</sup>  $\gamma$ -G globulin depleted fraction obtained by affinity column chromatography using column-bound goat anti-rat IgG antibody.

<sup>e</sup> Serum obtained from animals unexposed to *S. mansoni* cercariae.

<sup>f</sup> Decrease in number of recovered worms from unexposed animal (e).

<sup>g</sup> P value vs animals injected with serum from exposed animal (3) (Student's *t* test).

Additional protection afforded by adherent cell components might be inferred from these experiments; however, these mechanisms are not clear. The effects may be related either to incomplete fractionation and the transference of relatively adherent lymphocytes in the macrophage population or perhaps alternatively to the activation of the macrophages either by specific lymphoid cells or nonspecific stimuli.

The effector mechanisms of thymus dependent cell mediated immunity cannot be deduced from the experiments reported herein; direct lymphocytotoxicity, mediation through lymphokines, or collaboration with other cells and/or molecules are all possibilities. It would seem that collaboration with non-thymus dependent lymphocytes in such a manner as to bring about specific protective antibody synthesis is an unlikely candidate mechanism since the data presented above indicate that antibody is effective only against very immature worms shortly after challenge (see discussion below); it would seem that too little time would be available for synthesis of sufficient antibody to produce the observed effects.

Simultaneously with the operation of immunologically competent cells, the humoral antibody of the animal appears to influence the course of infection in an extremely complex manner. The serum can be shown to directly promote the development of worms and to block the protective effects of immunologically competent lymphocytes acting, perhaps, in a manner analogous to classic enhancement. The chemical character of this serum component is not known. Additional published studies indicate that



sera of infected animals contain *S. mansoni* antigen in the form of antigen-antibody complexes. Extensive studies are progress to evaluate this possibility. The formation of these complexes due to circulating antigen in a manner analogous to that seen with serum sickness might also explain the apparent inability of serum to provide significant protection at this time (3.5 weeks after initial infection). Protecting antibody would appear only after the bulk of antigen had been cleared and excess antibody could appear free in the serum.

Table XVII

DETECTION OF ANTIBODY ACTIVITY AGAINST SCHISTOSOME ANTIGEN IN SERA OF RATS PREVIOUSLY EXPOSED TO *Schistosoma mansoni* CERCARIAE

Assay	Weeks after initial exposure to cercariae				
	0	1	3	7	26
CF <sup>a</sup>	ns <sup>d</sup>	1	16	32	8
IHA <sup>b</sup>	ns	ns	ns	320	10
IF <sup>c</sup>	ns	ns	+	+	+

<sup>a</sup> Complement Fixation—reciprocal of highest positive dilution.

<sup>b</sup> Indirect Hemagglutination—reciprocal of highest positive dilution.

<sup>c</sup> Fluorescence binding (+ or -), detected by indirect immunofluorescence.

<sup>d</sup> No significant activity observed.

The present studies further indicate that the long-term role of antibody in schistosomiasis also appears to be very complex. On the one hand, antibody may modulate the initial cellular responses and the subsequent defense mechanisms of the animal. In this way the partial, but not complete elimination of parasites could be accomplished. Thus, the antibody can be shown to directly promote the development of viable parasites at certain times and to obviate cellular immunity. The antibody may be directly protective at other times (e.g., 7-12 weeks after initial infection). This protective effect of serum has been previously described by several investigators. A small number of surviving parasites (approximately 0.1% of the total initial number) coexist for prolonged periods of time in the presence of anti-schistosomal antibody. This antibody did not afford protection to recipients but has specificity for *S. mansoni* surface antigens as demonstrated by several serologic tests. This mechanism of balanced enhancement would allow small numbers of adult parasites to survive for indefinite periods and provide a constant stimulation to the animal's immune system, thus maintaining an immunologic readiness. The surviving adult form has already been implicated as the major stimulus for lasting immunity. The complex phenomena contributing to immunogenicity when antigen coexists with various concentrations of antibody has been the subject of much interest but is far from clear; however, prolonged low-grade schistosomal

infection often lasts many years in man and may provide the stimulus for the maintenance of the high degree of resistance to reinfection shown by persons living in endemic areas.

Additional observations to be described in an additional report indicate that the secondary immune response, that is, response of an animal to a second schistosomal exposure, is primarily mediated through protective antibody. Cell-mediated immunity is extremely difficult to demonstrate at this time. Evaluation of the CDF rat's response to re-exposure as well as precise in vitro quantitation of lymphocyte and antibody reactivity are in progress.

These studies may also help to delineate the parasite stages against which serum obtained from previously exposed animals may be protective. Since this serum was effective only if given within 72-96 hours of cercarial exposure, the implication is that it is the earliest stage of infection which is most susceptible to immune attack. Initial evidence for selective destruction of early forms has been provided by the work of Koppisch, who observed degenerating schistosomula in draining lymph nodes 4 days after infection. The findings are also consistent with the observations of Clegg and Smithers, who demonstrated that antibody is effective in in vitro cytotoxic tests only against very early schistosomula. Their explanation of this phenomenon is that worm-specific antigenic expression is modulated through a form of surface steric masking by host serum proteins. In this regard, it is of some interest that preliminary direct immunofluorescence studies failed to demonstrate rat IgG or albumen on the surface of rat schistosomes.

Finally, caution in interpreting these results must be emphasized. The rat is highly resistance to schistosomiasis and in this respect provides a poor model of human schistosomiasis. Whether or not the observations in experimental schistosomiasis accurately define the role of cells and serum in natural infections certainly is not clear. However, even with these reservations, the data clearly indicate the operation of acquired immunity in S. mansoni infection and suggest mechanisms which may well be operative, albeit perhaps with quantitative variations, in other host-schistosome systems.

Task 00 Communicable Diseases and Immunology

Work Unit 165 Parasitic Diseases of Military Importance

Literature Cited.

Publications:

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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-DR&E(AR)636	
3. DATE PREVIOUS <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DRGTH NSTRN <sup>a</sup>	9. SPECIFIC DATA - CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM <sup>a</sup>
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11. NO / CODES <sup>a</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
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B. CONTRIBUTING							
C. <del>XXXXXXXX</del>	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Viral Infections of Man							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002600 Biology 010100 Microbiology 003500 Clinical Medicine							
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B. NUMBER <sup>a</sup>				FISCAL		76	
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E. CUM. AMT.						448	
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: RUSSELL, COL Philip K.			
				NAME: BRANDT, Dr. Walter E.			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Viruses; (U) Immunology; (U) Arbovirus Infections; (U) Adenovirus Respiratory Diseases; (U) Influenza; (U) Human Volunteer							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRAM (Pursuit 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) 75 07 - 76 06 Influenza. In collaboration with Division of Preventive Medicine, WRAIR, an outbreak of a new strain of influenza virus, A/swine, was investigated at Fort Dix, NJ in February. A/swine strains caused the severe 1918 pandemic. In several companies, 20-60 percent of trainees were found to have A/swine infection, establishing that this new influenza strain was capable of human transmission. The WRAIR collaborated with other federal agencies in studies of the reactogenicity and immunogenicity of vaccines containing antigens of the A/swine strain, A/New Jersey/1976. One study showed that while persons over 25 years of age develop a good antibody response to A/swine antigens with few reactions, more febrile and constitutional reactions and fewer seroconversions occur in younger persons who lack experience with A/swine or related antigens. Except for vaccines of one manufacturer, a bivalent A vaccine with 400 CCA units each of A/New Jersey and A/Victoria, given with 500 CCA units of influenza B vaccine, induced acceptable reactions and immunogenicity. In both studies, split virus vaccines were less immunogenic than whole virus vaccines in young persons. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 July 1975 - 30 June 1976. Support in the amount of \$130,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76							

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b. <del>XXXXXXXX</del>	CARDS 114F						
c. CONTRIBUTING							
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Viral Infections of Man							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>							
002600 Biology 010100 Microbiology 003500 Clinical Medicine							
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
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				NAME: RUSSELL, COL Philip K.			
				NAME: BRANDT, Dr. Walter E.			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Viruses; (U) Immunology; (U) Arbovirus Infections; (U) Adenovirus Respiratory Diseases; (U) Influenza; (U) Human Volunteer							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>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.</p> <p>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.</p> <p>25 (U) 75 07 - 76 06 Adenovirus acute respiratory disease (ARD); adenovirus type 21 (Adv 21) emerged to cause ARD in military trainees for the first time since 1968; ARD rates of 2-4/100 trainees/week were common in Feb-Mar 1976 and Adv 21 persists in training posts. Protocols for summer and fall studies of the safety, immunogenicity, and efficacy of a live, oral, adenovirus type 21 vaccine have been approved by the AIDRB. The prevalence of neutralizing (N) antibody to adenovirus types 4, 7, and 21 in incoming trainees is 23 percent, 41 percent and 11 percent, figures similar to those in trainees in 1966. Throat swabs maintained in viral transport medium has been found to be as efficient as specimens maintained at -70C for adenovirus isolation; use of the carrier medium will eliminate requirements for ultra low refrigeration or dry ice in field studies. The usefulness of a hemagglutination-inhibition serological assay for Adv 21 is being evaluated to determine whether this test can replace the cumbersome and expensive N antibody assay in antibody prevalence surveys and in diagnosis of Adv 21 infections in ARD patients. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1975 - 30 June 1976.</p>							

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17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
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B. NUMBER <sup>a</sup>				FISCAL YEAR			
C. TYPE				CURRENT			
D. KIND OF AWARD				F. CUM. AMT.			
19 RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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22 KEYWORDS (Precede EACH with Security Classification Code) (U) Viruses; (U) Immunology; (U) Arbovirus Infections; (U) Adenovirus Respiratory Diseases; (U) Influenza							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish 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 scientific problems.							
25 (U) 75 07 - 76 06 Arboviruses. Determination of antibody responses to nonstructural soluble complement fixing (SCF) antigens of dengue viruses following first and repeated infection was carried out with a sensitive radioimmunoassay (RIA). The extensive purification required to prepare an effective solid phase SCF antigen limited the study to dengue types 1 and/or 2. Following documented primary infections, SCF antibodies were found in 20 percent of the cases. D-2 SCF antibodies were found in 80 percent of the secondary cases complicated by hemorrhagic fever (DHF); only 30 percent were positive by the standard CF test. Of the few that were negative by RIA, half were known to react with partially purified type 4 SCF antigen by the CF test; thus, probably all DHF sera contain antibodies to the dengue SCF antigens. As an alternative to live virus vaccines, separation systems for identifying and isolating cell associated viral proteins for their immunogenic potential are being carried out. Finally, arbovirus infection was found to modulate host specific cell surface antigens. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 75 - 30 June 1976.							

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

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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. Evaluation of the antibody response to structural and nonstructural antigens of dengue virus following infections in humans

Dengue infection causes high morbidity in military personnel, particularly in base camps in tropical countries. There are currently 4 accepted serotypes of dengue virus, any of which may produce hemorrhagic manifestations when it is responsible for a second (heterologous) dengue infection. Two or more infections with dengue virus are associated with very high hemagglutinin-inhibiting antibody titers to the virus structural antigens of all 4 serotypes. Sporadic appearance of complement fixing (CF) antibody to a virus-specified, nonstructural soluble (SCF) antigen was found only in second dengue infections.

(1974 Annual Report). Since we will investigate the simultaneous use of live attenuated viruses of all 4 serotypes for immunization against dengue, evaluation of the immune response would have to include a sensitive test to the nonstructural antigens. A solid phase radioimmunoassay (RIA) was developed for this purpose (1975 Annual Report). The RIA titers to the SCF antigen were about 100-fold higher than the CF titers, and of the dengue hemorrhagic fever (DHF) cases tested, most were positive for SCF antibody by RIA. It was not known which dengue serotype was responsible for the disease in the previous study, due to high titered crossreactive antibody and no virus isolation.

The following report is a study of the antibody response to dengue types 1 and 2 SCF antigens from: 1) primary and secondary dengue cases in Bangkok where the infecting virus serotype was determined by virus isolation in some cases (the secondary cases were hemorrhagic fever), 2) from primary and secondary dengue cases in the South Pacific (uncomplicated by hemorrhagic fever) where the sequence of infection was provided by Dr. Leon Rosen (data base unknown), and 3) from primary and secondary cases (not DHF) of dengue in Puerto Rico provided by Dr. Barney Cline. The primary and secondary cases from Puerto Rico were classified according to the magnitude of the titer and the magnitude of the cross reactions observed with the HI test, and virus isolation.

## 1. Methods

Purification of the structural and nonstructural antigens, iodination of goat anti-human gamma globulin, and the radioimmunoassay were described in detail in the previous Annual Report. Briefly, to perform the RIA: 1) antigen was allowed to absorb to the polyvinyl-chloride surface in the wells of flexible microtiter plates; 2) fetal bovine serum (10% in PBS) was used to fill the spaces on the plastic not occupied by antigen; 3) serum or serum dilutions were added to react with the antigen; 4) radioactive antiglobulin was added to react with antibodies that had bound to the antigen; 5) the wells were cut from the plate and assayed for radioactivity in a gamma counter; and 6) the raw data was evaluated for technical proficiency by plotting CPM bound versus serum dilutions. This test is summarized in Figure 1.

The structural antigens were hemagglutinins that were first pelleted from protamine sulfate clarified mouse brain suspensions, and then centrifuged in potassium tartrate-glycerol gradients. As higher concentrations of hemagglutinin were used as the solid phase antigen, a plateau was reached where no more antibody could be detected. The dilution of antigen used to coat the plastic surface for the solid phase RIA was that dilution at which the next higher dilution would not produce more counts per minute (CPM) of antiglobulin bound to the reference antibody.

The nonstructural soluble complement fixing (SCF) antigen was



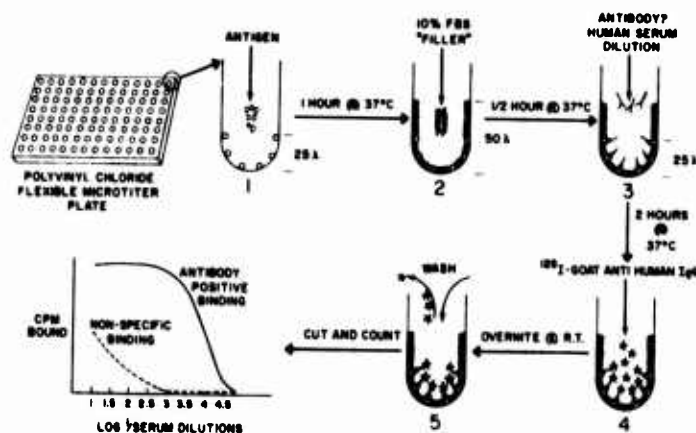


Figure 1. General procedure for the radioimmunoassay

purified in the following manner: 1) clarification of infected mouse brain homogenate in the high speed centrifuge; 2) chemical clarification with protamine sulfate; 3) removal of the hemagglutinins by ultracentrifugation; 4) concentration of the SCF antigen by precipitation with ammonium sulfate; 5) chromatography of the precipitate on Sephadex G-100 columns and collection of the 39,000 dalton peak; 6) reconcentration by pressure filtration; and finally, 7) the CF peak at pH 6.5 for dengue type 1 and pH 5.2 for dengue type 2 SCF from an isoelectric focusing column was used as the RIA nonstructural antigen in the initial studies. The antigen exhibited a prozone effect; undiluted and low dilutions of antigen did not appear to bind or absorb to the surface (Figure 2-A). As the antigen was diluted, it could then attach to the plastic surface, or be exposed in such a manner that antibody would react with it. Therefore, the antigen had to be carefully titrated, and that dilution producing a maximum RIA response with a constant dilution of specific antibody was the dilution used to sensitize the plastic wells in a microtiter plate. Subsequently, the antigen was refocused on a narrower pH gradient, and the prozone effect disappeared (Figure 2-B). Even the off-peak fractions that initially bound very little antibody, now behaved as a very effective RIA antigen at low dilutions or high concentrations after a refocusing procedure (Figure 2-A & B). This indicated that contaminating proteins may have been responsible for the prozone effect with antigens obtained from the first electrofocusing experiment; the second electrofocusing run was sufficient to minimize interference of antigen attachment by such contaminating proteins.

Paired sera that were simply screened for the presence or absence of antibody to SCF antigen were tested in the following manner. Four half-log<sub>10</sub> serum dilutions - 1, 1.5, 2, and 2.5 (e.g., 1:10, 1:30, 1:100 and 1:300) were placed in microtiter wells with antigen, and in 4

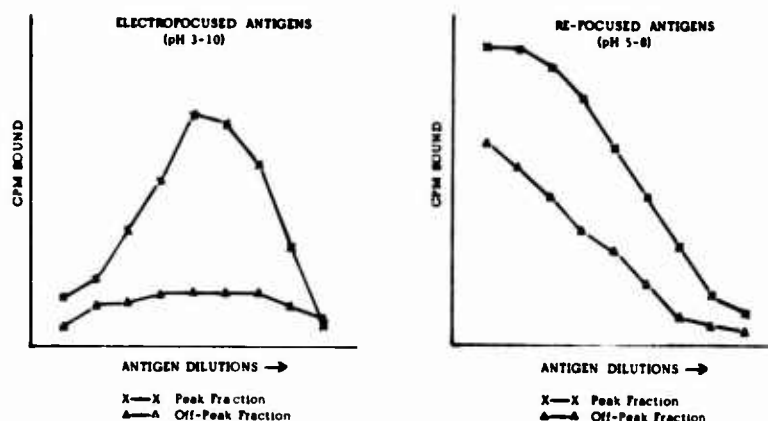


Figure 2 (A & B). Effect of purification on the ability of antigen to serve as a solid phase receptor for radioimmunoassays.

other wells without antigen in order to measure the nonspecific attachment of serum globulin to the plastic wells (background or control values). The radioactivity of the antiglobulin in the control wells was subtracted from that in the antigen wells and plotted as in Figure 3-A and B. The raw data from the first 2 dilutions in the control wells is included on the right portion of each panel in order to demonstrate the amount of nonspecific binding of serum globulins. Paired sera from a primary case of dengue is shown in Figure 3-A and illustrates a negative response to the SCF antigen. Most of the primary infection sera were negative by these criteria. Figure 3-B depicts a positive response to the SCF antigen, generally found only in second dengue infections. It can be seen that more antibodies were detected in the convalescent serum than in the acute phase serum. Most of the paired sera tested fell into one of the two groups shown in Figure 3, thereby giving us little difficulty in determining positive and negative sera.

Actual titers of antibodies to structural and nonstructural antigens determined by RIA were required to 1) follow the response in serial bleedings from surviving DHF cases (taken for over one year in some instances), and 2) compare the response with some standard serological tests such as the HI and the plaque reduction neutralization test (PRNT). The method of determining an RIA titer is shown in Figure 4. The raw data shown in Figure 4-A is converted to a single curve shown in 4-B by subtracting the CPM on the 'no antigen' curve from the corresponding CPM points on the 'antigen' curve. A least squares regression analysis is used to plot a straight line representing the background or control values of normal sera and/or nonspecific attachment of globulin to 'no antigen' wells. A parallel line representing 5 standard deviations was chosen as the line to intersect with the corrected curve to determine the titer of the particular serum.

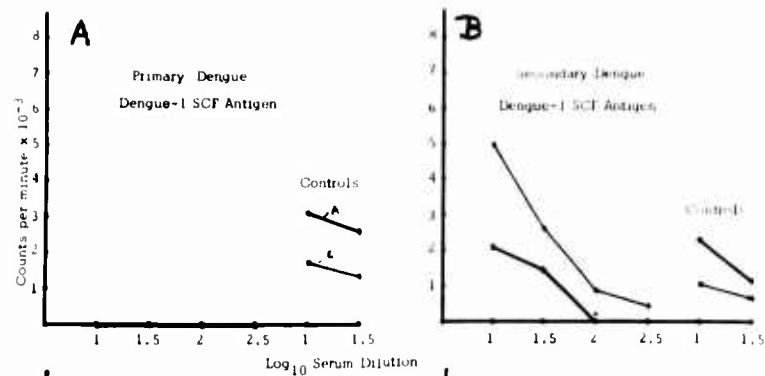


Figure 3 (A & B). Usual reactions encountered when testing paired sera from primary and secondary dengue infections against the nonstructural soluble complement fixing (SCF) antigen by radioimmunoassay.

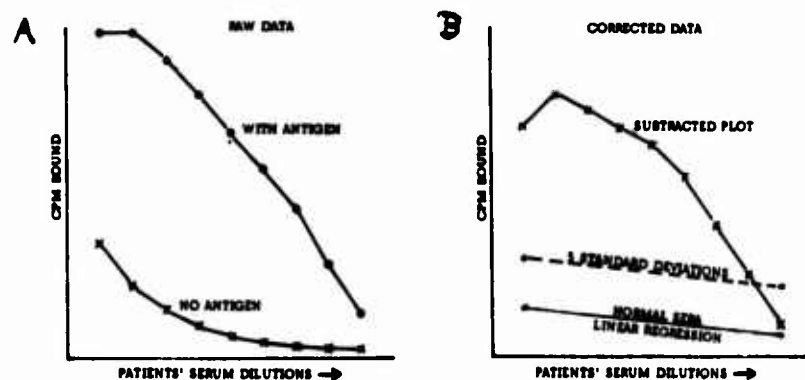


Figure 4 (A & B). Schematic depicting data corrections and one method of determining a serum titer by radioimmunoassay.

## 2. Antibodies to nonstructural SCF antigens in primary and secondary dengue infections

In preliminary experiments, groups of sera from Puerto Rico, the South Pacific, and Thailand were screened for antibody to type 1 and type 2 SCF antigens (Table 1). The primary infection sera from Puerto Rico were judged as such by low HI antibody titers (about 1:80 in the convalescent serum). They were determined to be dengue type 2 infection by virus isolation. The dengue 2 secondary infection sera from the same epidemic in Puerto Rico were grouped on the basis of very high HI antibody titers (up to or greater than 1:20,000). The group of

sera from dengue hemorrhagic fever patients in Thailand listed at the bottom of Table 1 were also second (or more) infection sera by the criteria of very high HI antibody titers. Those from the South Pacific were evaluated by Dr. Rosen by his criteria (unknown).

Table 1. Sera from dengue patients reacting with the dengue nonstructural soluble complement-fixing antigen (SCF) by radioimmunoassay

Infection	-	Location	SCF type 1	SCF type 2
Primary D-1		(S. Pacific)	0/5	1/5
" D-2		( " )	0/9	3/9
" D-2		(Puerto Rico)	2/10	2/10
Secondary D-2; D-1		(S. Pacific)	4/10	10/10
Secondary D-1; D-2		( " )	1/7	4/7
" D-3; D-2		( " )	8/10	7/10
" D-3; D-2		(Puerto Rico)	9/10	6/10
"		Unknown (Thailand) (Dengue hemorrhagic fever)		22/27

As shown in Table 1, sera from primary dengue cases seldom reacted with the SCF antigen, no more than 20 percent of the documented cases. Of the secondary cases, generally 80 percent of the sera reacted with at least one of the SCF antigens. The secondary DHF sera from Thailand had only been tested against type 2 SCF by RIA to specifically compare the RIA test to the CF test; only 30 percent of the DHF sera reacted to the type 2 SCF antigen by the CF test - performed in two laboratories, WRAIR and the Univ. of Hawaii. Over 80 percent (22/27) of this group reacted to type 2 SCF antigen by the RIA. Of the 5 negative sera, 3 reacted to the type 4 SCF antigen by the CF test in the Hawaii laboratory. Thus, the preparation of purified SCF antigen to all 4 serotypes would probably reveal the presence of antibodies in essentially all DHF sera if tested by RIA. The particular serotype causing primary dengue infection did not appear to affect whether the sera reacted with type 1 or type 2 SCF antigens. In sequential dengue infections, with one exception, there appeared to be more SCF reactors to the antigen related to the first infecting virus rather than the second (Table 1).

This question was examined more closely with better characterized

sera from Bangkok where virus isolation definitively identified the infecting virus - in this case, type 2 dengue virus. However, it was not known which of the dengue serotypes was responsible for one of the former infections. Listed in Tables 2 and 3 are the HI and SCF-RIA results for serial bleedings from DHF patients up to a year or more after infection. The rapid increase in broadly reactive HI antibodies to all 4 serotypes shown here is typical of second dengue infections. The SCF antibody response to dengue types 1 and 2 appeared to be independent of the finding that dengue type 2 was the infecting virus; 2 patients developed a type 1 SCF antibody response, 2 patients developed an antibody response to both type 1 and type 2 SCF antigens, and 2 more patients developed an antibody response only to the type 2 SCF antigen. It was also observed in several instances that as the HI titers went down, the SCF titers remained high. Thus, the antibody responses to the nonstructural SCF antigens and to the structural hemagglutinins are independent.

Secondary infection sera with titers as low as 1:80 in the HI test contained antibody titers to the SCF antigen as high as 1:600. Primary infection sera with HI titers as high as 1:320 did not contain any antibody to either types 1 or 2 SCF antigen (Table 4). Thus, the magnitude of the HI titer does not appear to be a factor in whether a particular serum will react to the SCF antigen. The serial bleedings shown in Table 4 were primary dengue 1 infections, and demonstrate the lower titers typical of primary infections. It can be seen that the primary HI antibodies listed here are also broadly cross-reactive.

### 3. Comparison of antibody titers measured by neutralization and radioimmunoassay

We investigated whether or not serum titers to structural antigens by RIA would be as broadly reactive as in the HI test, or more specific as the plaque reduction neutralization test when it is performed on primary infection sera. It appears that the RIA measures broadly reactive antibodies (to structural antigens). While the serial sera listed in Table 5 are from second dengue infections (DHF cases), the apparent specificity of the PRNT for one of the dengue serotypes in the acute phase serum is not matched by the RIA test. The PRNT measures antibodies that block infectivity, whereas the RIA measures antibodies that simply attach to virions. The lack of specificity of even the PRNT in second dengue infections can be seen in the broadly reactive titers of the convalescent second infection cases; there is generally no correlation with the infecting virus (listed in parenthesis in Table 5). There is even less correlation with the RIA titers to the structural antigens (virions). However, the RIA was found to be very sensitive for detecting dengue antibodies.

Table 2. SECONDARY INFECTIONS WITH DEN-2 (BANGKOK)

Pt. no. Disease day	HAI				RIA-SCF	
	D-1	D-2	D-3	D-4	D-1	D-2
<u>D73-02</u>						
2	20	10	20	40	< 10	< 10
10	> 10240	> 10240	> 10240	> 10240	80	< 10
24	> 10240	> 10240	> 10240	> 10240	240	< 10
398	160	80	160	320	< 10	< 10
<u>D73-62</u>						
4	160	40	160	160	< 10	< 10
8	> 10240	> 10240	> 10240	> 10240	> 640	< 10
35	> 10240	5120	> 10240	> 10240	> 640	< 10
296	320	160	160	160	60	< 10
<u>D73-91</u>						
2	< 10	< 10	40	10	< 10	< 10
4	80	40	160	80	< 10	50
16	> 10240	> 10240	> 10240	> 10240	> 640	> 640
31	1280	1280	> 10240	> 10240	> 640	> 640
240	20	20	80	40	10	640
<u>D73-68</u>						
4	40	20	40	40	< 10	< 10
27	> 10240	> 10240	> 10240	> 10240	320	320
40	> 10240	5120	> 10240	> 10240	320	640
270	640	320	640	320	< 10	< 10

Table 3. SECONDARY INFECTIONS WITH DEN-2 (BANGKOK)

Pt. no. Disease day	HAI				RIA-SCF	
	D-1	D-2	D-3	D-4	D-1	D-2
<u>D73-99</u>						
5	320	320	1280	2560	< 10	< 10
7	2560	5120	> 10240	> 10240	< 10	30
9	> 10240	> 10240	> 10240	> 10240	< 10	400
19	> 10240	> 10240	> 10240	> 10240	< 10	200
40	5120	1280	> 10240	> 10240	< 10	140
264	80	80	160	160	< 10	60
<u>D73-84</u>						
5	160	160	320	640	< 10	< 10
7	> 10240	5120	> 10240	> 10240	< 10	15
9	> 10240	> 10240	> 10240	> 10240	< 10	160
19	5120	> 10240	> 10240	> 10240	< 10	160
40	2560	2560	5120	> 10240	< 10	160
277	80	80	160	320	10	400

Table 4. PRIMARY DEN-1 INFECTIONS (BANGKOK)

Pt. no. Disease day	HAI				RIA-SCF	
	D-1	D-2	D-3	D-4	D-1	D-2
<u>D73-06</u>						
5	20	160	40	80	< 10	< 10
40	80	320	160	320	< 10	< 10
337	20	80	40	20	< 10	< 10
<u>D73-09</u>						
2	< 10	< 10	< 10	< 10	< 10	< 10
38	160	40	160	160	< 10	< 10
317	80	40	80	40	< 10	< 10
<u>D73-61</u>						
4	< 10	< 10	< 10	< 10	< 10	< 10
7	20	< 10	10	< 10	< 10	< 10
18	320	40	160	320	< 10	< 10
38	160	40	80	160	< 10	< 10
275	40	20	40	20	< 10	< 10



Table 5. COMPARISON OF VIRION RIA AND PLAQUE REDUCTION NEUTRALIZATION TITERS

Pt. no. Disease day	PRNT (50%)				RIA-Virion	
	D-1	D-2	D-3	D-4	D-1	D-2
<u>D73-91 (D-2)</u>						
2	< 10	11	23	< 10	100	100
6	170	340	> 1280	90	10,000	16,000
<u>D73-02 (D-2)</u>						
2	13	< 10	< 10	< 10	80	30
24	560	940	520	280	30,000	2,300
<u>D74-33 (D-3)</u>						
Acute	< 10	130	< 10	< 10	100	200
+17	350	890	1000	50	12,500	8,000
<u>D74-54 (D-3)</u>						
Acute	26	> 160	35	10	1,000	1,250
+17	390	840	800	23	3,000	20,000
<u>D74-137 (D-3)</u>						
Acute	< 10	< 10	< 10	< 10	200	100
+3	> 1280	> 1280	450	30	40,000	25,000

## B. Arbovirus enhanced modulation of cell surface antigens

Monkey kidney cells, upon progressive subculture, became refractory to complement (C) dependent immune cytotoxicity by anti-cell serum. Arbovirus infection restored these cells to a state of lytic susceptibility. Similar results were also obtained with antibody dependent cellular cytotoxicity (ADCC), which is C independent. Antibodies raised against different subcultures varied considerably in lytic efficiency, indicating changing patterns of host cell expression during continuous subculture. Taken together with the fact that arbovirus infection restored the lytic efficiency of all antibody preparations to the same degree suggested some form of host cell antigen re-expression as a mechanism. The results obtained in several exploratory experiments indicated that the antigenic re-expression responsible for the restoration of lysis was probably a local or selective, rather than a generalized, phenomenon. Thus, the amount of host cell surface antigen, measured by the use of mouse anti-cell serum and  $^{125}\text{I}$  anti-mouse globulin, was identical in both uninfected lytic susceptible and refractory cells, and decreased in both functional states following infection. Further, the binding of  $^{125}\text{I}$  concanavalin A, used to quantify surface glycoproteins, was similar in both lytic refractory and susceptible cells, and in both cases declined following virus infection. This result was incompatible with gross "masking" of cell surface antigens by exuberant production of surface coat material in lytic resistant cells. Finally, brief trypsinization of lytic resistant cells yielded an 8-fold increase in immune lysis, a result further consistent with local rather than generalized surface changes. The data was considered in terms of modulation of cell surface antigens affected both by repeated subculture and arboviral infection, and as a possible in vitro correlate of altered self-reactivity.

### 1. Background

A great deal of attention has focused on the appearance of virus specific antigens in the plasma membrane of infected cells due to the fact that these antigens have been shown to mediate a variety of immunopathological interactions (Allison, 1971; Notkins, 1974). Recently this laboratory described a surface membrane change of arbovirus infected cells unrelated (at least directly) to virus specific membrane antigens (Catanzaro, et al., 1975). It was shown that: 1) the established monkey kidney cell line, LLC-MK<sub>2</sub> (Russell, et al., 1967), after a period of susceptibility, became refractory to complement mediated immune cytotoxicity by antibodies directed against cell membrane antigens, and 2) productive arbovirus (either group A or B) infection restored the state of lytic susceptibility. The present report extends these studies and investigates possible mechanisms underlying this phenomenon. This report includes: 1) establishing the independence of this phenomenon of complement by carrying out parallel studies with antibody dependent cellular cytotoxicity (ADCC) (MacLennan et al., 1970; Yust et al., 1973); 2) monitoring the changes in antigenic expression occurring during continuous passage by comparing the relative lytic

efficiencies of antibodies raised against different subcultures; 3) quantifying the amount of host cell surface antigen by the binding of  $^{125}\text{I}$  antimouse globulin to lytic refractory and susceptible cells exposed to mouse anticell serum; 4) estimating the effects of arbovirus infection on the mechanical fragility of the LLC-MK2 membrane; 5) experiments to determine the role of surface glycoproteins by measuring the binding of  $^{125}\text{I}$  concanavalin A (Con A) (Sharon and Lis, 1972; Burger, 1973, 1974; Inbar and Sacks, 1969) to lytic susceptible and refractory cells; and 6) the effect of trypsinization of lytic resistant cells on immune cytolysis.

## 2. Methods

a. Cell cultures. LLC-MK2 cells were grown in stationary glass bottles (946 ml) or in flat bottomed multiple well culture plates (16 mm diameter; Linbro Chemical Co., New Haven, CT) and maintained with M-199 (Microbiological Associates, Bethesda, MD), containing 2% fetal bovine serum (FBS). The cells were subcultured by trypsinization (0.25%; Grand Island Biological Co., Grand Island, NY) and were passaged approximately 6 to 8 times each month. The same conditions of trypsinization were used in experimental procedures detailed below.

b. Infection of cultures. The LLC-MK2 cells were infected with a 20% (w/v) suspension of suckling mouse brain passage (SMBP) of the group A arbovirus Sindbis (AR 339 strain; SMBP 14) at a multiplicity of infection of 10, and the group B arbovirus dengue 2 (New Guinea C strain; SMBP 35) at a multiplicity of infection of 2. Control cultures were mock-infected with a 20% (w/v) normal suckling mouse brain suspension. Both viral and control cultures were incubated for 90 min at  $36^{\circ}\text{C}$ . The inoculum was then decanted and the monolayers were washed three times with Earle's balanced salt solution (Microbiological Assoc.). Finally, M-199, containing 2% fetal bovine serum (maintenance medium) was added and the culture was incubated at  $36^{\circ}\text{C}$  until the times selected for the studies detailed below.

c. Preparation of antibodies. A method for the preparation of antibodies to viral antigens in hyperimmune mouse ascitic fluid (HMAF) has been described in detail (Chiewsilp and McCown, 1972). Anti-LLC-MK2 HMAF was prepared in a similar fashion, using  $30 \times 10^6$  LLC-MK2 cells as the antigen. Normal ascitic fluid (NAF) was prepared in a similar fashion in unimmunized animals. The terms "antibody" and "HMAF" will be used interchangeably. Two separate preparations of HMAF were used in these studies: one obtained after immunization with LLC-MK2 cells used at the beginning of the present set of experiments, the other raised with LLC-MK2 cells approximately 100 subcultures before this. These will be referred to in the text as "new HMAF" and "old HMAF", respectively. Rabbit anti-LLC-MK2 antibody was prepared 24 subcultures before the start of the present experiments by the injection of complete Freund's adjuvant and LLC-MK2 cells in several intradermal sites. Following a set of intradermal boosting injections (without adjuvant) a

month later, the animals were bled from the ear vein 2 weeks after the last injection. The same animal was bled prior to immunization as a source of normal rabbit serum (NRS). Both rabbit and mouse antibody and normal serum preparations were stored in aliquots at  $-20^{\circ}\text{C}$ . These were adjusted to the appropriate dilutions for each experiment with M-199. All sera, except for fresh guinea pig serum, was heat-inactivated at  $56^{\circ}\text{C}$  for 30 min. The specificity of each antibody preparation was established by demonstrating that anti-LLC-MK2 activity, when present, could be abolished by absorption with LLC-MK2 cells but not with chick embryo cells or BHK-21 cells.

d. Immune cytotoxicity; complement mediated immune cytotoxicity.

Immune damage mediated by anticell antibody and guinea pig complement (C) was measured by the release of  $^{51}\text{Cr}$  from LLC-MK2 cells. These procedures have been described in detail elsewhere (Catanzaro, et al., 1974). Briefly,  $3 \times 10^5$  cpm of  $^{51}\text{Cr}$  (New England Nuclear Corporation, Boston, MA) were added to each well of a Linbro plate containing approximately  $3 \times 10^5$  LLC-MK2 cells in an adherent monolayer and allowed to incubate for 16 hr. The wells were then thoroughly washed with phosphate buffered saline (PBS). To each well was added appropriate dilutions of antibody, normal serum or PBS (0.1 or 0.2 ml). After 30 min an equal volume of a 1:1 dilution (PBS) of freshly prepared guinea pig C was added. The plates were then incubated in a humidified atmosphere of 5%  $\text{CO}_2$  in air on a rocking platform (Bellco Glass, Vineland, NJ) for 60 min at  $37^{\circ}\text{C}$ . The following controls were included: both infected and uninfected cells were incubated with anti-cell HMAF alone, C alone, or NAF plus C. We arbitrarily considered nonspecific release to be the highest of all the control values (calculated as described below) and refer to this collectively in the text and figures as "background". After incubation with the appropriate mixture, the fluid was carefully aspirated from the monolayer and clarified by centrifugation; both supernatant and pellet were assayed for radioactivity. The cell monolayers were dissolved in 2 ml of 2N NaOH and also counted. Comparisons between experiments were better appreciated by calculating the percent  $^{51}\text{Cr}$  release as follows:

$$\% \text{ lysis} = \frac{\text{CPM supernatant}}{\text{CPM (supernatant + pellet + dissolved cells)}} \times 100$$

Experiments were usually run in quadruplicate. Exceptions to this are noted in the text.

To insure uniformity and comparability of results of such experiments done at different times, the following internal controls were done: 1) Experiments were always done on cells on the 4th day following trypsinization and reculture. Such cells had reached confluency by the 3rd day; 2) Parallel cultures received  $^{51}\text{Cr}$  (as above) and the spontaneous release of  $^{51}\text{Cr}$  was monitored daily as a control for differential cell fragility at a particular subculture. It was found that the spontaneous release of  $^{51}\text{Cr}$  was small and constant throughout this entire group of experiments; and 3) The potency of the C used was

always checked by lysis of sheep red blood cells and most times by lysis of chick embryo cells and BHK21 cells. Again, in all cases, the C used in the present experiments was of similar potency.

e. Immune cytotoxicity; antibody dependent cellular cytotoxicity (ADCC). These procedures were modified from those described by Yust et al., (1973). In the present system, human lymphocytes were obtained by Ficoll hypaque separation (Perper et al., 1968) of peripheral blood from normal volunteers. Antibody or normal serum was added to the plate first and allowed to adsorb for 30 min at 37°C (all sera were heat inactivated at 56°C for 30 min). The antibody was then removed and the monolayer washed twice with PBS. The lymphocytes were subsequently added in 0.5 ml of M-199 containing 5% FBS, and their concentration adjusted to give an effector to target cell ratio of 100:1. The plates were incubated on a rocking platform as described above for 5 hr and the <sup>51</sup>Cr release was calculated also as described above. These experiments were performed in triplicate.

f. Iodination of proteins. Con A (Pharmacia Fine Chemicals, Piscataway, NJ) and goat antimouse gamma globulin (GAM; Cappel Laboratories, Downingtown, PA) were labeled with <sup>125</sup>I by the lactoperoxidase method of Marchalonis et al. (1969). The specificity of the GAM to mouse immunoglobulin was established by immunoelectrophoresis. Labeled <sup>125</sup>I Con A was separated from free <sup>125</sup>I by exhaustive dialysis with normal saline in the cold. Con A thus prepared retained its mitogenic potency with human lymphocytes. Powell and Leon found iodination neither affected the mitogenic potential or structural integrity (i.e., binding specificity) of Con A (Powell and Leon, 1970). GAM was iodinated and fractionated on a Sephadex G-25 column in a 50 ml syringe which permitted immediate separation of labeled protein from free <sup>125</sup>I.

### 3. Spontaneous variation of C-dependent immune cytotoxicity with progressive subculture.

Using three different antibody preparations, rabbit anti-LLC, "old" and "new" HMAF (see Materials and Methods), it was observed that the lytic efficiency of each preparation was not the same at each subculture in which it was used. This spontaneous variation of C-dependent immune cytotoxicity with progressive subculture is summarized in Figure 5. Antibody of varying dilution was used in the experiments shown in Figure 5, ranging from 1:1 to 1:50; the results shown in the figure represent the highest value of <sup>51</sup>Cr release obtained over this dilutional range. Figure 5 represents all the experiments of C-independent immune cytotoxicity carried out during this study and does not simply indicate representative results. Most of these experiments which monitored the variation of lytic efficiency with time were done in duplicate. No value of the replicate sample varied more than 3% from the mean. Also, it can be seen that not all antibody preparations were tested simultaneously. Although the latter would have been the optimal situation, the relationships indicated by this figure were only appreciated retrospectively. In addition, the pattern of variation of

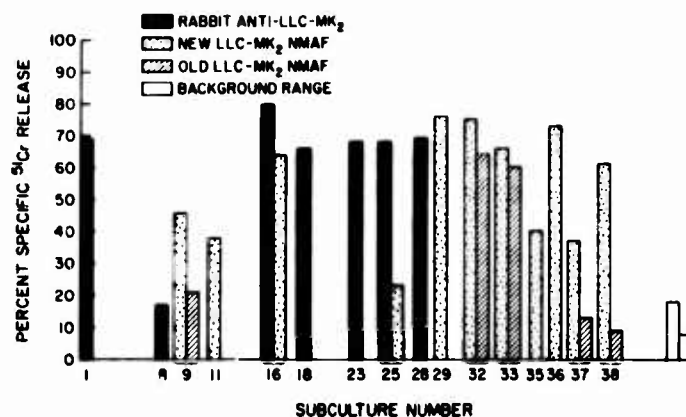


Figure 5. Variation of effectiveness of anticell sera from rabbit and mouse on different subcultures of LLC-MK<sub>2</sub> cells. Old HMAF and new HMAF represented anticell hyperimmune mouse ascitic fluid raised in mice immunized with remote and more recent passages of LLC-MK<sub>2</sub> cells. It can be appreciated that the variations of one antibody preparation cannot be predicted from the others.

each antibody was unique and changes in lytic efficiency with subculture did not occur in parallel. Thus rabbit anti-LLC-MK<sub>2</sub> serum yielded consistently high levels of specific <sup>51</sup>Cr release except at subculture 8, at which time specific release was only slightly greater than background. In contrast, results obtained with mouse antibodies were far more variable.

The results of several experiments when more than one antibody was used on the same subculture served to highlight the lack of parallelism. At subculture 16, specific <sup>51</sup>Cr release was almost equal between rabbit anti-LLC-MK<sub>2</sub> serum and new HMAF, while at subculture 25, the rabbit serum was approximately threefold more effective (i.e., 68% vs 23%). Experiments with rabbit serum were not carried out past the 28th subculture because the supply of this lot had been exhausted in these and ADCC experiments (described below). A similar lack of parallelism can be seen in comparisons with the two mouse antibody preparations, i.e., old and new HMAF. While at subcultures 9, 37, and 38, new HMAF was at least two-fold and at times, fivefold more efficient than old HMAF, the two preparations were about equally effective at subcultures 32 and 33. Finally, from Figure 5 it can be appreciated that changes in immune lytic efficiency may take place with great rapidity; e.g., in 4 subcultures, 25-29, <sup>51</sup>Cr release with new HMAF increases from 23% to 78% and in 4 subcultures, 33-37, <sup>51</sup>Cr release with old HMAF decreases from 60% to background levels.

#### 4. The influence of arboviral infection on C mediated immune cytotoxicity

The following relationship was established: If the LLC-MK2 cells exhibited high susceptibility to lysis, i.e., between 60% to 80% specific  $^{51}\text{Cr}$  release, no significant increases in C mediated immune cytotoxicity above these levels could be obtained by prior infection with arboviruses. If the LLC-MK2 cells, however, were resistant to immune lysis, without fail arboviral infection would restore high susceptibility to immune lysis. Figures 6 and 7 are typical examples of this phenomenon. With both old and new HMAF, this increase in lytic susceptibility occurred at least 24 hrs after dengue infection, or about the time of peak dengue release and remained at this level through several cycles of virus replication. At this latter time, cytopathic effects appeared and the experiments were terminated. Experiments with Sindbis infection were performed only at 18 hrs post infection, which is also the peak of viral release and which similarly restored lytic susceptibility to lytic resistant cells. It was observed that rabbit anti-LLC-MK2 serum was ineffective only at the 8th subculture (Fig. 5) and in similar fashion it was observed that dengue-2 infection restored the immune lytic susceptibility of LLC-MK2 cells to this rabbit antiserum (Fig. 6). Unlike the kinetic infection studies with HMAF, these experiments were carried out only at 72 hrs post dengue infection and, further, no comparable experiments with Sindbis infected cells were performed with this rabbit antiserum.

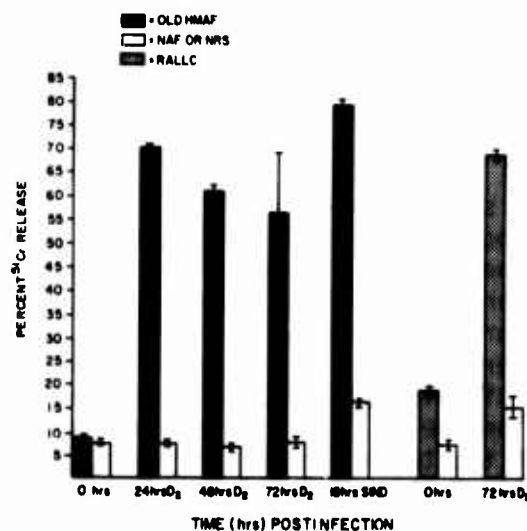


Figure 6. Restoration of lytic susceptibility by arbovirus infection to LLC-MK2 cells. Old HMAF and rabbit anti-LLC-MK2 serum (RALLC) are relatively ineffective in lysing uninfected (i.e., 0 hrs) LLC-MK2 cells (at 38th and 8th subculture, respectively). Infection with either dengue-2 (D2) or Sindbis (SIND) restored the state of lytic susceptibility. Ordinate = mean %  $^{51}\text{Cr}$  release + standard error of mean; abscissa = time in hrs post arbovirus infection.



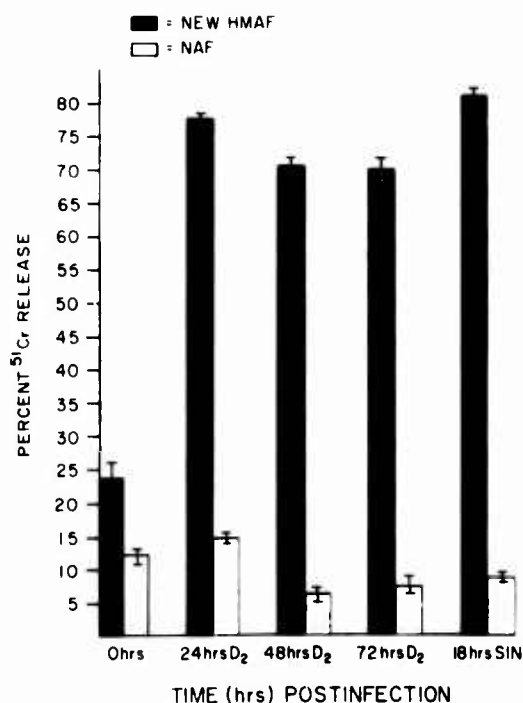


Figure 7. Identical experiment to that described in Figure 6 with the exception that new HMAF was used against LLC-MK<sub>2</sub> cells (25th subculture). Arbovirus infection restored the state of lytic susceptibility to the LLC-MK<sub>2</sub> cells refractory to lysis in the uninfected state (0 hrs) at this subculture.

##### 5. Effect of dengue infection on the mechanical fragility of LLC-MK<sub>2</sub> cells

Does dengue infection increase the mechanical fragility of infected cells and in this way contribute to the infection-induced restoration of lytic susceptibility described above? When uninfected and dengue infected LLC-MK<sub>2</sub> cells were subjected to several rapid freeze-thaw procedures, no difference in <sup>51</sup>Cr release from infected and noninfected cells was observed. In the experiment summarized in Figure 8, a gentler means of testing mechanical fragility was attempted; dengue infected and uninfected LLC-MK<sub>2</sub> cells were incubated in parallel in media of decreasing osmolarity for 1 hour and the <sup>51</sup>Cr released, measured, and compared. In isotonic media, it was observed that LLC-MK<sub>2</sub> cells, 24 hrs after dengue infection, were slightly (i.e., 4% greater <sup>51</sup>Cr release) yet significantly more fragile than uninfected cells (Figure 8).



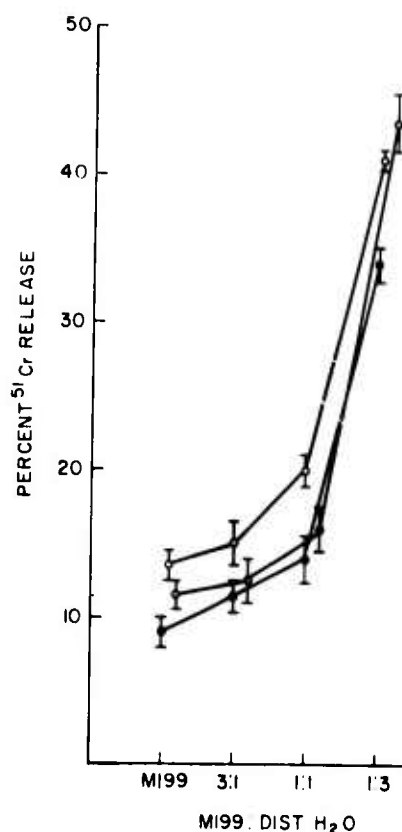


Figure 8. Uninfected LLC-MK<sub>2</sub> cells (● — ●); LLC-MK<sub>2</sub> cells 24 hrs (○ — ○) and 72 hrs (◻ — ◻) post dengue-2 infection are cultured for 1 hr in isotonic M-199 and solutions of decreasing osmolarity prepared by diluting M-199 with distilled H<sub>2</sub>O to the ratios indicated on the abscissa. Ordinate = mean % <sup>51</sup>Cr release ± S. E. of mean.

However, LLC-MK<sub>2</sub> cells 72 hrs after dengue infection did not exhibit significantly greater <sup>51</sup>Cr release than their uninfected counterparts (Fig. 8). This degree of difference between 24 hr infected cells and uninfected cells and the lack of a comparable difference with 72-hr cells was maintained as the osmolarity of the medium was decreased (Fig. 8). At the lowest osmolarity, however, the difference in <sup>51</sup>Cr release with 24 hr infected cells and 72 hr infected cells compared to uninfected cells, rose to 7% and 9%, respectively (Fig. 8). Although these differences were statistically significant as  $P < 0.05$  in the Student T test, they did not seem sufficient to account for the very large differences observed with C dependent lysis of uninfected and

infected cells.

#### 6. Effect of dengue infection on the quantity of LLC-MK2 cell surface antigen

The lytic refractory state could be due to net antigenic deletion. Arbovirus infection then might restore lytic susceptibility by re-expressing cell surface antigens. The amount of cell surface antigen was quantified by a binding of  $^{125}\text{I}$  goat antimouse globulin (GAM) to cells exposed first to anticell HMAF. To prevent phagocytosis of the mouse antibody or  $^{125}\text{I}$  GAM, all binding experiments were carried out at  $4^{\circ}\text{C}$ . These experiments were carried out during both periods of lytic susceptibility and lytic resistance to anticell HMAF (the 32nd and 25th subculture, respectively) and the results compared. In short, there was no difference in the behavior of cells during lytic refractory or lytic susceptible subculture with respect to binding of  $^{125}\text{I}$  GAM. Binding of anticell HMAF to uninfected lytic refractory or susceptible cells was similar; and following infection, a progressive decrease in the binding occurred. Figure 9 is an example of this phenomenon at the 32nd subculture, a period of lytic susceptibility to HMAF. The results for the 25th subculture, i.e., a period of lytic resistance to HMAF, were superimposable on the curves shown in Figure 9. Controls included incubating both normal and infected cells with nonimmune and antiviral serum before exposure to  $^{125}\text{I}$  GAM. These values were consistently much lower than anticell treated cultures. The slight increase in antiviral antibody binding to dengue infected cells probably reflected the appearance of dengue specific antigens during viral replication.

#### 7. Relation of cell surface to antigenic expression and immune cytolysis

Preliminary experiments were carried out to explore the relation of cell surface changes to immune lysis. 1) An experiment was carried out to determine whether surface glycoproteins were masking cell surface antigens. The binding of  $^{125}\text{I}$  Con A to cells at the 32nd subculture (Fig. 10), a period of lytic susceptibility, was virtually the same as that at the 25th subculture, a period of lytic resistance (not depicted). In both cases, following dengue infection, a progressive decrease in  $^{125}\text{I}$  Con A binding was observed with time. 2) It was also observed that when lytic resistant cells (49th subculture) were gently trypsinized and immediately subjected to C dependent immune cytolysis, an eightfold increase in lytic susceptibility was observed, in fact twice as great as that observed at 48 hrs post dengue infection.

#### 8. Influence of arbovirus infection on antibody dependent cellular cytotoxicity (ADCC)

Normal lymphocytes possess the ability to kill antibody-coated target cells in the absence of C, a process known as antibody dependent cellular cytotoxicity (ADCC) (MacLennan et al, 1970; Yust et

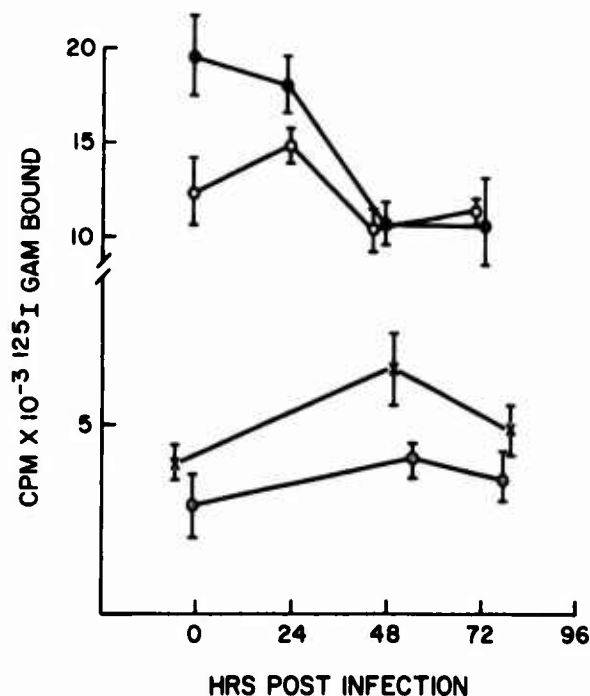


Figure 9. Effect of dengue infection on the binding of  $^{125}\text{I}$  goat anti-mouse globulin (GAM) to LLC-MK<sub>2</sub> cells first exposed to new HMAF (● — ●) and old HMAF (○ — ○). Controls included both uninfected cells incubated with anti-dengue antibody (X — X) and infected cells with nonimmune serum (○ — ○) before exposure to  $^{125}\text{I}$  GAM. Cells were taken from the 32nd subculture at which time they were lytic susceptible. Curves obtained with lytic resistant LLC-MK<sub>2</sub> cells are superimposable with those depicted above.

al., 1973). ADCC occurred when human peripheral blood lymphocytes were added to LLC-MK<sub>2</sub> cells coated with specific rabbit antiserum; the results obtained in such experiments were invariably lower than lysis effected by C dependent lysis (Figure 11). The single exception to this rule was observed at subculture 8, the only subculture (uninfected) relatively resistant to C dependent lysis with rabbit anti-LLC-MK<sub>2</sub> serum (shown in Figure 12). Optimal conditions for ADCC (Figure 22) included high dilutions of rabbit antiserum (1:250) and human peripheral blood lymphocytes in a ratio of 150 or 100 lymphocytes to 1 target cell. We were not able to effect ADCC with either of the mouse antibody preparations despite many variations of antibody dilution or lymphocyte source and concentration. Figure 12 shows that arbovirus infections increase lytic susceptibility to ADCC in a fashion similar to C mediated lysis.

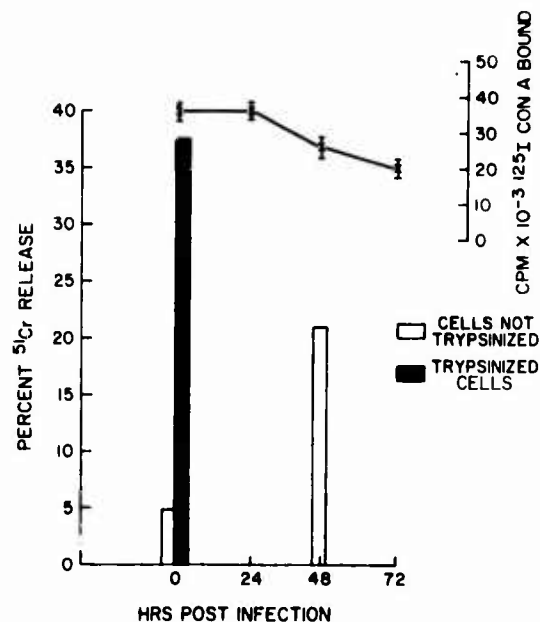


Figure 10. Preliminary experiments on the effect of dengue infection on surface glycoprotein and the influence of surface glycoprotein on immune cytotoxicity. The curve at the top shows the effect of dengue infection on the binding of <sup>125</sup>I Con A (X — X) to LLC-MK2 cells - when they were lytic susceptible; curves obtained with lytic resistant LLC-MK2 cells are superimposable with those depicted above. The bar graph in the lower part of the graph shows the eightfold increase in lytic susceptibility due to the anti-LLC-MK2 cell antibody following trypsinization of lytic resistant LLC-MK2 cells (tested only at 0 hr). Notice the increase due to dengue infection for 48 hrs is only fourfold. The replicates in this group of experiments were done in duplicate and varied less than 5% from the mean. Nonspecific <sup>51</sup>Cr release from trypsin treated cells was 12%.

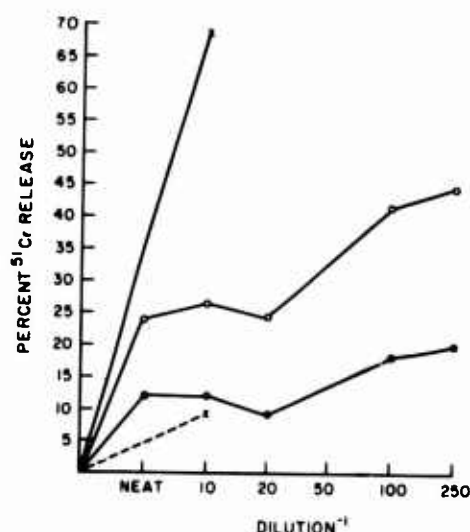


Figure 11. A comparison of the relative effectiveness of C-mediated immune lysis (X — X = rabbit anti-LLC-MK2 serum; X — X = normal rabbit serum); and ADCC mediated lysis, (O — O = rabbit anti-LLC-MK2 serum; O — O = normal rabbit serum). C-mediated lysis was carried out with a single antibody dilution (1:10), while ADCC was carried out over a wide range of dilutions. This figure illustrates the greater efficiency of C-mediated lysis versus ADCC.

Further, it can be appreciated in Figure 12 that the consistency of C dependent lysis with rabbit antibody was not mirrored with corresponding ADCC experiments. Although the bulk of lytic restoration experiments described above and in Figure 12 were carried out with dengue infection, a similar set of experiments was carried out with Sindbis-infected LLC-MK2 cells (25th subculture). Infection of lytic resistant LLC-MK2 cells by Sindbis resulted in increases in ADCC (Fig. 12).

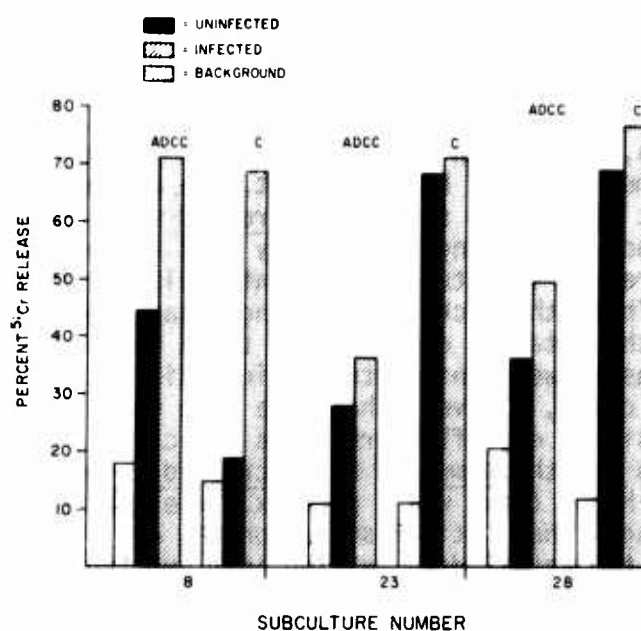


Figure 12. A comparison of the restoration of lytic susceptibility by ADCC and C-mediated lysis by arbovirus infection. Subcultures 8 and 28 indicate results obtained with uninfected LLC-MK<sub>2</sub> cells and those 72 hrs post dengue infection. Subculture 23 is a similar experiment obtained with uninfected LLC-MK<sub>2</sub> cells and those 18 hrs post Sindbis infection. Both ADCC and C lytic procedures utilized rabbit antibody (1:50 dilution).

The central conclusion of this report is the apparent modulation of cell surface antigens affected both by repeated subculture and arboviral infection. This conclusion was reached because each anticell serum preparation was capable of effecting maximal complement dependent lysis at some time; and further, when the use of one antibody source resulted in very little lysis, another was capable of producing higher levels of lysis (e.g., new HMAF vs old HMAF at subcultures 9, 37, 38, in Fig. 5). The fact that the lytic capability of the three antibody preparations considered in this report varied independently suggested that there existed multiple (at least 3) patterns of antigenic presentation which LLC-MK<sub>2</sub> cells possessed during continuous subculture. Since arboviral infection invariably resulted in the restoration of lytic activity (when it had been lost) of complement mediated lysis by each anticell antibody preparation, it suggested that arboviral infection must cause the re-expression of host cell surface antigens.

However, in both lytic refractory and susceptible cells, the amount of cell surface antigen, measured by  $^{125}\text{I}$  GAM directed against mouse anticell antibody adsorbed to the cell, was similar. Also surprisingly, the amount of this antigen decreased to the same extent in cells of both functional states following infection. In interpreting these results, it must be kept in mind that we have not presented any data to indicate whether the binding studies were performed at saturating concentrations of antibody. It can be readily appreciated that at less than saturation, cells with disparate amounts of surface antigen will exhibit the same levels of binding. But, the amount of cell surface antigen was not always the same and decreased with infection (Fig. 9). Such a difference in binding would imply that during infection at least antibody was present in saturating amounts relative to surface antigenic sites. Thus, the lack of a binding assay prevented us from determining with certainty only whether there was a difference of cell surface antigens between uninfected lytic resistant and susceptible cells. It was clear, however, that at a time when all cells were susceptible to lysis (i.e., after arbovirus infection), the amount of cell surface antigen was the same and had decreased from levels in the uninfected state. Here, then, was a paradox. The data up to this point suggested strongly that lytic restoration was due to a re-expression of antigen, while the actual data testing this hypothesis showed a decrease in cell surface antigen following infection. A possible solution to this paradox directs the central conclusion of this report - i.e., viral directed re-expression of host cell antigens must be a selective or local rather than generalized phenomenon. This local effect might involve conformational or steric changes allowing for better "fit" of antibody. It is suggested that this selective or local re-expression of these antigens takes place during the redirection of host cell synthetic processes which follows viral infection, especially those related to arbovirus maturation and release (Cardiff, et al., 1973).

A possible means for this local re-expression might be the selective "unmasking" of cell surface antigens by glycocalyx. In preliminary studies, when  $^{125}\text{I}$  Con A was used to quantify the amount of glycocalyx, no difference could be found in the gross amount of surface glycoprotein in lytic susceptible and lytic resistant states. This result was consistent with either local "unmasking" or redistribution of glycocalyx, perhaps eliminating steric interference with antigen-antibody reactions. Although the  $^{125}\text{I}$  Con A used in these experiments was mitogenically active, the only unequivocal means of proving the specific binding and structural integrity of Con A to the glycocalyx is by the demonstration of its reversibility of binding by methyl mannoside (Powell and Leon, 1970), which was not done during these experiments. This would rule out non-specific binding of structurally altered Con A as the basis for the similarity of binding in both lytic susceptible and resistant states.

It also was observed in a preliminary experiment that trypsinization resulted in an 8-fold increase in immune lysis (Fig. 10). There is a wide spectrum of cell surface changes effected by trypsin which

include: 1) increase in lectin agglutinability (Inbar and Sachs, 1969); removal of some surface glycoproteins (Hynes and Bye, 1974; Hynes and Humphreys, 1974), changes in cell shape (Revel et al., 1974), and the rearrangement of membrane proteins (Nicolson, 1971, 1972). The last effect of trypsin is of interest in light of our hypothesis of local or selective re-expression of cell surface antigens following viral infection. More experiments are clearly needed to uncover the precise mechanism of trypsin enhanced immune cytotoxicity. The trypsin treated cells were somewhat more fragile as evidenced by the higher levels of nonspecific  $^{51}\text{Cr}$  release, but this did not appear sufficient to account for the much larger C dependent specific release.

As an alternative explanation for the data, perhaps the metabolic effects of arbovirus infection might explain these results. Several authors have described that inhibitors of protein synthesis increase a cell's susceptibility to complement induced lysis (Segerling et al., 1974; Boyle et al., 1975). Experiments with actinomycin D on lytic refractory LLC-MK2 cells have not resulted in an increase in lytic susceptibility. Further, while group A arboviruses are known to inhibit cell protein and phospholipid synthesis (Strauss et al., 1969), group B arboviruses such as dengue do not exhibit this inhibitory effect (Shapiro et al., 1973).

Since recent reports implicated a selective role for complement in tumor cell resistance to immune lysis (Ohanian et al., 1973; Ohanian and Borsos, 1974), similar experiments were performed without complement, i.e., ADCC. It should be noted in these studies that the absolute  $^{51}\text{Cr}$  release in the ADCC versus C mediated lysis often differed (See Fig. 12, subculture 8 and 28). These differences in absolute release probably reflected the fact that normal human peripheral blood lymphocytes used in the ADCC experiments were obtained from a series of different donors. Our experience that there was a wide variation in the ability of lymphocytes from different individuals to effect an ADCC response was in agreement with the previous reports of others (Perlmann and Perlmann, 1970). In contrast, C-mediated lysis was carried out with aliquots from the same serum source stored under identical conditions. Because donors were not selected for the killing ability of their lymphocytes in ADCC, it was impossible to interpret Fig. 12 in the same kinetic terms (i.e., variations of immune cytotoxicity with subculture) as in Fig. 5. Further, the studies were not designed to compare the relative effectiveness of C-mediated cytotoxicity versus ADCC. The experiments simply showed that arboviral amplification of immune cytotoxicity occurred without complement, permitting us to exclude differential sensitivity to complement as the basis for the phenomenon. These results were also consistent with some form of antigenic re-expression.

In its broadest sense, these studies described a virus-enhanced alteration in self-reactivity of the cell, and as such, suggested this system as a potential *in vitro* correlate of this phenomenon. In this regard, it is of interest that tolerance is considered by some to be an active process, maintained by self-reacting, noncytolytic antibody rather than a simple deletion of self-reacting, or "forbidden" clones



(Hellstrom and Hellstrom, 1970; Ceppellini, 1971; Phillips and Wegman, 1973). In this schema, antibody, a product of host thymus independent cells, interferes with recognition of self by thymus dependent immunocompetent cells. Clearly an upset of this delicate balance would lead to self-reactivity or autoimmune disease and viruses have long been implicated in this process. On the basis of our *in vitro* data, we might speculate that membrane changes following virus infection may cause a change in the cells such that they now respond to blocking antibody with lysis. Further, host cell antigens may also be so altered as to abrogate the blocking effects of antibody.

### C. Characterizations of flavivirus specified proteins

One method of evaluating the immunological responses of the arbovirus infected host is to assess the antigenic and immunogenic roles of each of the virus specified proteins. Studies directed toward this goal must include an examination of both the structural components of the virion (V-3, V-2 and V-1) and the nonstructural but virus-specified proteins presumably in abundance within the infected cell. Previous experiments describing the presence of the nonstructural virus specified proteins employed chemical inhibitors of normal host cell protein synthesis and utilized radiolabeling techniques that would identify these virus proteins in preparations containing virtually all of the normal cell constituents. Studies of the type proposed are not simple extensions of this earlier work, but rather require the selective extraction of these antigens from the infected cell and subsequent purification from all host cell constituents using procedures sufficiently gentle to provide undenatured and antigenically unaltered proteins with full immunogenic potential. We might obtain the following information: 1) an antigenic characterization of both structural and nonstructural viral proteins with a particular emphasis on those which will induce a protective capacity in animals; 2) a description of the antigens responsible for common group reactivity, crossreactions associated with closely related virus complexes and type specificity; 3) information on the basic biology of flavivirus replication by using specific antisera to monitor each of the virus proteins in the replicative cycle; 4) methods for obtaining viral antigens in quantities sufficient to allow consideration of a subunit vaccine; and 5) as an alternative to immunization, identification of a critical step in the replicative cycle which might be susceptible to nontoxic blocking agents.

#### 1. Immunization of rabbits with infected crude cell extracts

Reference antiserum was prepared against all virus specified proteins as they exist in infected cells as a basis of comparison with antiserum against individual viral specified proteins. Rabbits were immunized with Japanese encephalitis virus (JEV) infected cell debris of three different cell types - suckling mouse brain (SMB), baby hamster kidney continuous liver (BHK-21), and a continuous Rhesus monkey kidney cell line (LLC-MK2). Primary chick embryo cell cultures (CEC) were used

to propagate the virus antigens to test these antisera to eliminate common host cell antigen reactions. Animals were immunized with two injections at a three week interval with infected cells pelleted by centrifugation and disrupted by repeated freeze-thaw cycles. The first injection contained Freund's complete adjuvant and animals were bled 10 days following the second injection. Plaque reduction neutralization (PRNT) and CF titers to JEV infected CEC suspensions (sonicated for disruption) are shown in Table 6. The neutralization titers appeared

Table 6. Rabbit antibody titers in animals immunized with infected cell suspensions

Animal no.	Infected cell type	PRNT titer	CF titer
667	SMB	5120	> 1024
670	SMB	720	1024
664	BHK	110	1024
669	BHK	410	> 1024
665	LLC-MK <sub>2</sub>	140	512
672	LLC-MK <sub>2</sub>	190	512

higher in the animals immunized with the infected SMB suspension but may well only reflect the additional virion concentration in these preparations. JEV replicates to a significantly higher titer in SMB than in any of the cell cultures. The CF titers were very encouraging and, at least under these conditions with infected CEC suspension antigens, are as good as the hyperimmune mouse ascitic fluids (HMAF) routinely employed as reference antisera.

## 2. Analysis of JEV proteins by continuous and discontinuous PAGE

A reference method for the detection of JEV proteins was required to monitor solubilization and purification of experiments. The earlier description of these proteins was accomplished by using 8% polyacrylamide gels in a continuous electrophoresis system (Shapiro et al., 1971). It appeared desirable to attempt modification of this method to a discontinuous system, employing a slab apparatus instead of cylindrical columns. This would allow greater sample volumes, increased protein load, and perhaps greater separation of certain of the JEV proteins previously shown to migrate close together. A comparison of the two methods is illustrated in Figures 13 and 14. Figure 13 illustrates the JEV intracellular distribution of virus specified polypeptides from a conventional 7.5% continuous PAGE cylindrical gel. The identical sample applied to a discontinuous 9% PAGE slab is shown in

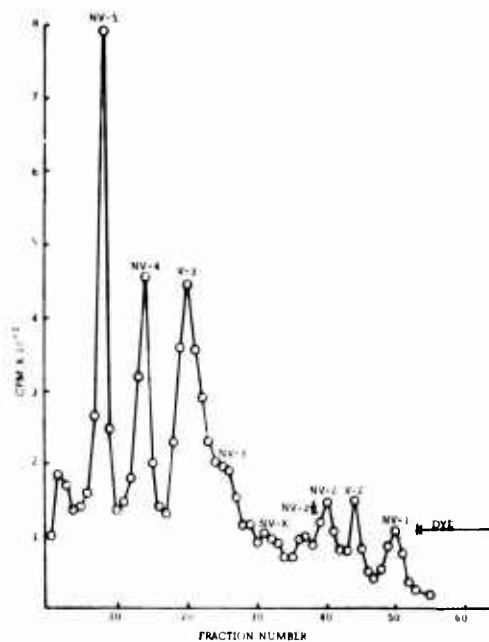


Figure 13. The intracellular virus specified polypeptides of Japanese encephalitis virus separated by electrophoresis through cylindrical 7.5% polyacrylamide in a continuous system.

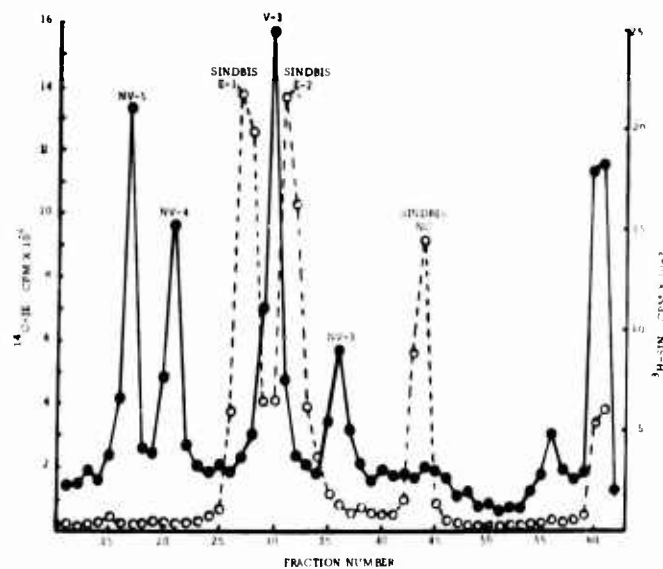


Figure 14. The intracellular virus specified polypeptides of Japanese encephalitis virus and the virion polypeptides of Sindbis virus separated by coelectrophoresis through a slab of 9% polyacrylamide in a discontinuous system.

Fig. 14. Sindbis (SIN) virus was added to this gel as a marker and the representative envelope polypeptides E-1 and E-2 and the nucleocapsid (NC) protein can be easily resolved. It would appear that the discontinuous slab gel system allows good resolution of the higher molecular weight (m.w.) polypeptides and gives even better separation of JEV proteins V-3 and NV-3; however, resolution of the low m.w. polypeptides is less. It is possible that a higher gel concentration would resolve these proteins; however, under these conditions the larger m.w. proteins are not clearly separated. It is difficult to understand the lack of resolution of the lower m.w. polypeptides when the nucleocapsid protein of SIN virus (30,000 m.w.) can be isolated so sharply.

Although not shown here, we have successfully separated each of the intracellular JEV specified proteins by discontinuous slab gel electrophoresis, eluted the proteins from the gel sections and demonstrated their purity by continuous PAGE in small columns. Immunization experiments using these fractions are in progress. An advantage of the slab gel procedure, in addition to larger sample capacity, is the ability to perform autoradiography on these preparations. An autoradiograph of a section of the gel from the slab gel of JEV intracellular proteins co-electrophoresed with Sindbis virions clearly identified the JEV intracellular proteins which were labeled with  $^{14}\text{C}$ -amino acids. The lower energy  $^3\text{H}$ -amino acid label of the SIN marker did not expose the film under these conditions.

### 3. Solubilization of infected cell membrane components

Solubilization of the virus antigens from infected cells was an essential prerequisite to subsequent purification and characterization studies. After infected cell preparations were sonicated and subjected to detergent treatment, complement fixing (CF) antigen can be recovered. Table 7 lists the results of Triton X-100 and sodium dodecyl sulfate (SDS) treatment of sonicated JE-infected cells. To determine the degree of solubilization, sonicated or sonicated plus detergent treated cell preparations were centrifuged at 100,000 xg for 1 hr. The supernatant fluids were assayed for antigen activity and compared to CF titers before centrifugation. Also, the supernatant fluids were analyzed by polyacrylamide gel electrophoresis (PAGE) to monitor the efficiency of detergent solubilization. From Table 7 it is clear that 1% SDS treatment solubilizes all JE intracellular proteins without loss of CF antigen activity. Treatment with 1% Triton X-100 partially solubilizes the proteins, leaving NV-4 and V-2 attached to some membrane or core structure that is sedimented at 100,000 kg. Recently, 0.1 M dithiothreitol, in addition to 1% SDS, has been used to solubilize cell sonicates; this resulted in significantly increased CF antigen titers.

A drawback to SDS treatment is the effect the detergent has on antigenic activity in some immunological tests. After 1% SDS treatment, JE cell extracts do not form precipitin lines in immunodiffusion tests. Also, radioimmune precipitation does not occur with SDS-treated antigens

Table 7. Detergent treatment of JE-infected chick embryo cell preparations

Treatment	% acid ppt material in sup	CF	Polypeptides in sup (PAGE)
None	22%	32 <sup>a</sup> / <sub>8</sub>	None
Sonicate	24%	128/ <sub>16</sub>	None
Sonicate +0.1% SDS	66%	64/ <sub>64</sub>	All intracellular
Sonicate +1.0% SDS	100%	128/ <sub>128</sub>	All intracellular
Sonicate +0.1% Triton-X	40%	64/ <sub>256</sub>	NV-5, V-3, NV-3, NV-2
Sonicate +1.0% Triton-X	31%	128/ <sub>256</sub>	NV-5, V-3, NV-3, NV-2

<sup>a</sup>Numerator = homogenate before centrifugation; demoninator = supernatant following 100 x 10<sup>3</sup> g for 1 hour

even after extensive dialysis of the antigen. Nonetheless, SDS has been the most efficient detergent used to date for membrane solubilization and recovery of JE polypeptides.

As a first step in the separation of SDS-solubilized JE proteins, gel filtration with Sephadex G-200 columns was used. Figure 15 depicts an SDS extract of JE infected cells chromatographed on G-200 with 0.05% SDS in the PBS eluent buffer. Detergent, either SDS or Triton X-100 was necessary for inclusion in the eluent buffer; otherwise, aggregation of the sample occurred and separation was impaired. Continuous PAGE analysis of the CF antigen zones (indicated by XXX in Fig. 15) revealed JE polypeptides V-3 and NV-3 associated with the first CF peak, while NV-2, NV-2½, and V-2 were associated with the second peak. (See Figure 13 for a typical electrophoresis used to identify these proteins). Radioactivity levels in the third CF antigen peak precluded PAGE analysis of this peak.

In order to further purify the JE-CF antigens from G-200 columns, SDS detergent had to be removed so that isoelectric focusing could be used as a purification procedure. A G-200 column equilibrated with 0.1% Triton X-100 in PBS was used to separate JE polypeptides solubilized with SDS. Figure 16 shows the resulting chromatograph of a JE preparation run under the same conditions as those used for Figure 15. Using the Triton X-100/PBS eluent, separation of CF antigens was comparable to a separation using an SDS/PBS eluent. Aggregates of high molecular weight material were not seen eluting in the void volume of the column. Low molecular size material was observed (by radioactivity) between fractions 70 and 80. This may have been missed in the SDS column due to slightly earlier termination of fraction collection.

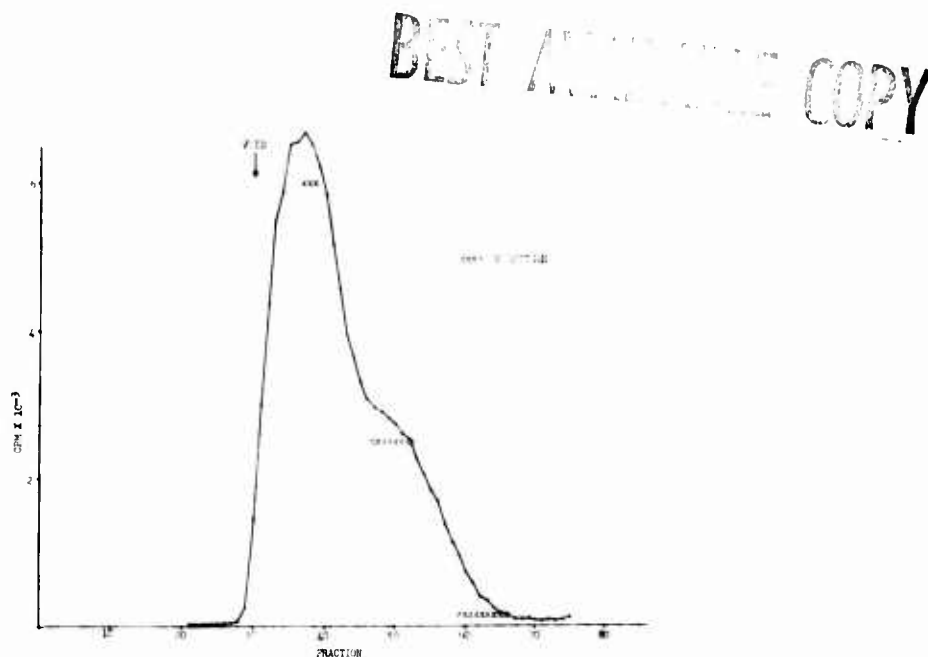


Figure 15. Gel filtrate chromatography of a JE infected cell sonicate solubilized with SDS; eluent buffer is 0.05% SDS in PBS, pH 7.2; XXX indicates detection of CF antigen.

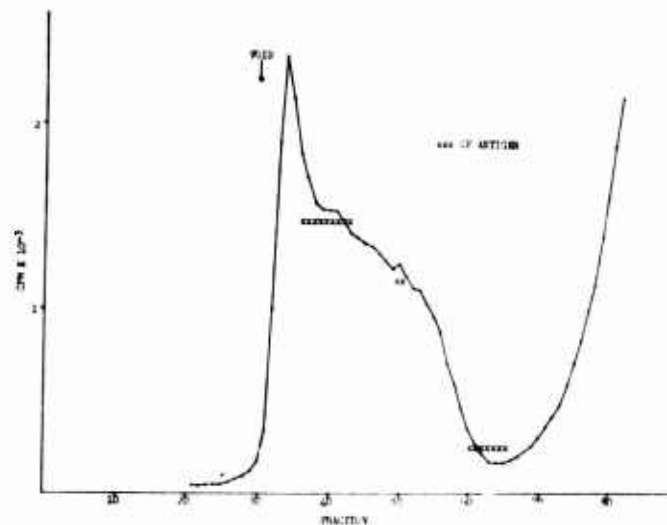


Figure 16. Gel filtration chromatography of a JE infected cell sonicate solubilized with SDS; eluent buffer is 0.1% Triton X-100 in PBS, pH 7.2; XXX indicates detection of CF antigen.

Radioimmune precipitation (RIP) tests of the column fractions containing CF antigen were negative. Apparently, the amount of SDS tolerated in a CF test was much higher than in a RIP test. This question was examined by incubating radioactive JE virions with various concentrations of SDS prior to addition of dilutions of JE HMAF and rabbit anti-HMAF in the RIP test described by Dalrymple et al (1972). The results (Figure 17) show that 0.05% SDS completely inhibits RIP of the virions, 0.01 percent partially inhibited the RIP reaction (plateau of RIP curve reduced by 35%) and 0.005 percent SDS was not detrimental (no more than 5 percent reduction of the RIP plateau). In addition, 0.005 SDS may have partially dissociated the virion preparation into a greater antigen surface area since the titer of the JE antiserum was increased (the reaction curve extended further to the right). The next question was whether or not the higher concentration of SDS required to solubilize virus specific membrane proteins (0.1 to 1%) could be dialyzed or diluted down to 0.005% SDS or less and allow the RIP to function. The virions were treated at the critical micelle concentration (0.2% SDS) and in one instance the sample was diluted to the lowest concentration of SDS where there was still significant radioactivity for counting purposes, and in the other instance the sample was dialyzed for one week in phosphate buffered saline (PBS) with and without Triton X-100. Triton X-100 is a nonionic detergent that does not solubilize as well as SDS, but which does not interfere with the RIP test at 0.1%. Dialysis against Triton was an attempt to exchange it for the SDS micelle, both by treating the sample with 0.2% Triton and then dialyzing

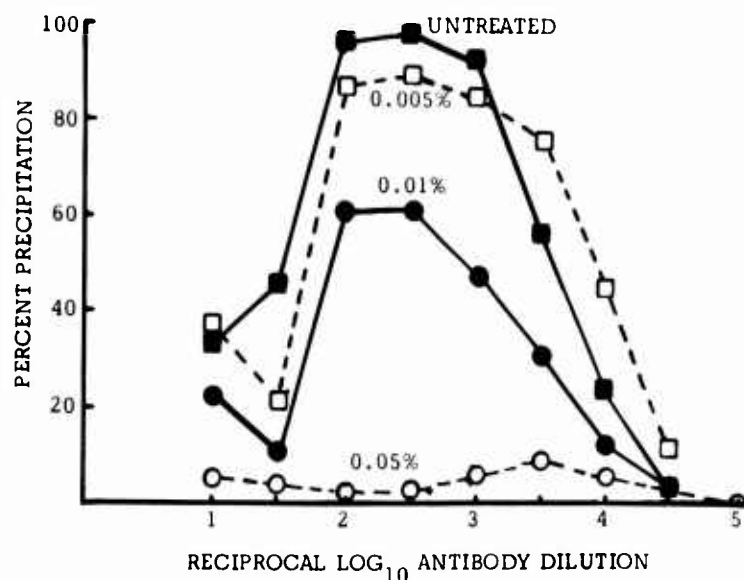


Figure 17. Radioimmune precipitation of Japanese encephalitis virions incubated with several concentrations of sodium dodecylsulfate.

against 0.1% Triton, or simply dialyzing the SDS sample against the 0.1% Triton X-100. With all of the above treatments, the SDS was apparently never removed; once the proteins came in contact with SDS, the RIP test would not work.

Solubilization, using the nonionic detergent Triton X-100, was investigated primarily because the products of such extractions were readily adaptable to subsequent isoelectric focusing procedures and could be assayed in RIP tests. Infected cells specifically radiolabeled in the JEV specified proteins were scraped, frozen and thawed and sonicated for 2 minutes prior to treatment. Criteria for protein solubilization consisted of TCA precipitable counts remaining in the supernatant following centrifugation at 100,000 g for one hour. The results of experiments designed to define the optimal conditions for Triton X-100 solubilization of membranes are shown in Table 8 (see next page).

The detergent Triton X-100 appears effective in solubilizing only an additional 20% of the radioactivity from JEV infected cells. Increasing concentrations of the detergent do not appear to effectively solubilize more protein and actually results in a decrease of the percentage that is protein bound (TCA precipitable). This could be the result of direct interference with the TCA precipitation step or continued degradation of the protein to very small m.w. components. Only slight increases in solubilization, if any, are observed upon the



Table 8. JEV infected cell membrane solubilization with the nonionic detergent Triton X-100

Membrane treatment	% Radioactivity soluble	% TCA precipitable
(Triton X-100 concentrations)		
PBS only	38	53
0.1% TX-100	55	52
0.5% TX-100	56	51
1.0% TX-100	56	34
1.5% TX-100	56	25
2.0% TX-100	56	35
(Triton X-100 plus high salt concentrations)		
1.0% TX-100 (no salt)	68	35
" (0.25 M KCl)	60	46
" (0.5 M KCl)	66	41
" (1.0 M KCl)	67	45
" (2.0 M KCl)	67	46
(Triton X-100 with high salt plus reducing agent)		
1.0% TX-100 (2.0 M KCl) + 0.05 M DTT*	93	61
1.0% TX-100 (2.0 M KCl) + 0.1 M DTT	88	50
1.0% TX-100 (2.0 M KCl) + 0.2 M DTT	88	40
1.0% TX-100 (2.0 M KCl) + 0.5 M DTT	94	28
1.0% TX-100 (2.0 M KCl) + 1.0 M DTT	79	24

\* DTT = dithiothreitol

addition of salt; however, the addition of a reducing agent such as dithiothreitol (DTT) solubilized the majority of the radioactivity. Further solubilization experiments are contemplated.

#### 4. Preliminary isoelectric focusing experiments

Isoelectric focusing of purified JE virions disrupted with Triton X-100 have yielded one major fraction (pI 7.6) and three minor fractions at pH 3.7, 8.5 and 10.0. The major fraction at pI 7.6 appears to represent the major envelope glycoprotein (V-3) in pure form. The composition of the other components is less clear but appears to represent V-2 together with varying amounts of V-3. The other envelope glycoprotein V-1 cannot be detected. Isoelectric focusing of solubilized JEV infected cell membranes will be feasible only after adequate solubilization in a non-ionic detergent has been accomplished.

#### D. Kinetics of California encephalitis protein synthesis in BHK-21 cells

Six viral proteins synthesized in BHK21 cells infected with BFS-283 California encephalitis (CE) virus were identified by polyacrylamide gel electrophoresis and designated as follows: Nucleoprotein (VP-1), three glycoproteins (VP-2, VP-3, and VP-4), and two nonstructural proteins (NVP-1 and NVP-2). Incorporation of <sup>3</sup>H-amino acids into each viral protein could be detected by the first hour after infection, and as early as twenty minutes for nucleoprotein and the two nonstructural proteins. Overall viral protein synthesis reached a peak approximately 3 hours after infection, but then continued at a relatively high rate throughout the infectious cycle. At approximately 12 hours after infection, the largest envelope protein (VP-4) began to decrease in the rate of synthesis. Short pulses of <sup>3</sup>H-amino acids revealed no uncleaved precursor peptides that could be chased into structural peptides. Antibody prepared against virus infected cell homogenates precipitated the four structural proteins, but not the two non-structural proteins.

#### 1. Background

Extensive studies have been conducted on structural and non-structural protein synthesis of group A and B togaviruses. Group A togaviruses have been shown to induce 5 non-structural and 3 structural proteins in infected cells (Hay et al., 1968; Morser et al., 1973). Also, group A togavirus proteins appear to be cleavage products synthesized from high molecular weight polypeptides that accumulate in infected cells (Snyder and Sreevalsan, 1973). Similar studies have been conducted on group B togavirus members (Shapiro et al., 1971; Westaway and Reedman, 1969). No evidence has been presented suggesting post-translational cleavage as the mechanism by which group B virus proteins are synthesized.

The biochemical and biophysical characteristics of the Bunyavirus members are less defined at this time. Evidence has been presented that some members of the California encephalitis (CE) complex contain three structural proteins. Other reports presented evidence that some CE group members contain four structural proteins (Kascsak and Lyons,

ASM Abstracts, 1975; White, 1975). There is at present no unanimity of opinion regarding the exact number and types of structural proteins these viruses contain. Recently, Obijeski (1975) reported the detection of six polypeptides in infected BHK21 cells specified by LaCrosse CE virus. This report describes the cellular synthesis of four structural proteins and two nonstructural proteins, specified by CE virus.

## 2. Methods

a. Infection and labeling. BFS-283 CE from infected suckling mouse brains (SMB) was used as seed virus to infect monolayer BHK21 - Clone 13 cells and assayed by plaque formation. Monolayers of BHK21 cells (106 cells/60 mm petri dish) were exposed for 10 hours to 5 ml of Medium 199 containing 1% fetal bovine serum (FBS) and actinomycin-D (0.5  $\mu$ g/ml). Cultures were drained, washed twice with Hank's balanced salt solution (HBSS) and infected with SMB grown virus (50-100 pfu/cell) for 15 minutes. Virus inoculum contained actinomycin-D (0.5  $\mu$ g/ml). Control monolayers were mock-infected with 20% SMB. Unadsorbed virus was poured off, the cells were washed twice with HBSS, and Medium 199 (5 ml/dish) containing actinomycin-D (0.5  $\mu$ g/ml) was added.  $^3$ H-amino acid mixture (20 uCi/ml) or  $^{14}$ C-amino acid mixture (2 uCi/ml) (New England Nuclear) was added to infected cells 15 minutes before harvest. For analysis of proteins synthesized 15 minutes after infection, radioactivity was added with virus inoculum.

b. Total cell lysates. Following the labeling period, the BHK21 monolayers were washed twice with HBSS, and drained. Cells in each monolayer were scraped into 0.5 ml of phosphate-buffered saline (PBS), pH 7.2, containing 1% sodium dodecylsulfate (SDS), 0.1 M mercaptoethanol (2-ME), and 0.5 M urea. After incubation for 1 hour at 37°C, the disrupted cell suspension was dialyzed at room temperature for 18-20 hours against 400 ml of phosphate buffer (pH 7.2) containing 0.1% SDS, 0.1 M 2-ME, and 0.5 M urea. Extracted protein samples were stored at -70°C for future use.

c. Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis procedures were the same as those described in previous Annual Reports. Cellular extract samples of 50  $\mu$  liters were layered on gels and electrophoresed. In double-labeling experiments,  $^{14}$ C-spillovers were corrected for.

d. Preparation of antiserum. Anti-viral antibodies were prepared in mice by the method of Brandt et al (1967). Adult mice were inoculated subcutaneously with 20% infected suckling mouse brain suspensions on days 1 and 3, and intraperitoneally on days 24 and 28. Sarcoma 180 cells were used to induce ascites fluid. Ascitic fluids were harvested on day 41. Anti-mouse antibodies were prepared in rabbit. Rabbits were injected intradermally at four separate sites with a total of 1 ml whole mouse serum mixed with equal volumes of Freund's complete adjuvant and again 1 month later with 1 ml mouse serum only. Immune serum was harvested 10 days after the last injection.

### 3. Results

#### a. Growth kinetics of BFS-283 CE virus in BHK<sub>21</sub> - clone 13 cells

When inoculated onto monolayer cultures of BHK<sub>21</sub> cells at a multiplicity of 50-100 pfu/cell, BFS-283 CE virus has an eclipse period of 2-3 hours. Figure 18 depicts the growth curve, showing cell-associated and released virus yields. Cell-associated virus production preceded released virus by approximately 5 hours, reaching a peak in 9 hours, then declining thereafter. Released virus was detectable at approximately 8 hours after infection, reaching a peak at 18-20 hours, then declining. Cytopathic changes began to appear at about 8 hours after infection, and by 18 hours most of the cells were rounded and detached from the surface of the petri dish. Thus the cessation of virus production coincided with the time when cytopathic effects became maximal.

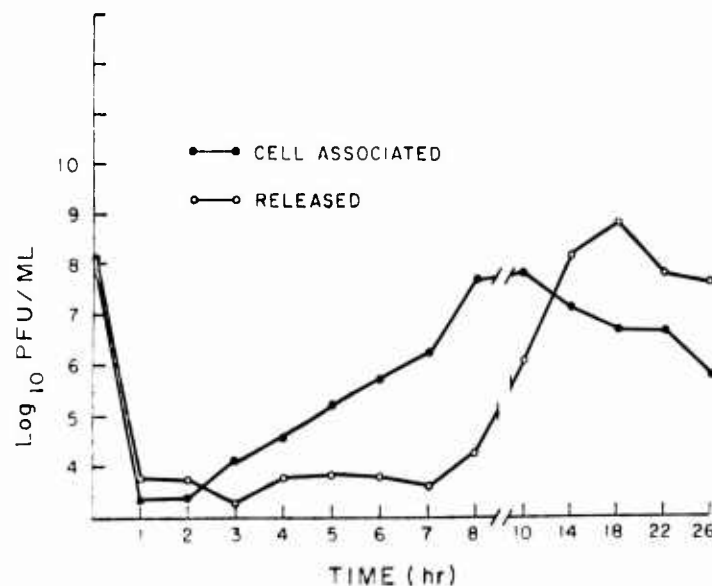


Figure 18. Growth curves of released and cell-associated virions grown in BHK<sub>21</sub> cells. Monolayers were inoculated at a multiplicity of 50-100 pfu/cell

b. Virus directed protein synthesis in BHK cells. The overall rate of viral-coded protein synthesis was demonstrated by measuring the incorporation of  $^3\text{H}$ -amino acid mixture into actinomycin-D treated BHK21 cells infected with BFS-283 virus at a multiplicity of 50-100 (Figure 19). Maximal incorporation occurred at 3 hours after infection, followed by a rapid decline, then leveled off at approximately 6 hours. A level of approximately 25% of maximal protein synthesis was maintained throughout the remaining 12 hour test period. Stimulation of uptake of  $^3\text{H}$ -amino acids by mock infected cells has yet to be done.

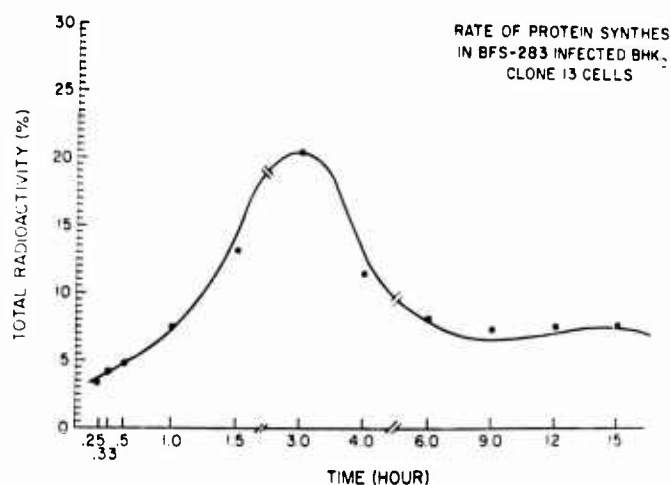


Figure 19. Overall rate of viral-coded proteins synthesized in actinomycin D treated BHK21 cells. Cells were pulsed with  $^3\text{H}$ -amino acid mixture for 15 minutes just prior to harvest.

c. Kinetics of protein synthesis. The kinetics of viral coded protein synthesis was studied by infecting BHK21 cells at high multiplicities ( $m \approx 50$ ) and labeling for 15 minutes with pulses of  $^3\text{H}$ -amino acid mixtures, at a concentration of  $20 \mu\text{Ci}$  per ml. Proteins were extracted from whole cells and analyzed by SDS-phosphate continuous electrophoresis, along with  $^{14}\text{C}$ -amino acid labeled BHK21 whole cells, mock-infected with normal suckling mouse brains. Figure 20 shows the electrophoretic profiles of total  $^3\text{H}$ -proteins recovered from  $4 \times 10^6$  BHK21 cells (two monolayer cultures) at various times after infection (15 minutes adsorption time). At 15 minutes after infection, two non-structural proteins (see Figure 21 for virion or structural proteins) were clearly detectable in nearly equal amounts. By 30 minutes after infection, nucleoprotein (VP-1) and the smallest glycoprotein (VP-2) could be detected. By 1 hour after infection, all four structural proteins were clearly identifiable. All of the viral coded proteins reached

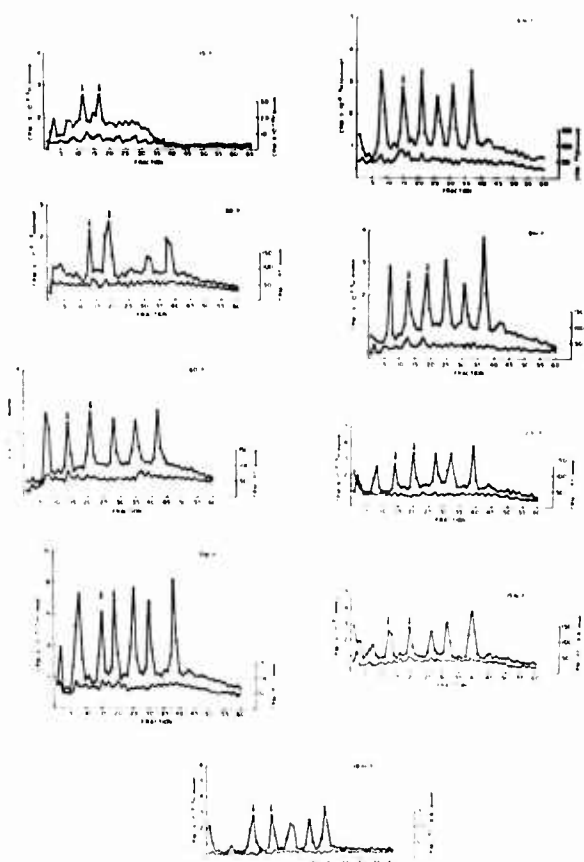


Figure 20. Electrophoretic profiles of viral-coded proteins derived from BHK21 infected cell extracts, pulse-labeled for 15 minute periods with a complete mixture of  $^3\text{H}$ -amino acids at various times after infection. Nonstructural proteins are indicated by arrows (▼▼). Infected cell extracts were co-electrophoresed with mock-infected BHK21 cell extracts labeled with a complete mixture of  $^{14}\text{C}$ -amino acids:  
 — ● — ● — ,  $^3\text{H}$  CPM, — 0 — 0 — ,  $^{14}\text{C}$ -CPM.

maximal synthesis at approximately 3 hours after infection, then abruptly declined; however, all proteins continued to be synthesized in substantial amounts, with the exception of the largest structural protein (VP-4). Incorporation of  $^3\text{H}$ -amino acids into VP-4 decreased beginning at approximately 12 hours and was essentially stopped at 18 hours post infection in order to gain greater confidence that the 2 nonstructural proteins observed at 15 minutes are viral specified. Mock-infected cells will be labeled with  $^3\text{H}$  amino acids, rather than  $^{14}\text{C}$  amino acids, since the  $^3\text{H}$  compounds have a higher specific activity. Since CE is a negative strand virus (Bouloy et al., 1973, 1975), the detection of

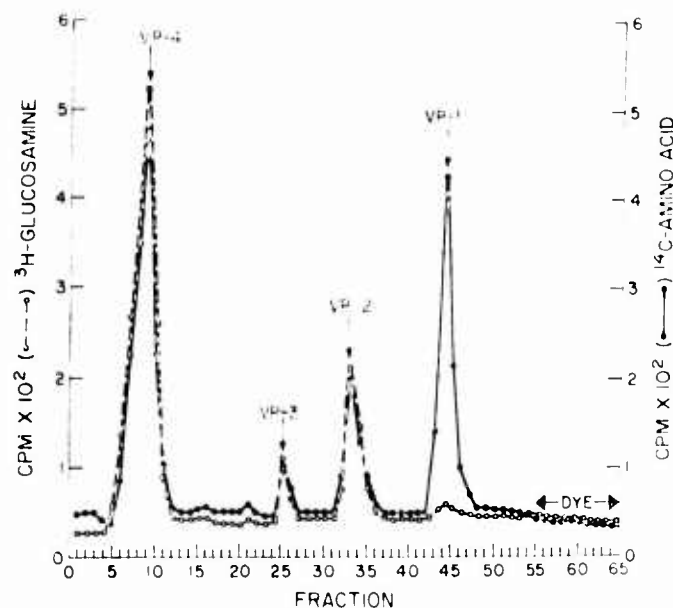


Figure 21. Electrophoresis of polypeptides of virions obtained from infected LLC-MK<sub>2</sub> cell culture supernates doubly labeled with <sup>3</sup>H-glucosamine and <sup>14</sup>C-amino acid mixture. —○—○—, <sup>3</sup>H CPM; —●—●—, <sup>14</sup>CPM.

virus specific proteins at 15 minutes in a surprising result, representing a most sensitive system.

The radioactivity in the virus isolated from cultures was compared with that in the induced proteins (Table 9). All structural protein in infected cells were produced in nearly equal amounts, whereas VP-3 on released virus was significantly lower than the other structural polypeptides.

Figure 22 illustrates the rates of protein synthesis of individual polypeptides for each time period (labeled with 20  $\mu$ Ci <sup>3</sup>H-amino acid mixture for 15 minutes just prior to harvest). The CPM associated with individual polypeptides at each time period tested was compared to the sum of the CPM associated with that polypeptide for all times tested. The CPM from the three highest points on acrylamide gel profiles were used for this calculation. All proteins reached a peak in the rate of synthesis at about 3 hours after infection. Structural proteins were

Table 9. Total percentage of each structural and nonstructural polypeptide synthesized in 18 hours

	All virus-specified polypeptides	Intracellular structural polypeptides	Extracellular virion polypeptides
NVP-1	18.1	--	--
NVP-2	16.1	--	--
VP-1	19.4	29.0	28
VP-2	14.6	22.3	23
VP-3	14.6	22.0	15
VP-4	16.7	26.7	34

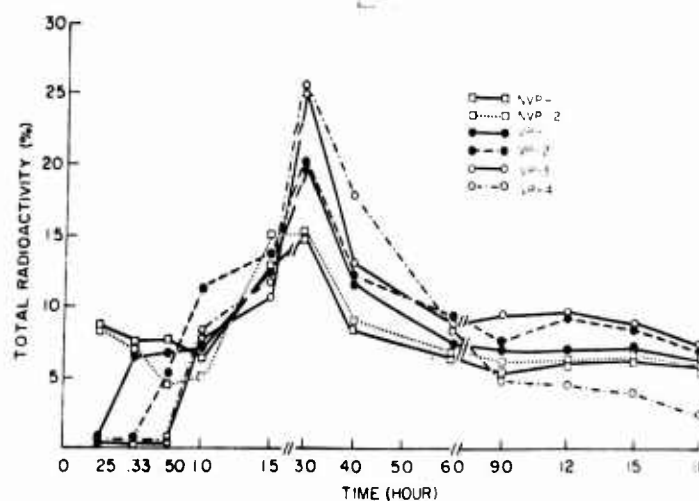


Figure 22. Distribution of the amount of radioactive label incorporated during each 15 min pulsing period in individual viral structural and nonstructural proteins. The amount of radioactivity in a given peak is expressed as a percentage of the radioactivity incorporated in that specific protein through the 18 hr growth cycle. — □ — □ —, NVP-1, -- □ --- □ --, NVP-2, — ● — ● —, VP-1, -- ● --- ● --, VP-2, — ○ — ○ —, VP-3, -- ○ --- ○ --, VP-4.



synthesized in the order of VP-1, VP-2, VP-3, and VP-4. The nonstructural polypeptides (NVP-1 and NVP-2) clearly appeared before the structural polypeptides and existed in very similar amounts throughout the cycle. The largest structural polypeptide, VP-4, declined in the rate of synthesis significantly more than the other polypeptides.

d. Pulse chase experiment. An experiment was designed to determine whether the polypeptides of BFS-283 CE virus were synthesized from a large precursor molecule such as observed with alpha togaviruses. To answer this question, BHK<sub>21</sub> cells were infected at a multiplicity of 50-100 and incubated with 5 ml of amino acid-deficient 199 Medium. At 1, 3 and 6 hours after infection, each culture was pulse labeled for 10 min with 20  $\mu$ Ci/ml of <sup>3</sup>H-amino acid mixture. The pulse was stopped by washing cells with HBSS containing 5 mg/ml of unlabeled amino acid mixture and chased by reincubation with unlabeled amino acid mixture in amino acid free 199 Medium for 60 minutes. Cells from two plates ( $4 \times 10^6$  cells) were harvested and proteins extracted and analyzed by polyacrylamide gel electrophoresis. Figure 23 shows that there is no evidence of a polypeptide precursor from which viral coded peptides were synthesized, since no preferential disappearance or accumulation of radioactive peaks occurred in any of the regions during the chase. Electrophoretic profiles are similar to the 1, 3 and 6 hour samples shown in Figure 21 (without a chase).

e. Radioimmune precipitation (RIP) of BFS-283 structural proteins. In order to identify viral proteins in infected cell extracts that would react with antibody to CE infected cell homogenates, the radioimmune precipitation technique of Dalrymple et al. (1972) was used. Equal volumes (0.05 ml) of virus antibody, diluent and test antigen were mixed and incubated for 1 hr at 37°C. Rabbit anti-mouse serum (0.05 ml) was then added and the mixture incubated for one hr at 37°C, followed by overnight incubation at 4°C. Mixtures were then centrifuged at 15,000 rpm for 5 minutes in a Beckman microfuge, 0.1 ml mixture of supernatant and precipitate. Controls included test antigen, normal ascitic fluid, and anti-globulin. Precipitation was calculated by using the difference in radioactivity (CPM) of the test and control samples. When infected cell extracts (1, 3 and 6 hrs after infection) were treated with antibodies, approximately 80% of the radioactivity was precipitated. These are the only experiments in this laboratory where RIP occurs in the presence of SDS. Control experiments using normal ascitic fluid precipitated no radioactivity. When precipitated extracts were analyzed by PAGE (Figure 24), four distinct radioactive peaks were obtained which corresponded to purified virion polypeptides shown in Figure 21.

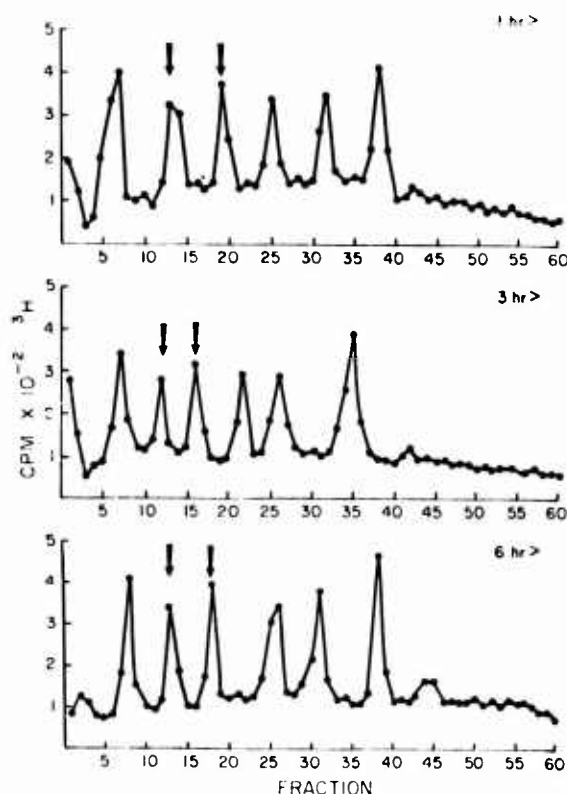


Figure 23. Virus specific proteins labeled during a pulse and subsequent chase. Two BHK<sub>21</sub> cultures were infected with BFS-283 virus and incubated at 1, 3, and 6 hrs in amino acid free 199 medium containing 5  $\mu$ g actinomycin-D per ml. Cells were then exposed to <sup>3</sup>H-amino acid mixture (20  $\mu$ Ci/ml) for 10 min. At the end of the pulse, cultures were washed twice with warm HBSS. Cultures were then incubated at 36°C for 60 min in amino acid free 199 medium containing 5 mg/ml of unlabeled amino acid mixture, — ● — ● —, <sup>3</sup>H CPM.

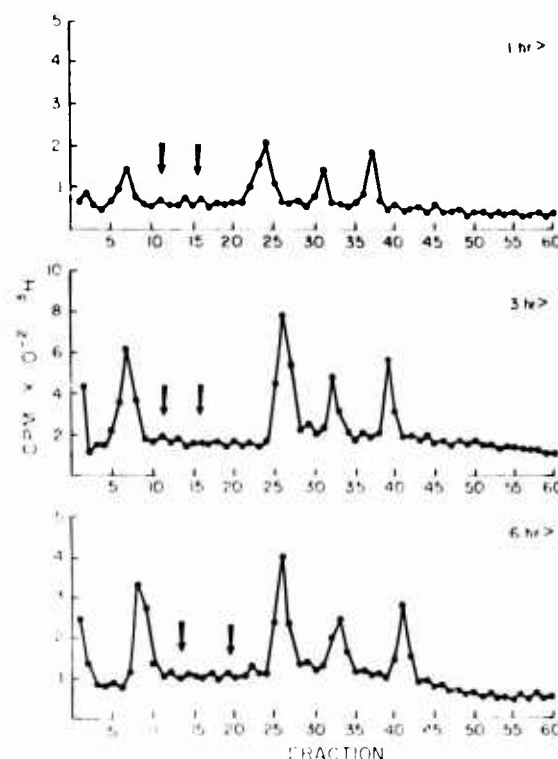


Figure 24. Polyacrylamide gel electrophoresis of radioimmune precipitates of  $^3\text{H}$ -amino acid-labeled infected BHK21 cell extracts. Cells infected with BFS-283 virus for 1, 3, and 6 hrs, were washed twice with warm HBSS, then solubilized in 0.5 ml phosphate buffer containing 1% SDS and 0.5 M urea. Extracts were dialyzed with 400 ml phosphate buffer, pH 7.2 at room temperature for 24 hrs. Extracts were reacted with mouse anti-BFS-283 ascites fluid, then subsequently reacted with rabbit anti-mouse serum, — 0 — 0 —,  $^3\text{H}$  CPM.

#### E. Morphogenesis of CE virus during the early stages of the replication cycle.

The development of BFS-283 California Encephalitis (CE) virus during the early stages (3 min to 4 hrs) of infection in BHK21 cells was studied by electron microscopy. Cells were infected at a multiplicity of 50-100 pfu/cell. In the initial stages of morphogenesis, ribosome-like rosettes formed at and apposed intracytoplasmic vacuolar membranes. After invaginations of the vacuolar membranes, membrane enclosed nucleoids (approximately 60 nm in diameter) with an electron dense core were

seen inside the vacuole. Nucleoids released by intracytoplasmic vacuoles were subsequently pinocytosed by other intracytoplasmic vacuoles, thereby obtaining a second membrane which became the viral envelope of mature viral particles. The mature viral forms with a diameter of 95-100 nm were detected as early as three hours after infection.

Although numerous electron microscopic studies have been conducted on the morphology and development of Bunyavirus, none has revealed nucleoids or previral forms. One possible reason for this was that all studies were done with cells harvested late (18-36 hrs) in the infectious cycle. This report is an electron microscopic study of the morphogenesis of BFS-283 CE virus during the early stages (3 min - 4 hrs) of the infectious cycle in BHK21 - clone 13 cells.

### 1. Electron Microscopy

Infected cells were examined for virus development at frequent intervals, commencing one minute after infection and ending 4 hrs after infection. Infected and control monolayer cultures in 60 mm Petri dishes were prefixed in situ with 2.5% phosphate buffered glutaraldehyde (pH 7.2) for 1 hr at 40°C. Cells were rinsed three times in phosphate buffer (pH 7.2) and postfixed for 1 hr at 40°C in 1% phosphate buffered osmium tetroxide (pH 7.2). After dehydration in graded aqueous ethanol and propylene oxide, specimens were embedded in Epon. Ultrathin sections were cut with a diamond knife on a Porter-Blum MT-2 ultra microtome, mounted on bare 300 mesh cooper grids, stained for 30 min with 4% uranyl acetate and counter-stained 15 min in 0.5% alkaline lead citrate (Lyons and Heyduk, 1973). Specimens were examined on a Hitachi HU-11C electron microscope.

### 2. Electron microscopy of infected BHK21 - clone 13 cells

The earliest changes observed in infected cells occurred approximately 3 min after infection, as uncoated entering viral particles were detected in the cytoplasm of infected cells (Figure 25). Newly synthesized viral-precursors were not detected until approximately 30 min after infection. However, as early as 10 min after infection, numerous vacuoles were readily seen in the cytoplasm and between the nuclear membranes (Figure 26). The first observation of pre-viral structures was seen approximately 30 min after infection (Figure 27). Ribosome-like pre-nucleoid clusters arranged as rosettes, with each cluster consisting of 12-15 electron-dense particles, were seen on the cytoplasmic surface of intracytoplasmic vacuoles (Figure 28). These clusters were endocytosed into the vacuoles' interior, forming nucleoids. Many types of nucleoids were seen inside vacuoles (Figure 29), as some were compact sphericles (60 nm) with a uniform electron-dense core, while others were larger (100-200 nm) with less uniformity in electron-dense cores (Figure 30). Occasionally seen were elongated particles (Figure 30) similar to those reported by others (Murphy et al., 1968).



Figure 25. Uncoated virions detected 3 min after infection, resulting from infecting input virus.

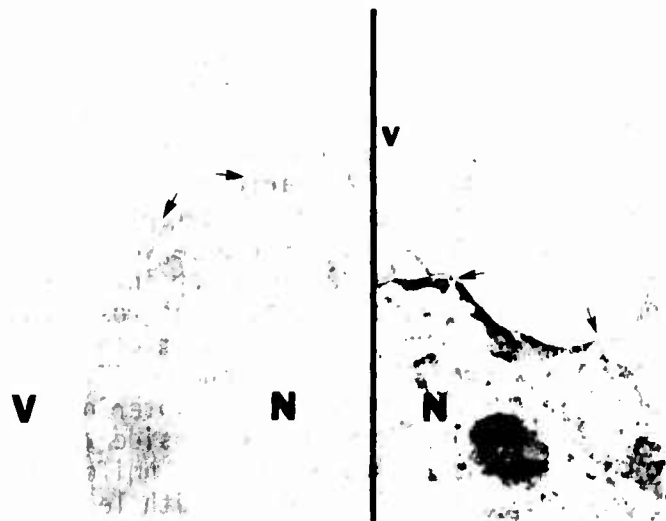


Figure 26 (A & B). Fifteen minutes after infection, cells show vacuolation (V) in the cytoplasm. Frequently, vacuoles involved the outer nuclear membrane (arrow).



Figure 27. Prenucleoid particles have formed approximately 30 min after infection at the vacuola surface and are migrating to the interior. Circular arrays of particles are seen (PN), and each array contains 12-15 electron dense particles. One 60 nm nucleoid is shown near the right center of the pack (arrowhead).

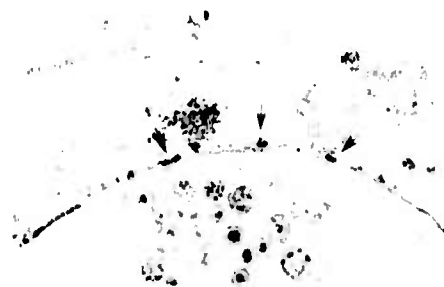


Figure 28. An intracytoplasmic vacuole with rosettes at sites on the outer vacuolar membrane (arrows). Inside the vacuole are different forms of nucleoids.

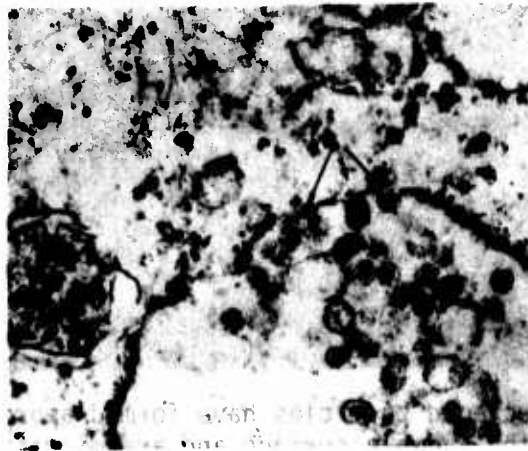


Figure 29. Approximately 45 min after infection, pre-nucleoid particles can be seen entering a vacuole (arrows).



Figure 30. After 1 hr infection, many nucleoid forms are seen in vacuoles (arrows), including elongated, rod-like forms (arrowheads).

The maturation process of cell-associated virions occurs very early when cells are infected at a high MOI, as the two final stages of virion development was observed in a single cell only 3 hrs after infection (Fig. 31). This correlates well with the production of BFS-283, cell-associated virus (Fig. 18). During this period, we observed numerous virions in the process of obtaining outer envelopes (Fig. 31). The enveloped areas had surface filaments, whereas the nonenveloped areas were smooth (Fig. 31). Furthermore, mature virions produced prior to cytopathic changes had uniform surface filaments over the entire particle (Fig. 31), whereas mature virions observed in the late stages of the growth cycle (post cytopathic stage) were enclosed by a halo-type membrane. These observations suggest the morphogenesis scheme depicted in Figure 32.



Figure 31. Virions are shown in the final stages of development after 3 hr infection. These particles are budding through vacuolar membranes, and the membrane becomes the viral envelope (ve). Inside the vacuole are nucleoids (n), and mature virus particles (mv).

Differences in pre-cytopathic and post-cytopathic forms (also observed by Lyons and Heyduk, 1973), could possibly mean a differential staining of virions due to the absence or presence of a particular protein molecule in the viral envelope. Shapiro, et al. (1972) showed that cell-associated virions contained two membrane glycoproteins, whereas released virions lost the sugar moiety of one of the glycoproteins.



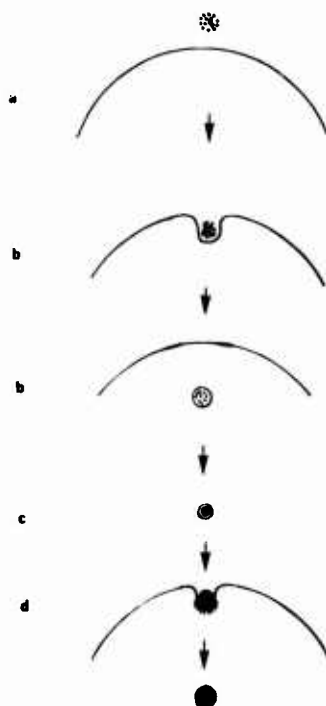


Figure 32 (A,B,C,&D). A schematic diagram illustrating the formation of CE virus. 32-A) Pre-nucleoid particles assemble at vacuolar membranes in the cytoplasm, and 32-B) endocytose to the vacuole's interior. 32-C) These particles condense into a smaller nucleoid, with electron-dense cores. 32-D Finally, the nucleoid is released by the host vacuole and endocytosed by other vacuoles. The nucleoid becomes completely enclosed with this membrane and is the mature virus particle (virion).

Three independent steps in the formation of CE virus can be distinguished morphologically in BHK<sub>21</sub> - clone 13 cells; (i) the assembly of pre-nucleoid-like structures at sites on the intracytoplasmic vacuoles, (ii) migration into these vacuoles forming nucleoids, and (iii) release of nucleoids and subsequent budding of these nucleoids through a second intracytoplasmic vacuolar membrane, where that membrane

becomes the viral envelope. Numerous empty viral envelopes (Fig. 33) were observed throughout the cytoplasm.



Figure 33. 14-hr post-infection. Mature virions (mv) are seen throughout the cytoplasm (arrows). Also seen are empty envelopes (ee). X20,000.

Although the development of nucleoids now seems quite clear, there yet appears to be many different morphological types of nucleoids. One possible explanation might be that at high multiplicities, as used in these experiments, CE virus could produce high levels of defective particles (Kascsak & Lyons, 1975). Friedman, et al. (1974), recently studied the assembly of Herpes simplex virus after removal of a hydroxyurea (hu) block from infected cells. Hydroxyurea is a drug that reversibly inhibits both virus and host DNA production and when removed from infected cells, complete synchrony results with the appearance of developing virions. When they removed Hu from infected cells, nucleoids appeared in a sequence of developmental forms, which resembled many of the forms shown in this study (Figs. 28, 29, 30). Thus, another explanation might be that heterotypic forms of nucleoids presently observed may be due to virions at different stages of development.

The rapidity in viral development noted in this report correlates well with the production of cell-associated virus (Fig. 18) and the kinetics of viral protein synthesis analyzed under the same conditions

(previous section). We have shown that all viral coded proteins were synthesized by one hour post-infection and reached a peak at 3 hrs PI. Furthermore, immune cytolysis experiments with the same system showed that viral coded antigens were detectable on the surface of BHK21-clone cells as early as 3 hrs post-infection (future report).

## II. Respiratory viruses

### A. Adenovirus

#### 1. Adenovirus ARD in basic combat trainees

During fiscal year 1976, acute respiratory disease (ARD) rates in basic combat trainees (BCTs) were higher than in FY 1974 and 1975. Two developments accounted for this increase: 1) outbreaks of influenza A virus during January through March 1976, and 2) the emergence of adenovirus type 21 (Adv 21). This was the first appearance of Adv 21 in BCTs since 1967.

As in past years, ARD due to adenovirus types 4 and 7 was well controlled by immunization of live, oral adenovirus type 4 and 7 vaccines. Type 4 vaccines used (Wyeth Lots 06701 and 06901) titered 5.4 and 5.1 log<sub>10</sub>, respectively, while the type 7 vaccines (Wyeth Lots 06801 and 07001) titered 6.7 and 6.4 log<sub>10</sub>. The former lot of each type was used from the beginning of immunization at BCT posts into February 1976 and the latter lot was used thereafter.

Influenza A strains were isolated from BCTs at all but one post (Fort Sill) in winter 1976. Aside from the outbreak of influenza A/swine virus identified at Fort Dix (see following), all isolates resembled A/Victoria/1975. While most strains were isolated in January through March 1976, isolates were obtained from Forts Bliss and Knox in April and from Fort Bliss in May. Influenza A isolates were temporally associated with ARD rates of 1.5 - 2.6/100/week at Forts Ord, Gordon, Dix, and Jackson, and with rates between 3.0 - 4.0/100/week at Forts Bliss, Wood, and Knox (adenovirus type 21 was prevalent at all posts during the peak of influenza A activity and undoubtedly contributed to these elevated rates). In addition, 2 strains of influenza B were isolated from Fort Ord trainees in February.

In the absence of an unimmunized control group, it is impossible to gauge the efficacy of the military influenza vaccine containing 350 CCA units each of A/Port Chalmers/73 and A/Scotland/74, as well as 500 CCA units of influenza B/Hong Kong/72 against the A/Victoria/75 strain. It is of interest that 64 of the 82 (78%) A/Victoria-like strains isolated in the Adenovirus Surveillance Program were from BCTs in the reception center or during the first 2 weeks of training. Only 22% of isolates were from trainees in later weeks of training (which comprise the larger part (approximately 5/8) of the total BCT population). These findings are similar to WRAIR's virus isolation experience of Fort Dix this

February during which 22 (80%) of 25 A/Victoria isolates in BCTs were obtained in trainees before the 3rd BCT week. The preponderance of A/Victoria isolates among BCTs in early weeks of training is consistent with a high degree of protection conferred by this year's influenza vaccine approximately 3 weeks after immunization.

Adenovirus type 21 was first isolated in trainees at Fort Knox in October 1975 and at Fort Wood in November. By February 1976, the virus had been identified at all Army BCT posts and it has been just recently isolated from recruits in San Diego Naval Training Center. ARD rates associated with adenovirus type 21 peaked in February and March and attained 1.0 to 1.8 at Forts Bliss, Gordon and Sill, 2.0 to 2.6 at Forts Dix, Jackson and Knox, and 4.1 at Fort Leonard Wood. Most distressing is the persistence of adenovirus type 21 at all BCT posts adequately sampled into June 1976.

The following is a summary of adenovirus surveillance data at various BCT posts:

a. Fort Bliss. Basic combat training began at this post in January and BCTs were immunized with both type 4 and 7 adenovirus vaccines from January through 1 May. Only one strain each of Adv 4 and Adv 7 was isolated from the 308 throat swabs of ARD hospitalizations submitted through 1 June (0.6%). Influenza A/Victoria isolates were associated with peak ARD rates of 3.0/100/week in early February. Adv 21 was first isolated in January, caused peak rates of 1.0/100/week in March when isolated from 38% of hospitalized BCTs, and is still present on post in June.

b. Fort Dix. Adenovirus type 4 and 7 vaccines were used from 1 September 1975 through 1 May 1976; only one strain each of Adv 4 and Adv 7 were isolated from 742 throat swabs submitted during this period. Coxsackievirus A-21 was isolated from 44 of the 466 throat swabs obtained from July through December 1975 (9.4%), but was never associated with ARD rates greater than 1.3/100/week, and was not recovered after December 1975. Influenza A/swine virus caused ARD in January and A/Victoria virus caused ARD from January through March; the peak ARD rate was 2.2/100/week, but rates were much higher in the early weeks of training. Adv 21 was first isolated in January, caused a peak rate of 2.0 associated with recovery of virus in 45% of throat swabs in March, and remains at Fort Dix in June 1976.

c. Fort Gordon. This post first received BCTs in October 1975. Adenovirus vaccines were used from 1 October through 1 May 1976. Two isolates of Adv 4 and 9 isolates of Adv 7 were made from the 261 throat swabs submitted during this period (4.2%). Adv 21 appeared in January and although it was associated with 24% of ARD hospitalizations from mid-February to mid-March, peak rates were but 1.8/100/week. Sporadic isolates of A/Victoria were made in February and March 1976.

d. Fort Jackson. Occasional isolations of both Adv 4 and 7 were made in November and December, 1975; the ARD rate fluctuated between 0.9 - 1.7/100/week during these months. Adv 4 and 7 vaccines were used from 1 October 1975 through 1 May 1976; only 7 isolates of Adv 4 and 17 isolates of Adv 7 were made from the 416 throat swabs submitted during that period (5.7%). Most isolates were obtained in the month immediately after beginning immunization. Influenza A/Victoria virus was isolated from trainees in March. Adv 21 first appeared in February, was isolated from 33% of 85 throat swabs submitted in March when it carried ARD rates between 1.7-2.6/100/week, and has persisted into June at Fort Jackson.

e. Fort Knox. Coxsackievirus A-21 was isolated from 12% of the 420 throat swabs obtained from trainees with ARD from July through September but was rarely associated with ARD rates greater than 1.0/100/week. Scattered isolates of Adv 7 and Adv 4 were made from August through November and Adv 21 was first isolated from Fort Knox in October. Adv 4 and 7 vaccines were used from 1 October through 1 May 1976; during that period only 5 type 4 and 9 type 7 strains were isolated from the 749 throat swabs submitted (1.9%). Adv 21 was associated with peak rates of 1.7 - 2.0/100/week in February and March when 28% of all throat swabs yielded this pathogen. Adv 21 has persisted at Knox through June 1976. Sporadic isolates of influenza A/Victoria virus were made in February, April and May.

f. Fort Ord. This post closed basic training in April 1976. Adv 4 and 7 vaccines were used from 1 July through April. ARD rates were consistently below 1.7/100/week and only 2 strains of Adv 4 and 15 strains of Adv 7 were made from the 320 throat swabs submitted (5.3%). Both influenza virus A/Victoria and Adv 21 isolates were most prevalent in February and March, with a peak ARD rate of 1.6.

g. Fort Polk. Fort Polk ceased basic combat training in January 1976. Sporadic isolations of Adv 4 (32 of 150 throat swabs submitted) were made from July through September 1975; however, ARD rates were always below 1.5/100/week. Only 1 strain of Adv 4 was isolated from the 20 specimens submitted (5.0%) after beginning adenovirus vaccines on 1 Oct 76.

h. Fort Sill. This post began basic combat training in February. Weekly ARD rates between 1.4-4.7/100 were likely associated with influenza A/Victoria virus in March. Three isolates of Adv 21 were made from the 43 samples submitted during March and April 1976.

i. Fort Wood. An early Adv 7 epidemic beginning in August led to beginning adenovirus vaccines in early September rather than 1 October as planned; immunization was continued through June 1976. Adv 7 caused a median ARD rate of 2.8/week in September when Adv 7 was isolated from 38% of submitted samples. Only incoming trainees were immunized with adenovirus vaccines to abort the Adv 7 epidemic; the

ARD rate declined to 1.0/100/week 7 weeks after beginning adenovirus vaccines. After mid-November, only 3 isolates of Adv 4 and no isolates of Adv 7 were made from the 402 throat swabs submitted up to 1 May (0.7%). Adv 21 was first isolated in November, was obtained from 45% of throat washes in late February and early March when the ARD rates ranged between 3.4-4.1/100, and remained on post into June. Only rare isolates of influenza A/Victoria virus were made in January and February.

## 2. Prevalence of adenovirus neutralizing antibody in incoming basic combat trainees

Military trainees are particularly susceptible to epidemics of adenovirus acute respiratory disease. In addition to the ubiquitous adenovirus types 4 and 7, outbreaks of ARD due to types 3, 11, 14 and 21 have occurred in military trainees.

In order to design a proposed study of possible adenovirus vaccine interference when the 3 adenovirus vaccines - types 4, 7 and 21 - are given simultaneously, it was necessary to determine the prevalence of neutralizing (N) antibody in incoming basic combat recruits. BCTs from 6 different posts studied in 1965 had the following prevalence of N antibody to the indicated virus: type 3 - 72%, type 4 - 27%, type 7 - 48%, and type 21 - 15%. Whether this N antibody prevalence was accurate in 1976 trainees was unknown.

In collaboration with members of the Division of Preventive Medicine, WRAIR, incoming BCTs at 4 posts - Forts Jackson, Knox, Wood, and Dix - were studied. At the first 3 posts, all trainees with social security administration numbers (SSANs) ending in digits 1, 6, or 9, and who were seen for medical processing in the respective reception stations between 22-24 March, were studied. All but 6 of the 444 trainees selected for study contributed blood specimens. In addition, sera from 59 reception center trainees with a terminal SSAN digit of 9, bled from 23-27 February 1976 in conjunction with the influenza A/swine outbreak at Fort Dix, were included in these tests.

Sera were tested at a 1:2 dilution against prototype strains of adenovirus type 3, 4, 7, 11, 14, 16 and 21. Serum, heated at 56°C for 30 min, was diluted 1:2 and mixed with a dose of virus standardized to yield 32-320 tissue culture infectious dose<sub>50</sub> (TCID<sub>50</sub>) in human embryonic kidney cell (HEK) culture within 2 to 4 days after inoculation. After incubation for 1 hr at room temperature, 0.1 ml of the serum virus mixture was inoculated into each of 2 HEK tubes. Each tube was scored for presence or absence of adenovirus cytopathic effect at a time when titration of the virus inoculum showed between 32-320 TCID<sub>50</sub> in the test. Results of this study to the present are shown in Table 10 on the next page.

There has been no appreciable change in the prevalence of N antibody to types 4, 7 and 21 in BCTs over the decade since 1966. The

Table 10. Prevalence of adenovirus neutralizing antibody in BCTs

Adenovirus type	No. sera tested	N antibody prevalence
3	387	55.3%
4	495	23.4%
7	456	41.4%
11	496	10.7%
14	462	5.8%
21	496	11.3%

prevalence of N antibody to type 3 seems to have decreased from 72 to 55%. Upon completion of N antibody tests to adenovirus type 16, the presence of N antibody to a given serotype will be correlated with N antibody to other adenovirus types and the trainee's geographical origin, level of education, family size, and other variables. In addition, the sera will be used to determine the correlation, if any, between N antibody and hemagglutination inhibition (HI) antibody to adenovirus type 21, 7 and 4, in order to determine whether the HI test can replace the more cumbersome and expensive N antibody test in antibody prevalence surveys to adenoviruses of military interest.

### 3. Use of a charcoal viral transport media in isolation of adenovirus type 21

Leibovitz (1969) described a charcoal viral transport media (CVTM) which permitted survival of adenovirus type 4 and 7 viruses at ambient temperatures for several weeks' time. This medium permitted collection and shipment of throat swabs obtained from trainees hospitalized with ARD without the necessity of ultralow freezers or dry ice, and eliminated significant transportation problems in the Army's Adenovirus Surveillance Program.

The present study attempted to determine whether throat swabs maintained in CVTM provide as efficient a tool for Adv 21 isolation as throat washings maintained at -70°C, the technique used in previous WRAIR adenovirus field studies.

Forty-eight BCTs admitted to the ARD annex at Fort Leonard Wood, MO, in February 1976, were studied. The retrovular area was swabbed with 2 swabs which were immediately inserted into a screw-capped tube containing CVTM prepared as described by Leibovitz. These tubes were maintained at ambient temperature for 14 days prior to inoculation into cell cultures. In addition, each trainee gargled with 10 ml of Hank's



balanced salt solution containing 0.4% bovine plasma albumin. This throat washing was frozen on dry ice and maintained at a temperature of 70°C prior to inoculation.

One of the 2 throat swabs was agitated and incubated in 5 ml of L-15 media containing 2% FBS, glutamine and antibiotics; then 1.5 ml of the media was inoculated into each of 3 HEK cells. After incubation with antibiotics for 30 min, 0.3 ml of the throat wash was inoculated into each of 3 HEK cells. HEK cells were maintained in L-15 medium with 2% FBS and examined every other day for adenovirus CPE until positive or for 21 days before being discarded.

Adenovirus type 21 was isolated from 25 of the 43 trainees sampled. In 22 trainees, both throat swabs and throat washings yielded Adv 21. In 2 trainees, the swab yielded virus while the throat washing was negative, while in one trainee, the washing was positive and the swab negative. In the remaining 23, both swab and washings were negative for Adv 21.

Generally, viral CPE was evident earlier with throat swabs than with throat washings. The only major problem with the CVTM was the presence of yeast contamination in 14 of the 48 specimens. However, this was easily circumvented by filtration or Amphotericin B treatment. It should be noted that the extreme conditions used to test the CVTM (14 days at ambient temperature) will not likely be duplicated in an actual field study where swabs in CVTM would be refrigerated before inoculation and inoculated immediately after arrival at the WRAIR.

In summary, throat swabs maintained at ambient temperature for 2 weeks in CVTM proved at least equally efficient in isolating Adv 21 as the standard throat washing maintained at ultra low temperature. Use of the CVTM transport medium would permit virus isolation studies to be performed at the WRAIR rather than at a field lab and would obviate the necessity for an ultralow freezer and/or a dry ice supply at the BCT post study site.

## B. Influenza

### 1. Influenza A/swine infection in Fort Dix trainees

In February 1976, the New Jersey State Board of Health isolated a new strain of influenza virus, A/swine, from throat swabs of hospitalized basic combat trainees at Fort Dix, NJ. The isolation of this virus from humans was of considerable importance since the appearance of a novel influenza strain with radically different hemagglutinin and neuraminidase antigens from contemporary strains has always been associated with a pandemic. Also, A/swine strains are considered to have caused the severe 1918 pandemic. For these reasons, the Department of Virus Diseases and the Division of Preventive Medicine began an immediate epidemiologic investigation of A/swine influenza at Fort Dix,



a study which involved nearly all personnel of both departments during February and March 1976. This study proved that influenza A/swine strains had infected approximately 500 trainees at Fort Dix and thus established the epidemic potential of this virus. A detailed report of this study is included in the Annual Report of Project No. 3A762760-A806, Task 00, Military Preventive Medicine, Work Unit 034, Epidemiologic Studies of Military Diseases (Preventive Medicine).

## 2. Influenza A/swine vaccine studies

Since April, 1976, the Department of Virus Diseases, WRAIR, has collaborated with other federal agencies under the sponsorship of NIAID, NIH, in evaluating the reactogenicity and immunogenicity of vaccines containing antigens of the A/swine strain, A/New Jersey/8/1976. Both Chief, Department of Virus Diseases and Director, Division of Communicable Diseases and Immunology collaborated with investigators from the National Institute of Allergy and Infectious Disease, NIH, Center for Disease Control, and the Bureau of Biologics, FDA, in designing and implementing A/swine vaccine studies to provide a data base for rational decisions for the National Influenza Immunization Program. USAMRDC laboratories and contractors collaborated on 2 studies of influenza A/swine immunization with direct military relevance: 1) evaluation of a monovalent A/swine vaccine, and 2) evaluation of a bivalent A/swine and A/Victoria vaccine given with a monovalent B/Hong Kong vaccine. Below is a summary of the preliminary data from these studies:

### a. Evaluation of monovalent A/swine vaccines in adults.

Two USAMRDC contractors, Dr. Theodore Eickhoff of the University of Colorado School of Medicine and LTC Creed Smith, Pathology Reference Laboratory, LAMC, Fort Baker, CA, participated in this study. Dr. Eickhoff used student volunteers at Lowry Air Force Base and Dr. Smith used military volunteers at Fort Baker, CA. Additional investigators were from the University of Rochester, Baylor University, BOB, NIAID, and CDC. A total of 1920 volunteers participated in this study and were divided randomly into 13 vaccine groups - one placebo group, and for each of the 4 manufacturers, Wyeth, Parke Davis, Merrell-National, and Merck, Sharp and Dohme, 3 vaccine groups receiving 200 CCA, 400 CCA, and 800 CCA, respectively. In this and the following study, volunteers were examined at 1 and 2 days after immunization, their temperature recorded in addition to a history of constitutional and local reactions, and their inoculation site observed for tenderness, erythema, and induration. All reactions were recorded in a standard form used by all investigators. Blood was drawn prior to and 4 weeks after immunization. Paired sera were forwarded to the CDC which performed HI tests to A/NJ/8/75, A/Mayo Clinic/103/74, and A/swine/1976/31 (and in the following study to A/Victoria/3/75 and B/Hong Kong/72). Vaccines used were coded by BOB personnel not involved in the study so that neither clinical investigators nor serologists were aware of the vaccine received by individual volunteers until the study was completed.

Febrile reactions were not common, 1.7% of the placebo group had fever  $\geq 100^{\circ}\text{F}$  and all vaccine groups had less than a 5% incidence of fever, with the exception of the recipients of the 800 CCA vaccines of Parke-Davis (5.1%), Merrell-National (5.4%) and Merck (12.8%). The latter vaccine induced fever of  $102^{\circ}\text{F}$  or greater in 3.7% of vaccines. Using a systemic reaction score (awarding 1 point each for headache, malaise, nausea, and febrile response), the placebo group averaged 0.40 and all vaccine groups averaged below 0.60 except the Merck 400 CCA (0.90) and 800 CCA vaccine (2.15). Generally, reactions were more frequent in recipients of whole virus vaccines. In volunteers receiving either the 400 or 800 CCA Merck vaccine or the 800 CCA unit Merrell-National vaccine, systemic reactions were more common in persons lacking antibody to A/swine. Two or more moderate systemic reactions occurred in 12.4% of the 362 volunteers without pre-existing A/swine antibody, but in only 0.9% of the 110 volunteers with pre-existing A/swine antibody ( $p = 0.0008$ ). This would suggest that systemic reactions are not mediated by immunological mechanisms or endotoxin, but are an inherent characteristic of the influenza antigens themselves.

The immunogenicity of the 3 doses of the vaccines tested on all volunteers lacking A/swine antibody is shown in Table 11. The Merck

Table 11. Monovalent A/swine influenza immunization in adults; effect of vaccine dose on participants without pre-existing HI antibody

Manufacturer	Dose (CCA)	No. subjects	Percent seroconverting	
			$\geq 20$	$\geq 40$
Wyeth	200	125	62	50
	400	131	76	65
	800	121	77	69
Parke-Davis	200	119	77	68
	400	116	68	61
	800	125	79	76
Merrell-National	200	128	70	56
	400	120	80	69
	800	122	82	67
Merck-Sharp & Dohme	200	140	90	78
	400	124	96	86
	800	116	96	91

vaccines were clearly more immunogenic than the other vaccines, but little difference was apparent between the 2 split virus vaccines (Wyeth

and Parke-Davis) and the Merrell-National whole virus vaccine. If, however one examines the post immunization antibody status of all participants 23 years and under (those with no previous experience with A/swine strains or influenza A and A-1 strains whose hemagglutinin shares a common antigen with Hsw), a different picture emerges (Table 12). A much smaller proportion of recipients of split virus vaccines seroconverted to HI antibody titers of  $\geq 1:20$  or  $\geq 1:40$  than did recipients of whole virus vaccines. In persons 24 years or older, all vaccines were acceptably immunogenic with the 400 CCA vaccines, producing  $\geq 1:40$  titers in 83-92% of recipients and the 200 CCA vaccines producing  $\geq 1:40$  titers in 71-92%. No difference in immunogenicity between split virus and whole virus vaccines was evident in persons older than 24.

Table 12. Monovalent A/swine influenza immunization in adults; post vaccination antibody status of all participants 23 years and under

Manufacturers	Dose (CCA)	No. subjects	% with HI antibody titers			
			< 10	$\geq 10$	$\geq 20$	$\geq 40$
Wyeth	200	43	56	44	35	28
	400	42	26	74	64	41
	800	40	20	80	50	33
Parke-Davis	200	41	54	45	44	37
	400	41	46	54	32	22
	800	38	29	71	50	45
Merrell-National	200	46	20	80	54	46
	400	32	9	91	75	56
	800	42	10	91	67	48
Merck, Sharp & Dohme	200	38	8	92	74	58
	400	49	0	100	96	86
	800	29	0	100	93	90

This study uncovered a paradox of considerable military importance. Not only are A/swine influenza vaccines less immunogenic in the age group under 24, but they tend to cause more febrile and constitutional reaction in this group which has no prior experience with A/swine or related antigens. Since a large proportion of military personnel is younger than 24, the military will experience greater problems with reactions and immunogenicity than the older civilian population.

Fortunately, the reactions experienced with all but the Merck 800 CCA vaccine were mild and of less than 48 hrs duration and clearly acceptable in military personnel.

b. Evaluation of immunization with bivalent A/New Jersey/1976 and A/Victoria/1975 vaccine given with monovalent B/Hong Kong/72 vaccine. Influenza vaccines containing relevant influenza A and B antigens have been mandatory immunizations for active duty military personnel; these vaccines generally contain 700 CCA units of influenza A antigen and 500 CCA units of influenza B antigen in the 0.5 ml dose. Since the Department of Health, Education, and Welfare has sole authority to procure and distribute influenza A vaccines under the National Influenza Immunization Program this year, the military will receive influenza A vaccines from that Department and procure influenza B vaccines separately. Thus, military personnel are expected to receive 2 separate influenza immunizations this year, a 500 CCA unit B/Hong Kong/72 and the bivalent vaccine containing 400 CCA units each of the A/New Jersey/76 and A/Victoria/75 antigens. This study determined reactions and antibody response induced by simultaneous immunization of adults with the bivalent A and immunovalent B influenza vaccines.

The study was conducted by investigators at the BOB, USAMRIID, and the WRAIR. Volunteers were obtained from FDA and NIH employees, Fort Detrick military personnel and employees, and WRAIR military personnel. The age distribution of the 531 volunteers in this study is shown in Table 13. There were 11 vaccine groups in this study. The three manufacturers - Parke-Davis, Merrell-National, and Merck, Sharpe and Dohme (MSD) each had 3 vaccine groups: one receiving the bivalent influenza A vaccine in the left arm and a placebo inoculation in the right arm, a second group receiving the monovalent B vaccine in the right arm and a placebo in the left arm, and the third receiving both bivalent A vaccine in the left arm and monovalent B vaccine in the right arm.

Table 13. Influenza bivalent A and monovalent B immunization of adults; distribution of subjects by age

Age	Number	Proportion
< 24	72	13.6%
24 - 34	166	31.3%
35 - 51	173	32.6%
> 52	120	22.6%
Total	531	100.1%

Febrile and moderate or severe systemic symptoms encountered are shown in Table 14. The MSD bivalent A vaccine caused a significantly higher incidence of fever and systemic symptoms than the placebo group. There was no significant difference between placebo and the monovalent influenza B vaccines in regard to fever or systemic symptoms. Volunteers immunized with the trivalent MSD vaccine combination had a significantly higher incidence of fever (20%) and systemic symptoms (31%) than the placebo group. Subsequent studies by BOB showed that MSD 400 CCA A/swine vaccine contained 1.6 times the total viral mass of the equivalent 400 CCA Merrell-National vaccine. The MSD bivalent A vaccine caused more reactions than did its monovalent B vaccine. Although a high proportion of those immunized with MSD A vaccines experienced some fever or constitutional symptoms, few reactions were sufficiently severe to keep the volunteers from work.

Table 14. Influenza bivalent A and monovalent B immunization of adults; fever and constitutional reactions in recipients

Vaccine group	Number	Proportion of recipients with		Systemic symptoms
		Fever $\geq 100^{\circ}\text{F}$	Fever $\geq 101^{\circ}\text{F}$	
Placebo	97	1%	0	4%
PD* AA	47	0	0	6%
MN** AA	46	4%	2%	6%
MSD*** AA	47	13%	11%	19%
PD B	49	8%	0	6%
MN B	48	2%	2%	10%
MSD B	48	4%	2%	13%
PD AA + B	47	2%	0	4%
MN AA + B	48	6%	0	15%
MSD AA + B	51	20%	8%	31%

\*Parke Davis

\*\*Merrell-National

\*\*\*Merck, Sharp & Dohme

Preimmunization HI antibody titers to the three A/swine, A/Victoria, and B/Hong Kong antigens were similar in all vaccine groups. HI antibody responses to A/swine antigens were similar in recipients of any of the three bivalent A vaccines and in recipients of the three trivalent vaccines. Shown in Table 15 are the relevant A/swine HI antibody titer distribution in recipients of the trivalent vaccine combinations. Each vaccine raised the proportion of volunteers with HI antibody titers  $\geq 1:40$  to 80-87% after immunization. Post immunization geometric mean antibody titers were similar to vaccines of all manufacturers, both among volunteers with and among those without pre-existing A/swine antibody. However, as in the monovalent A/swine study, the split virus vaccine was less immunogenic than whole virus vaccines in younger age groups; only 57% of the 35 persons younger than 35 years seroconverted to a  $\geq 1:20$  titer after immunization with the Parke Davis vaccine, while 92% of 64 recipients of the 2 whole virus vaccines seroconverted to  $\geq 1:20$  titers. This difference was not apparent in volunteers over 35 years of age.

Table 15. Influenza bivalent A and monovalent B immunization of adults; distribution of A/SW HI antibody titer pre and post immunization in recipients of bivalent A and monovalent B vaccines

Vaccine	Serum	Percent with HI titer of					
		$\geq 10$	$\geq 20$	$\geq 40$	$\geq 80$	$\geq 160$	$\geq 320$
Placebo (n = 92)	Pre	45	35	24	17	9	1
	Post	46	34	24	19	7	1
Parke-Davis* (n = 45)	Pre	44	31	22	9	4	2
	Post	87	80	80	72	50	37
Merrell-National (n = 45)	Pre	36	24	16	9	4	2
	Post	93	93	87	67	44	29
Merck, Sharp, Dohme (n = 48)	Pre	48	31	19	13	6	4
	Post	98	96	85	58	38	25

\* A/SW/1976/31 antigen used in HI test for Parke-Davis vaccine recipients (A/MC/103/74 antigen used in HI tests for all other vaccine groups)

Although the proportion of vaccines with pre-existing antibody to A/Victoria was similar to that with pre-existing A/swine antibody, antibody titers induced to A/Victoria were much lower than to A/swine (Table 16). In persons with pre-existing antibody to the relevant antigen, the post immunization geometric mean titer to A/Victoria induced by the various vaccines (range 31-80) was considerably lower than that induced by A/swine antigens (range 182-346). With the exception of the Parke-Davis trivalent combination, however, no significant difference in antibody response to A/Victoria was apparent in the recipients of the bivalent A vaccine.

Table 16. Influenza bivalent A and monovalent B immunization of adults; distribution of A/VIC HI antibody titer pre and post immunization in recipients of bivalent A and monovalent B vaccines

Vaccine	Serum	Percentage with HI titer of					
		≥ 10	≥ 20	≥ 40	≥ 80	≥ 160	≥ 320
Placebo (n = 93)	Pre	47	22	11	4	1	0
	Post	44	19	10	3	1	0
Parke-Davis (n = 45)	Pre	51	27	7	2	0	0
	Post	92	70	41	20	9	2
Merrell-National (n = 45)	Pre	56	33	24	9	0	0
	Post	98	84	64	36	16	9
Merck-Sharp, Dohme (n = 48)	Pre	46	27	17	10	0	0
	Post	96	79	63	42	15	4

Approximately 70% of volunteers had pre-existing antibody to B/Hong Kong antigen. The antibody response induced by all 6 vaccine combinations containing the B/Hong Kong antigen was similar, with 82-94% of recipients having ≥ 1:40 HI titers after immunization (Table 17).

Table 17. Influenza bivalent A and monovalent B immunization of adults; distribution of B/HK HI antibody titer pre and post immunization in recipients of bivalent A and monovalent B vaccines

Vaccine	Serum	Percentage with HI titer of					
		≥ 10	≥ 20	≥ 40	≥ 80	≥ 160	≥ 320
Placebo (n = 93)	Pre	71	56	45	28	13	4
	Post	70	60	41	28	12	5
Parke-Davis (n = 46)	Pre	74	59	37	28	15	11
	Post	100	91	85	63	50	33
Merrell-National (n = 44)	Pre	66	55	36	23	11	5
	Post	98	96	89	67	42	24
Merck, Sharp, Dohme (n = 45)	Pre	58	47	29	22	9	4
	Post	100	98	90	67	46	21

### 3. Early events in ortho and paramyxoviral infection measured by immune cytolysis

Studies of the early events in influenza A/WSN virus and Sendai viral infection were continued using a method similar to that described in last year's Annual Report. Several problems were encountered which required minor changes in the procedure. First, it was discovered that if the cells were not allowed to remain confluent for at least 12 hours, background cytolysis (without specific antibody) increased to unacceptable levels with either virus. Secondly, it was discovered that non-specific cytolysis increases shortly after warming up the cells; this may be due to greater cell fragility caused by the 1 hour cold adsorption period. Backgrounds were reduced by incorporating 1% bovine plasma albumin (BPA) in all wash solutions and diluents. The BPA must be screened before use, as some lots interfere with specific lysis. Third, most guinea pig complement obtained from the WRAIR laboratory animal supply appeared to contain low levels of antibody to Sendai virus and WSN virus. This interfered with the experiments late in infection. However, lyophilized complement derived from Baltimore Biological Labs, Inc. gave much better results. In most of the experiments reported here, egg grown Sendai or WSN was purified by tartrate gradient centrifugation as described in the last Annual Report.



An experiment designed to determine the amount of spontaneous release of radioactive chromium during WSN or Sendai viral infection showed that in the absence of antibody and complement CR<sup>51</sup> is released at a rate of about 1.5% per hour. This rate does not increase because of cytopathic effect until at least 36 hours post infection with multiplicities of infection greater than 1. Therefore increased spontaneous release of chromium is not a problem during immune cytotoxicity experiments which lasted for a maximum of 24 hours.

Figure 34 shows a plot of the percent immune cytotoxicity at various times following a WSN viral infection. At three hours after infection, cultures infected with an M.O.I. of 30 TCID<sub>50</sub> per cell began to show immune cytotoxicity. With a lower MOI, immune cytotoxicity first appeared at four hours post infection. It should be emphasized that no immune cytotoxicity at all is seen before 3 hours post infection in spite of the fact that the immune cytotoxicity system functioned well as evidenced later in infection.

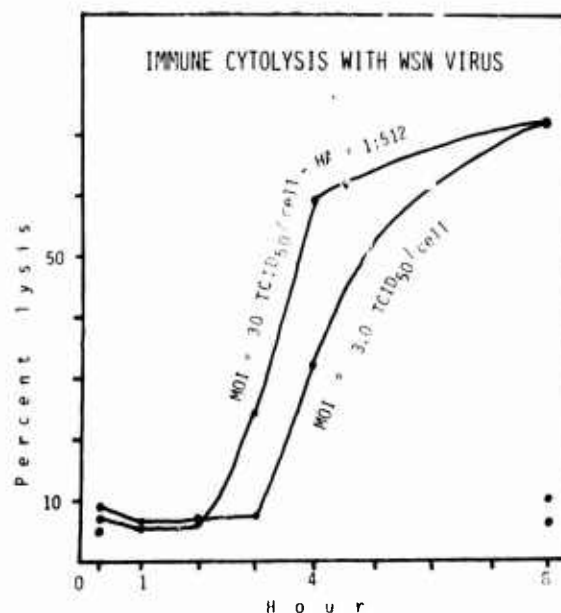


Figure 34. Immune cytotoxicity with the WSN strain of influenza virus in BHK-21 cells.

Figure 35 shows the results of similar experiments done with the paramyxovirus Sendai virus. In strong contrast to the results obtained with WSN virus, immune cytotoxicity could be demonstrated as soon as 15 minutes after the cultures were warmed. One hour after warming, there was substantially more lysis. In some cases, the amount of cytotoxicity

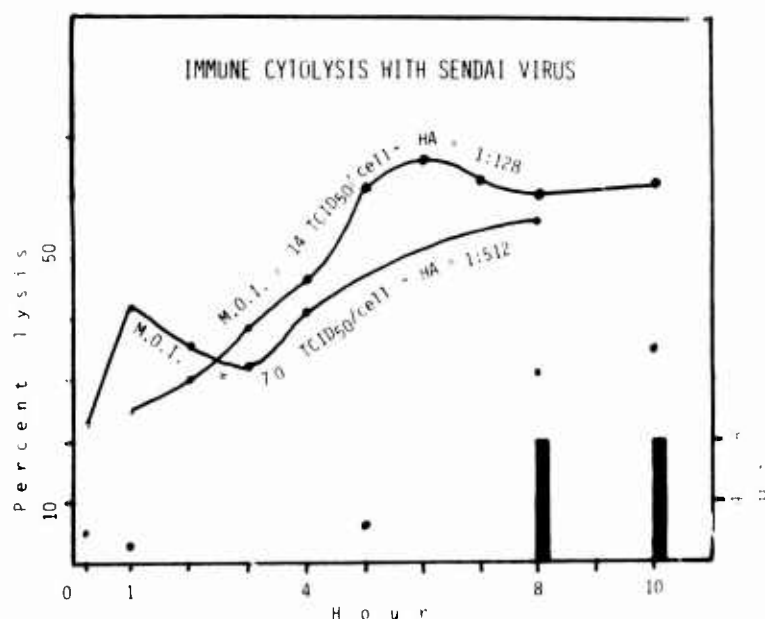


Figure 35. Immune cytotoxicity with parainfluenza virus type 1 (Sendai) in BHK-21 cells.

remained constant or dropped after one hour. Later, presumably as progeny virus buds through the cellular membrane, lysis increased. In some experiments, we obtained a second type of curve where immune cytotoxicity increased steadily after one hour. This occurrence is not a direct function of the M.O.I., as might be interpreted from this Figure. When an M.O.I. of 14 TCID<sub>50</sub> per cell was used, we saw the initial appearance of HA activity at 8 hours post infection.

Figure 36 shows the results of experiments designed to trace the fate of the surface antigens of the infecting inocula, unobscured by insertion of antigens into the cellular membrane by replicating virus. Sendai virus was irradiated with ultraviolet light for twenty minutes, at which time infectivity was not detectable, but HA activity remained at a titer of 1:256. When we added this virus to cells, 30% immune cytotoxicity occurred one hour after infection. With time, cytotoxicity slowly decreased from this value. Cytotoxicity with the infectious virus control increased with time as expected.

Figure 37 shows the results of an experiment, monitored for a shorter time period, where cells were treated with various dilutions of an ultraviolet irradiated stock virus having an original HA titer of 1:2048. The curves shown in this experiment are representative of an

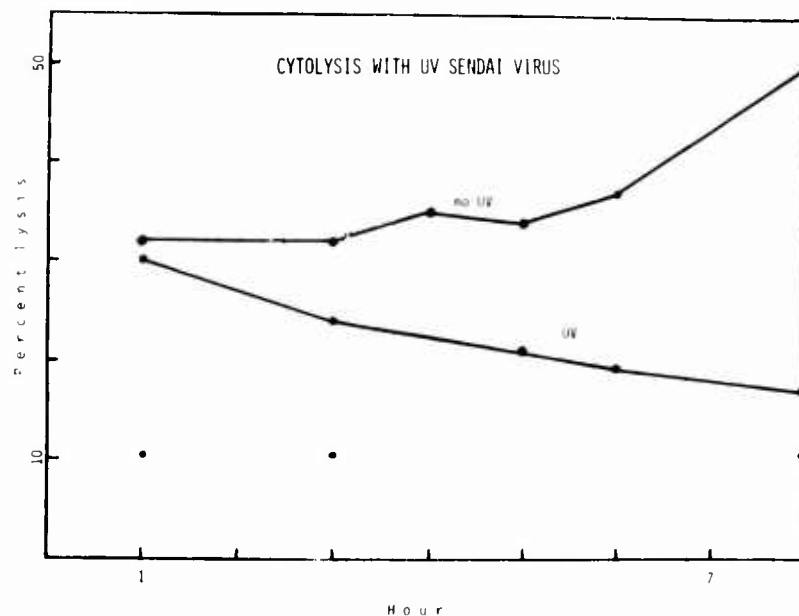


Figure 36. Immune cytotoxicity with ultraviolet light (UV) inactivated Sendai virus.

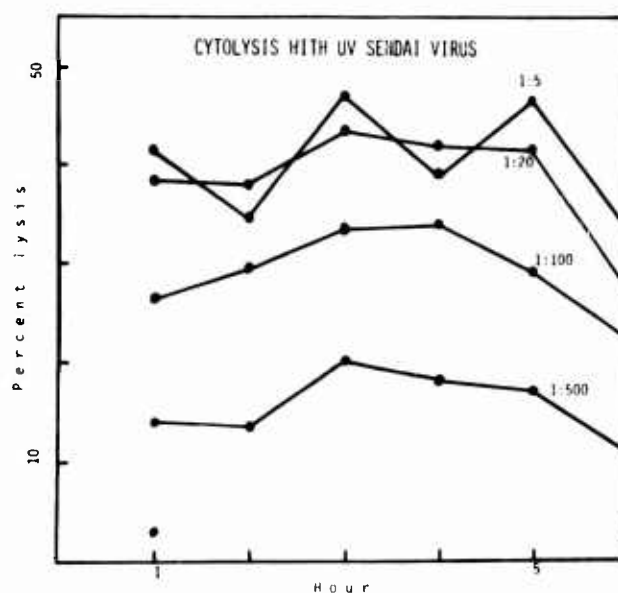


Figure 37. Immune cytotoxicity with varying dilutions of ultraviolet light (UV) inactivated Sendai virus.

often seen variation from the curves represented in Figure 36. Here, immune cytolysis continued to increase to a maximum value at approximately three hours post-adsorption. Then the amount of lysis begins to fall off. This type of curve occurred regardless of the input virus dilution.

Table 18 quantitates the amount of specific Sendai viral immune cytolysis at one hour and correlates this with the amount of input virus. The stock virus had an HA titer of 1:32 and an EID<sub>50</sub> of 10<sup>6.5</sup>. It can be seen that the technique is sensitive enough to detect the presence of as little as one infectious unit per cell or even less.

Table 18. Dependence of early lysis on M.O.I.

M.O.I.	% Specific lysis at one hour
10 . . . . .	44
2 . . . . .	29
1 . . . . .	21
0.5 . . . . .	8
0.25 . . . . .	3
0.125 . . . . .	1

There are several possible ways to explain the presence of early immune cytolysis with Sendai virus in contrast to WSN virus. It is possible that the immune cytolysis system is detecting virions which have fused with the surface of the cell as well as those which are only attached to the cell. If this were true, one must hypothesize some difference in the WSN and Sendai viral surface antigens which allows immune cytolysis by antibody to attached Sendai virions but not to attached WSN virions.

A more likely possibility, for which Eaton and Scala (1971) have provided some evidence, is that antibodies to attached virions are not able to participate in the immune cytolytic reaction, but, that antibodies to viral antigens incorporated into the cellular membrane by a fusion process are able to participate in the cellular lysis process. In agreement with this hypothesis, we have shown that cells infected with Sendai virions, attached to the cell at 40°, require at least one hour at 37° before maximum early cytolysis is obtained. We interpret these data as evidence for fusion as a requirement for early immune

cytolysis.

If immune cytolysis did measure only fused virions, it could serve as a powerful tool to determine whether or not various enveloped viruses were able to enter cells by a process of fusion with the external cellular membrane.. Experiments are continuing in this laboratory using various techniques to inactivate the Sendai viral fusion factor but not the hemagglutinin, in order to obtain further evidence which will elucidate whether or not fused virions are the only virions capable of rendering a cell susceptible to immune cytolysis.

#### 4. Role of the viral carbohydrate

Studies are continuing to investigate the role of the carbohydrate moiety which is covalently bound to the surface proteins of influenza virus. The approach described last year, involving a glycosylation of carbohydrate deficient virus which was devoid of HA activity, was abandoned. A new approach was begun which involves specifically cleaving the terminal sugars of influenza virus with highly purified glycosidases and looking for a decrease in HA activity. Bikel and Knight (1972) described the use of partially purified exoglycosidases from *Aspergillus* on PR8 influenza virus. They showed a large reduction of HA activity after treatment. We used the unpurified or partially purified enzyme preparation from the identical source used by these investigators and no reduction in HA activity of PR8 virus could be obtained. The discrepancy could have been caused by the fact that we assayed for viral HA activity directly after incubation with the enzyme mixture, whereas they centrifuged the virus before the HA assay. This centrifugation could have caused nonspecific aggregation of viral particles which might have fewer charged molecules after glycosidase treatment. Also, we found the pH used by these authors to be at the borderline of where destruction of HA occurs. We were able to demonstrate, however, the complete abolition of HA activity of high titered, gradient purified WS virus and Sendai virus after treatment with unpurified or partially purified glycosidases at a pH where the virions are stable. Using the procedures described by Bahl and Agrawal (1969) and Bikel and Knight, we have obtained a preparation of N-acetylglucosaminidase which is greater than 99% free of contaminating glycosidases and a B-galactosidase preparation which is greater than 85% free of contaminating glycosidase. Both preparations are completely free of proteases. We are attempting to completely purify these enzymes in usable quantities. At the same time, preparation is being made to monitor the results of the action of glycosidases on influenza and parainfluenza viruses using gas-liquid chromatography.

Should the purified glycosidases impare the biological activity of these viruses, a study will determine if this effect is due to simple aggregation of the viruses or to another effect.

### III. Clinical virology

Case Report: The isolation of Herpes simplex virus from spinal fluid of a patient with radiculoneuropathy

A thirty-eight year old Army Sergeant was admitted to a hospital in Korea because of the spontaneous onset of back pain that radiated to the posterior aspect of his right leg. His pain was reproduced by straight leg raising and there was weakness in ankle dorsiflexion ipsilaterally. There was scattered hypesthesia of the L4-S1 distribution on the right. He did not improve after one month of bed rest and myelography demonstrated small filling defects at the L3 - L4, L4 - L5 levels. Two days later an exploratory laminectomy was performed and a L5 - S1 disc was removed. One day post operatively his pain persisted and a vesicular eruption appeared on the back of his right wrist. Two days after the operation burning pain developed in the right C6 - C8 dermatomes. He had right triceps weakness, a diminished right triceps reflex, hypesthesia in the C6 - C8 area, and continued sciatic pain. In the seven weeks after laminectomy, he was transferred to the Walter Reed Army Medical Center. Here he had bilateral sciatic pain and radicular pain in the right arm. The straight leg raising test was positive on the right. The right triceps reflex was diminished; scars consistent with healed vesicles were seen on the dorsum of the right wrist. He had lost 25 pounds since surgery. Laboratory studies which included complete blood count, post prandial serum glucose, BUN, serum creatinine, serum electrolytes, antinuclear factor, VDRL, SMA-12 chemistry screen, and serum protein electrophoresis were normal. Radiographs of the lumbosacral spine demonstrated changes consistent with a recent laminectomy, and cervical spine radiographs were interpreted as normal. A lumbar puncture yielded crystal clear CSF under normal pressure; Herpes simplex virus type 1 was isolated in human embryonic kidney cell cultures. Results of this and subsequent CSF examinations are shown in Table 19. Serum specimens taken in the 8th and again in the 10th week, post laminectomy, had CF titers to Herpes simplex virus of 1:16 or 1:32, respectively. The patient continued to have partially disabling, radicular pain in the right arm and both legs during the subsequent fourteen months. Additionally, hyperalgesic bands were present in the right C6-C7 and L5 dermatomes.

Table 19. Tabulated CSF findings

Week post laminectomy	Cells	CSF protein	Sugar	Bacteria	Virus	Virus titer/ml CSF
8	0	54 mg%		sterile	HSV-1	10 <sup>5.0</sup>
10				"	"	10 <sup>5.0</sup>
11				"	sterile	-
56	2	29 mg%	69	"	"	-

Although HSV-1 is a frequent cause of clinically apparent encephalitis, it has not often before been associated with overt disease of the peripheral nervous system and reported cases have had virus recovered from typical recurring cutaneous vesicles which appear in the field supplied by the involved nerve. These cases have not reported recovery of virus from the nervous system.

Initially the patient reported here had no skin lesions and laminectomy was performed for relief of a presumed herniated nucleus pulposus. Postoperatively, a vesicular eruption preceeded an upper extremity radicular syndrome which in this case is believed to be due to an HSV infection; unfortunately, the vesicles were not cultured for virus.

Later, HSV-1 was isolated in high titer from CSF at a time when neither encephalitis nor meningitis was clinically apparent. Inadvertent contamination of the specimen is unlikely since virus was recovered from two separate specimens obtained by lumbar puncture two weeks apart. Each specimen was processed by a different technician working in different rooms of the laboratory. Furthermore, virus was reisolated from both CSF specimens.

One can only speculate as to the presence of HSV in the cerebrospinal fluid. It is unlikely that HSV was introduced by surgery, since the dura arachnoid was not entered. Although it may have been introduced at myelography, it is curious that it should have been first manifest by centripetal spread to the dorsal ganglion rather than CNS infection. The most plausible explanation is that HSV, latent in cervical sensory ganglia, was activated by the stress of surgery and moved centripetally into the peripheral nervous system. That high titers of HSV-1 were detected in the CSF over 2 weeks in the absence of CNS pathology or symptomatology is unusual and postulates an unusual host virus relationship since latent HSV is more often than not present in the general population, but the CSF remains free of infectious virus. This case is unique and important because it documents the not previously reported association of a radicular syndrome with an otherwise occult involvement of the central nervous system with HSV type 1.

Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable Disease and Immunology

Work Unit 166, Viral Infections of Man

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Project 3A161102B71Q COMMUNICABLE DISEASE AND IMMUNOLOGY

Task 00 Communicable Disease and Immunology

Work Unit 166, Viral Infections of Man

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<p>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 control of meningococcal, gonococcal, pseudomonas and staphylococcal infections in military forces.</p> <p>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.</p> <p>25 (U) 75 07 - 76 06 Procedures for isolating meningococcal group Y capsular polysaccharide for testing as a candidate vaccine have been developed. Polysaccharides isolated from P. aeruginosa were characterized chemically and immunologically and found to provide protection in a mouse model. The presence of antigenic determinants in the lipopolysaccharide (LPS) of P. aeruginosa, N. meningitidis and N. gonorrhoeae that cross react with the rough LPS of the E. coli J5 and Re mutants was demonstrated. A solid phase radioimmunoassay (SPRIA) has been used to quantitate antibody to the E. coli trials and evaluation of active immunization. The SPRIA was shown to be clinically useful in measuring the antibody response of patients with streptococcal and staphylococcal infections to cell wall teichoic acids. Inhibition of attachment of N. gonorrhoeae to epithelial cells by specific IgA and IgC antibodies in genital secretions was demonstrated. The killing of gonococci by normal human sera was found to be primarily due to IgM antibodies directed against the LPS antigens. The gonococcal capsule was shown to be antiphagocytic and, therefore, of functional significance. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$100,000 from FY 7T funds is programmed for the period 1 Jul - 30 Sep 76.</p>							

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168 Bacterial diseases of military importance

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I. Support of EPICON-monitoring of meningococcal disease in military populations.

Data on meningococcal disease in military populations is derived from three sources: 1) Meningococcal disease (MgcD) in active duty (AD) Army personnel is reported weekly by the Health and Environment Division, OTSG. This data is extracted from Special Telegraphic Reports (STR) (RCS Med-16). 2) Copies of all STR's generated by Army facilities are forwarded to the Department of Bacterial Diseases, Walter Reed Army Institute of Research. From these reports, meningococcal disease in non-active duty populations can be monitored. Under-reporting is an obvious possibility which cannot be estimated. 3) Strains of meningococci isolated at military hospitals are to be routinely forwarded for confirmation to the department. In fact, only a small fraction are. From this sample and information contained in the STR's, group-specific disease can be monitored.

A. From 1 Jul 75 through 19 Apr 76 there were 17 cases of MgcD in AD personnel with one death (6% case fatality rate). This compares with 21 cases and one death (5%) for the same period of FY 75. During FY 73 through FY 75 (Table 1) there were 27, 22 and 29 cases of MgcD respectively. Thus, MgcD continued to occur at a level much reduced from that reported prior to active immunization of all incoming personnel with the group C capsular polysaccharide vaccine: 176-451 cases/year from 1966-1970. The case fatality ratio, however, remains unchanged: 4-15%.

B. During FY 76 there were 54 cases of MgcD reported by STR's to this department from Army generating facilities, worldwide (Table 2). Twenty-seven (27) cases occurred in active duty personnel, which is roughly

\*Died 9 Mar 76.

Table 1. Cases of meningococcal disease in active duty  
Army personnel - CONUS: by Fiscal Year.

Installation	Cases/Deaths/Mortality (%)			
	FY 76*	FY 75	FY 74	FY 73
Ft. Knox	1/10	2/10	5/10	2/0
Ft. Dix	3/1	5/0	2/0	3/1
Ft. Jackson	5/0	5/1	3/0	7/1
Ft. Polk	0/0	4/1	3/1	4/1
Ft. LW	1/0	1/0	2/0	1/0
Ft. Ord	1/0	1/0	2/1	6/0
Other CONUS	6/0	11/0	5/0	4/1
Total	17/1/6	29/2/7	22/2/9	27/4/15

\*Complete through 17 April 76.

Table 2. Cases of meningococcal disease by area and military status:  
1 June 75 - 24 May 76.

Area	Active Duty	Dep. Child	Dep. Spouse	Unknown*	Total
Ft. Dix	5	1	-	1	7
Ft. Jackson	4	1	-	-	5
Ft. Sill	2	1	-	-	3
Ft. Lewis	2	-	-	-	2
Ft. LW	2	-	-	-	2
Ft. Knox	2	-	-	-	2
Ft. Polk	1	2	-	-	3
Ft. Carson	1	1	-	-	2
Ft. Ord	1	1	-	-	2
Ft. Hood	-	3	-	-	3
Ft. Rucker	-	-	-	-	2
Ft. Campbell	-	-	1	1	2
Ft. Gordon	-	-	1	1	2
Other CONUS**	3	2	-	-	5
Europe	4	7	1	2	14
Korea	-	1	-	-	1
Total	27	22	3	5	57

\* Case isolate only - no STR available.

\*\* Fts. Bliss, Lee, Sam Houston, Aberdeen Prov. Grounds and Redstone  
Arsenal - one case each.

equivalent to the figures reported by the OTSG, discrepancies being accounted for by the slight difference in reporting period and the inclusion of overseas generating facilities in the above figures. Twenty-two (22) cases were in dependent children and three (3) cases in dependent spouses.

There were five additional cases from whom case isolates were submitted for confirmatory identification, but for whom no STR's were received, and whose status, therefore, cannot be determined. Two of these cases occurred in Europe and one each at Fts. Dix, Gordon and Campbell (Table 2).

There was no unusual geographic or temporal distribution of cases except for 10 cases which occurred in Europe from January to May 1976. These cases occurred in all three population categories, were scattered throughout the European theatre, and were of at least three different serogroups (A, B and C). The civilian population of Europe has been experiencing wide-spread, focal increases of MgcD for several years, with well defined outbreaks of A and C MgcD in Finland and of B MgcD in England, Wales, Belgium and The Netherlands. The clustering of cases among military personnel in Europe may or may not be related. Data for prior years is not available.

C. The serogroup of 40 of the 57 causative organisms (70%) of cases of MgcD reported world-wide were determined. Twenty-eight (28) were reported by STR's; 12 by this department. Eight strains were reported by STR's and confirmed by WRAIR - a distressingly low number. The serogroup determination by the local hospital or Area Laboratory was confirmed in five of eight (62.5%); three were found to be of a different serogroup by WRAIR. Where disagreement occurred, the WRAIR determination was used. Table 3 breaks down the serogroup of the 40 strains by area and patient status.

Among active duty patients within CONUS, 11/16 (69%) cases were caused by group Y and 4/16 (25%) by group B. This is essentially the same distribution seen in active duty military populations since the routine use of group C vaccine in all incoming recruits began in late 1971. Since then group C disease has been virtually absent and group Y has been the dominant serogroup. There was only one case of group C disease reported in active duty personnel. When active duty cases OCONUS are included, group Y remains the dominant serogroup (55%), with group B accounting for most of the remainder (35%) (Table 3).

For non-active duty patients, the distribution of causative serogroups is more uniform, reflecting the absence of vaccination in this population. The percentage of MgcD caused by group B is the same for both active duty and dependent populations - within and without CONUS (Table 3).



Table 3. Serogroup of MgcD by status and area - 1 June 1975-24 May 1976.

Status	Area	Serogroup (%)						Total
		A	B	C	Y	W135	NG*	
Active Duty	CONUS	-	4(25)	1	11(69)	-	-	16
	OCONUS	1	3	-	-	-	-	4
	Total	1	7(35)	1	11(55)	-	-	20
Dependent	CONUS	-	3	2.	2	2	-	9
	OCONUS	1	1	2	-	-	2	6
	Total	1	4(26.7)	4	2(13.3)	2	2	15
Unknown	CONUS	-	1	-	1	1	-	3
	OCONUS	-	2	-	-	-	-	2
	Total	-	3	-	1	1	-	5
Total		2	14(35)	5(12.5)	14(35)	3	2	40
All	CONUS	-	8(29)	3	14(50)	3	-	28
	OCONUS	2	6(50)	2	-	-	2	12
	Total	2	14(35)	5(12.5)	14(35)	3	2	40

\*NG = non-groupable

The occurrence of group A MgcD in two patients in Europe is noteworthy. Group A disease has been absent from CONUS for over 30 years, when a focal outbreak occurred during this FY in the Pacific Northwest. Because of this rarity, group A capsular polysaccharide vaccine is not routinely used in military personnel. Should group A MgcD continue in Europe, its use may become warranted.

It is also noteworthy that two of three cases caused by group W135 occurred in dependents at Ft. Gordon, Ga. Group W135 is thought to be a nonepidemic serogroup. Cases caused by this serogroup are currently occurring in New York City and it was the most prevalent serogroup carried by recruits at Ft. Ord, Calif. prior to closing this recruit training center.

An additional 27 strains of N. meningitidis were received from various civilian sources for confirmation of serogroup determination.

## II. Meningococcal vaccine studies in humans.

### A. Experience with meningococcal polysaccharide 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.

Two studies have been performed to determine the effect of storage on the group C meningococcal polysaccharide vaccine. In both studies Army recruits in the first week of basic training received a 50 µg dose of the test vaccine subcutaneously. The number of subjects in any vaccine group varied from 25 to 50 depending upon the total number of recruits in the company unit on the day of the study. In accordance with Army regulations informed consent was obtained from all volunteers. Sera specimens and pharyngeal cultures were obtained prior to and two weeks after vaccination.

The first study has followed the antigenicity of Lot C-9 meningococcal polysaccharide vaccine prepared in March of 1970 by the Squibb Institute for Medical Research for 5.8 years. From 1970 to 1973 the vaccine was stored at 4 to 8°C and from 1973 to 1975 at -20°C. The July 1975 data is the final testing of this lot of vaccine since the supply has been depleted. The sera specimens from these trials were analyzed for an antibody response by the Radioactive Antigen Binding Assay (RABA) (3). These data are summarized in Table 4. Visual examination of the data would suggest that the vaccine had become somewhat less antigenic. However, when the seven sets of data were examined by analysis of variance, the differences were found significant only at the 5% level, probably indicating no difference.

Table 4. Antibody responses (RABA) to group C meningococcal vaccines over 5.8 years (Lot C-9 Squibb).

No. of months stored	No. of men tested	Geometric mean titer increase*
5	30	335
20	25	389
32	38	351
35	35	430
48	45	427
63	50	181
69	45	250

\* Increase of serum binding capacity (post minus pre-vaccination) expressed in nanograms of antigen bound.

The second study was performed for Merck Sharp and Dohme (Meningovac Type C Vaccine Stability Study #350). Two lots of group C vaccine (95934/32412/C-B837 and 95935/32413/C-B837) prepared by Merck Sharp and Dohme were injected into Army volunteers after 0 and 16 months of storage at 2-8°C. The pre- and post-vaccination sera were sent to Merck Sharp and Dohme where they were assayed for antibody content by the serum bactericidal assay. The data from these studies is given in Table 5.

Table 5. Bactericidal serum conversion after vaccination with group C meningococcal vaccine stored for 16 months (Merck Sharp and Dohme).

Lot Number	Percent Conversion**	
	Initial	16 months
95934/32412/C-B837	95.6	93.6
95935/32413/C-B838	93.6	94.6

In the Bureau of Biologics, Food and Drug Administration guidelines for release of meningococcal polysaccharide vaccine it is stated that 90% of the subjects tested for antibodies after vaccination should have a 4-fold or greater rise in bactericidal antibody in order for the vaccine to be released. As the data in Table 5 show, both of these vaccine lots gave a sera conversion rate of greater than 90% even after 16 months of storage. An additional trial on these vaccines will be performed after 28 months of storage at 28°C.

Acknowledgement: The following non-WRAIR personnel participated in the above studies: LTC Creed D. Smith, Chief, Bacteriology Department, Sixth USA Medical Laboratory, Ft. Baker, Calif.; COL H. A. Leighton, Chief, Preventive Medicine Activities, Ft. Ord, Calif.; members of the Sixth USA Medical Laboratory Respiratory Disease Investigation Team; and Mr. Angus Hull, Sixth USA Medical Laboratory.

#### B. Preparation of a group Y polysaccharide (Ysss) vaccine.

Group Y meningococci continue to cause appreciable disease among military recruits and military dependent populations. Although group Y meningococci have never been responsible for epidemic disease in either military or civilian populations since the control of epidemic group A and C disease by vaccination, they have accounted for over 50% of endemic disease in military recruits (see preceding section). Additionally, small (≤15 cases) outbreaks of group Y disease have occurred on numerous occasions in recruit camps (4). Consequently, a Ysss vaccine, similar to the Asss and Csss vaccine, will be prepared and tested.

Production of a pilot lot of group Y meningococcal polysaccharide vaccine is now underway at the Forest Glen Section of WRAIR. The methodology for the isolation of this polysaccharide is similar to the method of Gotschlich (5) with the following modifications: 1) the polysaccharide was extracted from the cetavlon:polysaccharide complex with 0.5M NaCl rather than 1M CaCl<sub>2</sub>; and 2) protein, which appears to be more tightly bound to this polysaccharide, was removed by treatment with pronase, followed by a phenol water extraction, rather than multiple extractions with chloroform butanol.

A radioactive antigen binding assay using <sup>14</sup>C labeled group Y polysaccharide has been developed to measure the polysaccharide specific antibodies (3). Sera from a few individuals before and after pharyngeal carriage of a group Y N. meningitidis show an antibody response to the group Y polysaccharide. The antibody response to the Ysss following carriage of the organisms is analogous to that following group C carriage, which also gives rise to group specific antibodies, but unlike that following carriage of a group B organism, whose capsular polysaccharide is non-immunogenic (6). These results, we believe, give an indication that the Ysss, like the Csss, will be antigenic in humans.

### III. Investigations on the control of *Neisseria gonorrhoeae*.

The overall goal of the gonococcal research projects is to develop effective control measures for *Neisseria gonorrhoeae* (GC). The development of an immunoprophylactic agent 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. A second objective has been to investigate the nature of the immune response to GC in humans, with particular reference to the surface structures identified above, in the hope that an immunochemical model of resistance and susceptibility could be developed which would indicate a fruitful point for immunoprophylactic intervention. The studies detailed below further these two objectives.

#### A. Phagocytosis of encapsulated and non-encapsulated *Neisseria gonorrhoeae* (GC).

When grown under certain suitable conditions GC appear to elaborate a capsule which can be identified by both India-ink staining and by the Quellung reaction with appropriate antisera. To determine if this apparent morphologic structure is of functional significance its effect upon phagocytosis was investigated.

The differences between the interaction of encapsulated (E) and non-encapsulated *N. gonorrhoeae* (GC) with human polymorphonuclear leukocytes (PMN) were examined using a slide phagocytosis system. Log phase type 4, non-pilated GC were induced to form capsules when grown

in association with Streptococcus viridans (SV) on enriched agar. Non-encapsulated GC from the same log phase culture were grown on regular GC agar without SV. Encapsulation was determined by a wet India ink method. Serial dilutions of encapsulated and non-encapsulated organisms were simultaneously inoculated onto chamber slides coated with adherent PMN's and incubated on a rotating shaker at 37°C for 20 min. The degree of attachment and/or phagocytosis was calculated as the percentage of 400 PMN's associated with one or more bacteria. The percent association was dependent on the concentration of organisms incubated with PMN's but at each concentration the percent association of encapsulated GC was significantly less than for non-encapsulated organisms. For example, strain No. 108 was associated with only 11% of the PMN's when encapsulated, as compared to 50% when unencapsulated, at a concentration of  $10^6$  organisms/ml; while at a concentration of  $10^7$  organisms/ml the respective figures were 40% and 90% (Fig. 1). These findings suggest that encapsulation of GC confers some resistance to phagocytosis by human PMN's by interfering with the physical association which precedes and triggers the phagocytic event.

#### B. Blocking of adhesion of GC to epithelial cells.

Since gonorrhea, per se, is a local mucosal surface infection it is assumed that attachment of GC to the mucosal surface is the initial pathophysiologic event and that inhibitors of this attachment may play a key role in determining resistance to infection.

In the preceding year a simple in vitro assay for adhesion of attachment of GC to epithelial cells was developed and local genital secretions were found to contain inhibitors of attachment. The exact nature of these inhibitors was identified during the current reporting period.

In this study the stimulation of local antibodies by gonococci was detected by immunofluorescence (Table 6,7). Both IgG and IgA classes of immunoglobulin were involved and the IgA component was primarily of a secretory antibody (11S) nature (Table 8). The ability of these antibodies to inhibit attachment to epithelial cells was demonstrated (Table 9,10), appears to persist at least for a short period of time (Table 11), and to be relatively strain specific (Table 11).

The specificity of the inhibiting antibody was further demonstrated by using rabbit antisera. Inhibition of epithelial cell adhesion (attachment) of individual strains of N. gonorrhoeae by specific rabbit antisera was quite antigenically distinct (Table 12).

#### C. Studies on the nature of bactericidal activity in human sera.

1. In previous studies defining parameters of a complement dependent serum bactericidal assay for N. gonorrhoeae (GC) serum from five species of mammals were studied for their ability to act as sources of complement for the test. In the absence of known specific antibody, guinea pig, rabbit, dog and horse sera were lethal to some degree for the six strains of N. gonorrhoeae tested. However, human serum was lethal for only three of the strains, the remaining three being resistant. A larger

Fig. 1.

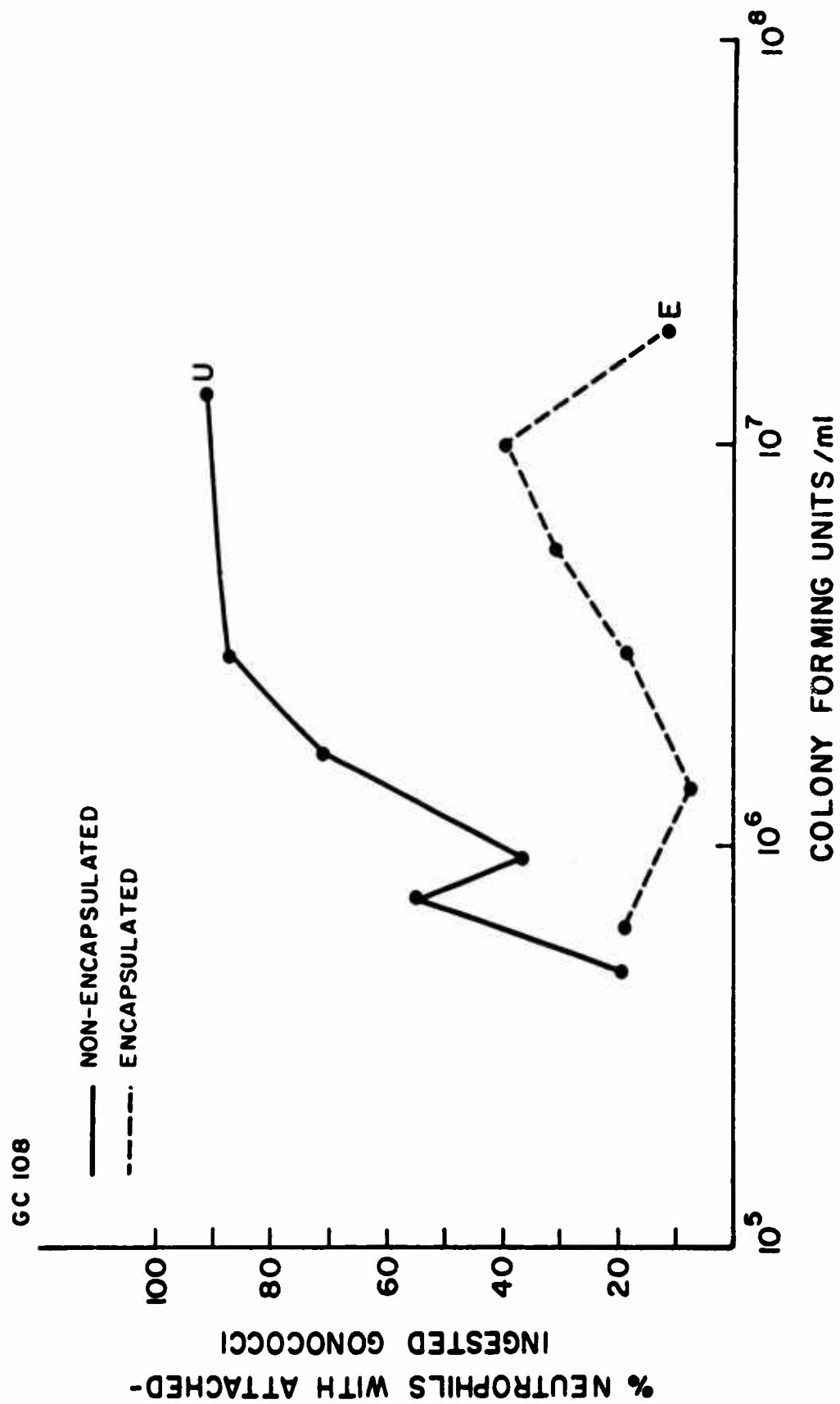


Table 6. Antibody concentrations and indirect immunofluorescent antibody (IFA) titers in vaginal secretions.

Patient	Date	Immuno fluorescent titers <sup>1</sup>		Conc. immunoglob. (mg/100 ml)		Specific ab titers <sup>2</sup>		IgG/IgA
		IgG	IgA	IgG	IgA	IgG	IgA	
135	9 Jun	<2	<2	2.15	0.4	(0)	(0)	5.4:1
	17 Jun	<2	<2	2.15	0.4	(0)	(0)	5.4
	23 Jun	<2	<2	2.15	0.5	(0)	(0)	4.3
	7 Jul	<2	<2	1.80	0.4	(0)	(0)	4.5
152	7 Jul	32	<2	40	4.0	0.80	(0)	10.0:1
	25 Jul	128	<2	42	3.9	3.05	(0)	10.8
	8 Aug	16	<2	38	3.2	0.42	(0)	11.9
136	17 Jun	8	4	9.8	0.8	0.82	5.00	12.3:1
	22 Jun	32	16	13.5	23.0	2.37	0.70	0.6
	30 Jun	128	<2	3.5	5.9	36.57	(0)	0.6
148	14 Jul	8	64	8.7	4.2	0.92	15.24	2.1:1
	28 Jul	8	8	8.5	3.9	0.94	2.04	2.2
	4 Aug	16	8	17.3	7.8	0.92	1.03	2.2
174	11 Jul	64	64	8.4	1.4	7.62	45.71	6.0:1
	18 Jul	128	64	18.3	5.5	6.99	11.64	3.3
	11 Sept	64	16	9.2	2.7	6.96	5.93	3.4
	19 Sept	32	8	4.3	1.4	7.44	5.61	3.1
134	9 Jun	4	2	33.0	2.2	0.12	0.91	15.0:1
	17 Jun	16	4	8.7	1.7	1.84	2.35	5.1
	23 Jun	32	4	17.0	2.6	1.88	1.54	6.5
	30 Jun	64	16	33.0	13.1	1.94	1.22	2.5
143	7 Jul	128	16	21.1	13.3	6.07	1.20	1.6:1
	14 Jul	64	32	17.0	7.4	3.76	4.32	2.3
	21 Jul	64	8	28.2	12.4	2.27	0.65	2.3
N1	Control	<2	<2	4.2	2.3	(0)	(0)	1.8
N2	Control	<2	<2	3.6	1.9	(0)	(0)	1.9
N3	Control	<2	<2	2.8	1.6	(0)	(0)	1.8
N4	Control	8	<2	7.4	3.0	1.08	(0)	2.5

<sup>1</sup> Patients vs. homologous strain; control vs. GC9.

<sup>2</sup> Specific IFA titer ÷ immunoglobulin concentration.



Table 7. Local antibodies to N. gonorrhoeae in male urethral secretions.

Patient	IFA titer <sup>(1)</sup>		Immunoglobulin conc.		Specific titer		IgG/IgA
	IgG	IgA	IgG	IgA	IgG	IgA	
7051	32	<2	14.2	4.0	2.25	(0)	3.55
7105	32	4	10.2	1.3	3.14	3.08	7.85
7102	4	<2	1.1	0.6	3.64	(0)	1.83
7107	8	8	6.2	1.8	1.29	4.44	3.44

(1) Reciprocal titer, indirect immunofluorescence.

Table 8. Immunofluorescent IgA antibody titers using 7S and 11S conjugates.

Patient	Date	IgA conjugate	
		7S	11S
134	9 June	2 <sup>(1)</sup>	2
	17 June	4	2
	23 June	4	4
	30 June	16	16
136	17 June	4	4
	22 June	16	8
	30 June	2	<2

(1) Reciprocal titers

Table 9. Blocking of epithelial cell attachment (IEA) by vaginal secretions.

Patient	Date	IEA		Immunofluorescence	
		Homol. (1)	Heterol. (2)	IgG	IgA
134	9 Jun	64	8,8,8	0.12	0.91
	17 Jun	256		1.84	2.35
	23 Jun	128	8,8,8	1.88	1.54
	30 Jun	8		1.94	1.22
135	9 Jun	<4		(0)	(0)
	17 Jun	<4		(0)	(0)
	23 Jun	<4		(0)	(0)
	7 Jul	<4		(0)	(0)
136	17 Jun	128	<4	0.82	5.00
	22 Jun	128	<4	2.37	0.70
	30 Jun	128	<4	36.57	0.00
143	7 Jul	8		6.07	1.20
	14 Jul	8		3.67	4.32
	21 Jul	<8		2.27	0.65
152	1 Aug	<4		0.80	(0)
	8 Aug	<4		3.05	(0)
	21 Aug	<4		0.42	(0)
149	7 Jul	8		ND	
	25 Jul	8		ND	
	8 Aug	8		ND	
N1			<4 (3) <4 <4 <4		
N2			<4 <4 <4 <4		
N3			<4 <4 <4 <4		
N4			<4 <4 <4 <4		

(1) EAI vs. infecting strain

(2) EAI vs. heterologous strain

(3) EAI vs. one of the above infecting strains

Table 10. The ability of IgA and IgG fractions in vaginal secretions to inhibit epithelial cell attachment (IEA).

Date	Unfractionated secretions <sup>(1)</sup>	Fractions		
		11S (Pool 1)	7S (Pool 2)	Pool 3 <sup>(1)</sup>
		IgA	IgG	
29 Jan	64 <sup>(3)</sup>	8	2	<2
9 Feb	64	4	2	ND <sup>(4)</sup>
19 May	64	4	16	ND

(1) Unfractionated vaginal secretions tested the same day

(2) Pool 3 tested as a control

(3) Reciprocal titers

(4) Not done

Table 11. Inhibition of epithelial cell attachment of homologous strain over a 4-month period.

Date	IEA (1)	IgG conc. (2) mg/100 ml	IFA (3)		Specific IgG
	GC418		IgA	IgG	
29 Jan	64 <sup>(4)</sup>	5.6	16 <sup>(4)</sup>	4 <sup>(4)</sup>	0.71
31 Jan	64				
6 Feb	16	4.4			
8 Feb	32	4.4			
19 Feb	32	4.8	8	32	6.67
4 Mar	64	15.0	8	64	4.27
23 Apr	32	10.4	8	64	6.15
19 May	64	5.5	4	32	5.82
<u>Uninfected secretions</u>					
N 9	2	>12.5	<2	<2	0.00
N 12	8	1.2	<2	2	1.67
N 14	4	1.6	<2	<2	0.00
N 15	4	7.4	<2	4	0.54
N 16	2	2.2	<2	<2	0.00

(1) Inhibition of epithelial cell adhesion.

(2) Secretions were standardized to 50% of an IIS standard; therefore, IgA titers can be compared directly and need not be standardized.

(3) Indirect immunofluorescence.

(4) Reciprocal of titer

Table 12. Inhibition of epithelial cell adhesion (EAI) by rabbit antisera.

Rabbit antisera (1)	Organisms														
	9	101	103	104	105	108	110	113	120	121	125	129			
9	16 <sup>(2)</sup>	2	2	2	<2	<2	2	4	<2	<2	2	<2			
101	<4	512	4	16	2	4	<2	<4	64	2	16	<2			
103	4	2	512	2	2	2	2	2	<2	2	4	<2			
104	<4	<4	4	64	2	<4	<2	2	<4	<2	<4	<2			
105	2	2	2	2	128	4	2	4	<2	4	2	<2			
108	4	8	2	4	<2	32	2	<2	4	<2	2	<2			
110	2	2	4	2	<2	2	64	2	<2	2	<2	<2			
113	2	2	<2	<2	2	2	<2	32	2	2	2	<2			
120	ND <sup>(3)</sup>	4	<2	8	4	2	<2	2	32	2	8	4			
121	ND	2	4	<2	2	2	32	4	<2	512	4	<2			
125	ND	4	2	8	2	8	<2	2	<4	4	1024	2			
129	ND	2	2	<2	2	<2	<2	<2	<2	<2	<2	256			

(1) Antisera to piliated organisms absorbed with unpiliated organisms

(2) Reciprocal titer

(3) Not done

number of strains were, therefore, studied. Approximately two-thirds were relatively or markedly insensitive to fresh human serum.

Since purported correlations have been shown between resistance to human serum and the ability of strains to produce disseminated gonococcal infections, which, in turn, have been correlated with specific amino acid auxotrophy, the nature of serum resistance/sensitivity was investigated further.

Chemical treatment with TRIS-EDTA has been shown to reverse serum resistance of gram-negative enteric bacteria by unfolding endotoxic LPS molecules and exposing core antigens against which bactericidal antibody was directed. Similar treatment of "resistant" GC strains, however, had no effect.

Studies completed during the previous year had shown that lipids extracted from normal human sera were equally toxic to serum sensitive and serum resistant strains of GC and that removal of lipids from serum had no effect on its bactericidal activity against serum sensitive strains.

These two studies indicated that the phenomenon of serum sensitivity probably results from specific antibodies in human serum directed against antigens present on sensitive strains but absent from resistant strains. To further develop this hypothesis, we absorbed human serum with both serum sensitive and resistant strains and found that serum sensitive strains were at least two times more efficient in absorbing bactericidal activity than were serum resistant strains. Thus, it is apparent that serum sensitivity is the result of "natural antibodies" present in human serum, specific for discrete antigens of the sensitive strains, and possibly resulting from antigenic stimulus by unrelated organisms which share these antigens. The chemical nature of these antigens will be investigated in future studies.

2. The reported correlation between serum resistance and potential for dissemination by GC strains was questioned by an investigation of the bactericidal activity of convalescent case sera from male patients with acute urethritis (3-21 days of infection), male and female patients who were asymptomatic carriers, and female patients who had symptomatic pelvic inflammatory disease or disseminated disease (Table 13).

Independent of the clinical category (including noninfected individuals) some strains were sensitive to the action of serum and complement in virtually every instance, some strains were completely resistant, and some strains were variably susceptible. However, sera from infected patients were more broadly bactericidal. Sera 135 and 152 were particularly potent and killed the largest number of strains, most of which were resistant to the remainder of the 30 sera tested including the homologous strain.

In order to determine whether titers varied over time, sequential sera was studied from patients in each group (Tables 14,15,16). Of the 15 patients with acute urethritis, acute PID or disseminated gonococcal infections, five developed progressive increases in bactericidal titers,

Table 13. Bactericidal activity of human sera obtained from patients with various clinical presentations.

[illegible]

Straight lines represent titers <4. Reciprocal titers presented whenever strain was killed

Table 14. Bactericidal antibodies in male patients with acute urethritis.

Patient	Day <sup>1</sup>	Bactericidal titer
137	5	- <sup>2</sup>
	19	-
	16	-
	24	128 <sup>3</sup>
139	2	-
	15	32
	23	32
186	3	-
	10	-
146	7	-
	12	-
	20	-
	28	-

<sup>1</sup> Day after treatment - patients cultured with 3-8 days of acquisition

<sup>2</sup> - = <1:4

<sup>3</sup> Reciprocal titer



Table 15. Bactericidal antibodies in female patients with acute inflammatory disease.

Patient	Day <sup>1</sup>	Bactericidal titer
132	4	32 <sup>2</sup>
	12	512
	18	512
	25	256
201	6	-
	27	-
	53	-
128	5	-
	13	-
	20	-
129	4	-
	9	-
	17	-
135	4	4
	12	32
	18	64
	32	16

<sup>1</sup> Approximate day after onset of symptoms

<sup>2</sup> Reciprocal of titer

Table 16. Bactericidal antibodies in patients with disseminated gonococcal disease.

Patient	Day <sup>1</sup>	Bactericidal titer
127	28	<sub>2</sub>
104	14	-
	21	-
317	18	-
432	6	>16
	10	>16
	26	>16
	47	>16
433	5	-
	10	-
	19	-

<sup>1</sup> Approximate day after infection

<sup>2</sup> Reciprocal of titer

and three of those strains (139, 135, 432) were killed by sera from non-infected individuals (Table 13). However, none of seven patients who were asymptomatic carriers developed bactericidal antibodies.

All of the patients had immunoglobulin binding to the homologous organism as determined by immunofluorescence. When mean titers were compared against common strains, sera from infected patients had mean titers 2-3 x higher than noninfected individuals.

### 3. Antigens involved in the bactericidal reactions:

**Endotoxin:** The importance of endotoxin as an antigen in the bactericidal reaction was demonstrated by both inhibition and absorption studies. Partial chemical analysis verified the presence of 2-keto-3-deoxyoctonate (KDO) and the absence of protein (by Lowry determination and SDS gel filtration) and nucleic acid.

Specific and complete inhibition of the bactericidal reaction of serum from patients 135 and 127 with endotoxin from strain 139 occurred with greater than 20 µg of endotoxin (Fig. 2). Activity against a sensitive strain used as a control was unaffected. Absorption with endotoxin absorbed on red cells gave identical results.

However, absorption of a heterologous serum (127) with endotoxin 139 removed most but not all of the antibodies. Adsorption of this serum with a protein preparation (devoid of endotoxin as evidenced by KDO determinations) removed the remainder of the bactericidal antibodies, suggesting that a small amount of activity was directed against a protein antigen (5).

### 4. Antibody classes involved in the bactericidal reaction:

Serum from three infected and two noninfected individuals was fractionated into IgG and IgM fractions and IgA was removed from the sera by immunoabsorption of five infected patients. All of the bactericidal activity in the serum resided in the IgM fraction. The bactericidal activity was unaffected by removal of IgA.

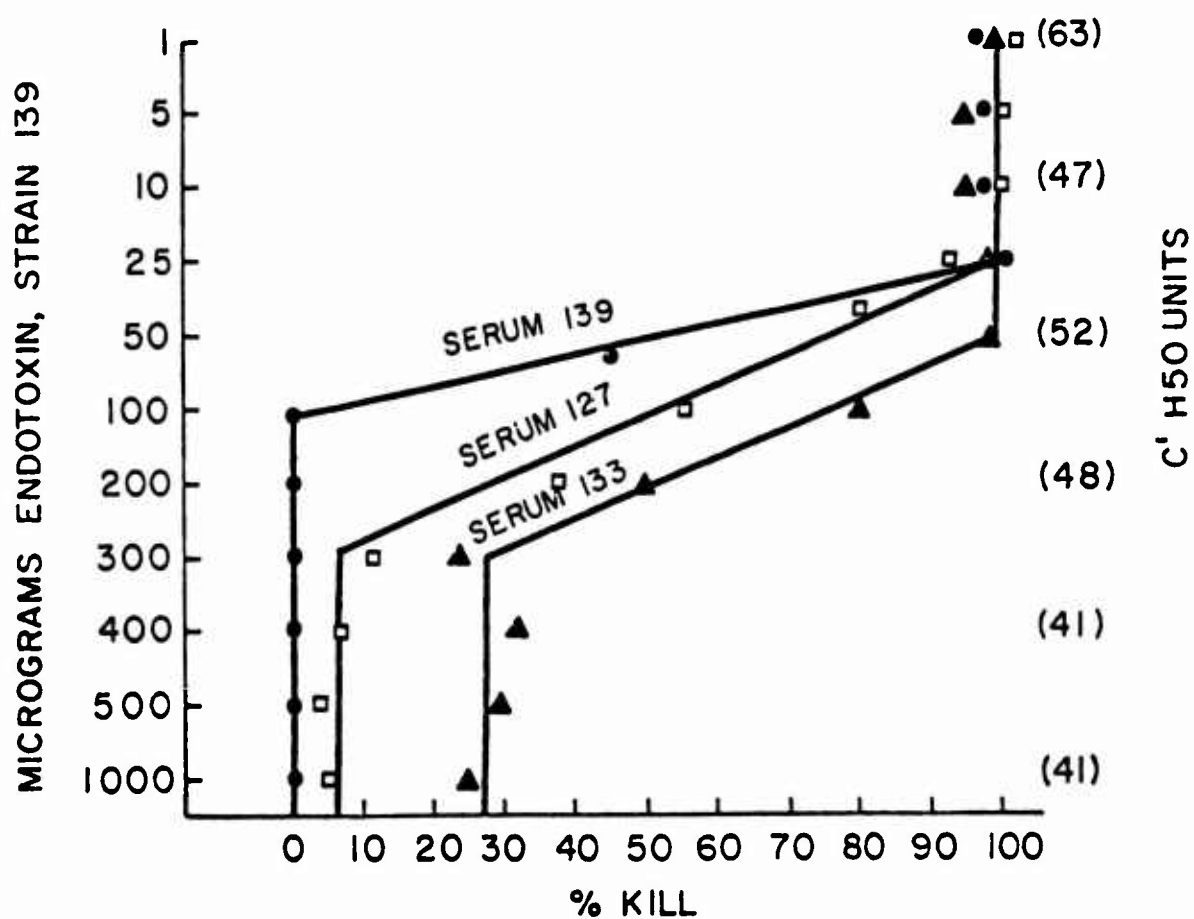
## IV. Pyocin sensitivity as a typing system for *N. gonorrhoeae* (GC).

Epidemiological studies of GC require a system for identifying individual strains. The finding that GC strains were sensitive to the bacteriocins of *Pseudomonas aeruginosa* (Ps), or pyocins was utilized to develop such a system.

Mitomycin induced pyocin extracts of Ps were unable to inhibit the growth of GC. Crude pyocin extracts were applied to agar plates which were then seeded with a lawn of GC. Zones of inhibition of growth of sensitive GC strains were demonstrated. Pyocin extracts were active

Fig. 2.

# INHIBITION OF BACTERICIDAL ACTIVITY OF HUMAN SERA BY ENDOTOXIN



in dilutions as high as 1:64. Pyocin activity of extracts was reduced by heating at 37° or 56° for 10 minutes and was completely lost when heated at 100° for 10 minutes (Table 17).

Table 17. Effect of temperature on pyocin activity.

	Indicator strains			
	GS 120	Ig 209	Ps W	Ps X
Pyocin M untreated	16*	16	4096	4096
Pyocin M 37°C	4	4	256	512
Pyocin M 56°C	4	4	256	1024
Pyocin M 100°C	0	0	0	0

\* Reciprocal of highest dilution able to inhibit growth.

Extracts were stable as long as four months at 4°C (Table 18). Pyocin activity against both GC and Ps was nondialyzable and was reduced by pronase treatment (Table 18), but was not affected by starch or BSA. Absorption of pyocins with sensitive Ps removed activity toward GC and Ps while absorption with insensitive Ps did not remove activity (Table 19). One hundred and six GC strains were tested for patterns of inhibition using a battery of 18 pyocin extracts (Table 20). Eighty-five percent of GC were separated into four major groups. Fifteen sets of GC strains thought to be similar or different on epidemiologic grounds were tested by pyocin typing. A positive correlation with epidemiologic data was observed (Tables 22,23,24).

#### V. Immunochemical relationships between lipopolysaccharide of gram negative bacteria.

Active and passive immunization directed against common antigens found in the core structures of the lipopolysaccharide from gram negative organisms is an approach to control of infections caused by these organisms in man. The immunochemical basis of this approach is dependent on demonstrating common antigenic determinants in these core structures. A solid phase radioimmune assay was used to investigate whether lipopolysaccharides (LPS) from P. aeruginosa, N. meningitidis and N. gonorrhoeae share antigenic core determinants with LPS of Enterobacteriaceae. The J-5 mutant of Escherichia coli 0111 is a galactose epimerase negative mutant whose LPS is rough in that it consists of lipid A, 2-keto-3-deoxyoctonic acid (KDO), heptose and glucose, but no galactose or O-side chain. Salmonella minnesota Re 595 (re) is a rough mutant whose LPS contains only lipid A and KDO and is, therefore, rougher than the J-5 mutant LPS.

Table 18. Test of stability and reproducibility of pyocin preparations.

Pyocin Preparations																				
Date induced	Date tested	GC																		
		Strains	B	C	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	W	V
4/74	8/22/74	218	+	+	+	n	0	0	+	n	+	n	+	+	0	+	+	+	0	0
9/74	10/9/74	218	+	+	+	0	0	0	+	0	+	+	+	+	0	±	+	±	0	0
4/74	7/31/74	202	+	+	+	n	0	0	+	n	+	n	+	+	0	+	+	+	0	0
9/74	10/9/74	202	+	+	+	0	0	0	±	0	+	±	+	±	0	±	+	+	0	0
4/74	8/22/74	214	±	+	+	n	0	0	+	n	+	n	+	+	0	+	+	+	0	0
9/74	10/9/74	214	+	+	+	0	0	0	+	0	+	+	+	+	0	±	+	+	0	0
4/74	6/12/74	265	+	+	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0
4/74	8/27	264	+	+	+	n	0	0	0	n	±	n	0	0	0	0	0	0	0	0
9/74	10/2/74	265	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

+ = Killing

0 = No kill

± = Light overgrowth

n = Not tested

Table 19. Enzymatic treatment of pyocins.

	Gonococcal Indicator Strains					Pseudomonas Indicator Strains	
	GC 273	GC 210	GC 263	Ig 209	Ps V	Ps W	
Pyocin 0 control	8*	16	4	32	4096	4096	
Pyocin 0 treated with pronase	2	4	0	8	64	32	
Pyocin 9 treated with trypsin	8	16	4	32	4096	4096	
Pronase alone	0	0	0	0	0	0	
Trypsin alone	0	0	0	2	0	0	

\* Reciprocal of highest dilution able to inhibit growth.

Table 20. Absorption of Pyocin P.

	<u>N. gonorrhoeae</u>						<u>P. aeruginosa</u>				
	GC 120	GC 263	GC 134	GC 230	Ps P	Ps X	Ps W	Ps I			
Pyocin P unabsorbed	8*	4	16	4	0	1024	128	256			
Pyocin P absorbed with GC 120**	0	0	0	0	0	64	2	4			
Pyocin P absorbed with GC 120 native complex**	4	2	8	2	0	64	2	4			
Pyocin P absorbed with Pseudomonas X	0	0	0	0	0	0	0	0			
Pyocin P absorbed with Pseudomonas P	32	32	64	16	0	1024	512	1024			

\* Reciprocal of highest dilution having activity

\*\* Pyocins were absorbed with live washed organisms or native complex as described in Materials and Methods



Table 21. Distribution of pyocin activity.

Pyocin	No. of strains +	No. of strains ±	No. of strains -	% +	% ±	% -
A	53	2	0	96	4	0
B	103	3	0	97.2	2.8	0
C	106	0	0	100	0	0
D	1	0	105	0.9	0	99.1
E	103	2	1	97.2	1.9	0.9
F	3	0	103	2.8	0	97.2
G	102	3	1	96.3	2.8	0.9
H	5	7	43	9.1	12.7	78.2
I	5	5	96	4.7	4.7	90.6
J	5	12	87	4.8	11.6	83.6
K	61	17	28	58	16.0	26
L	0	1	53	0	1.9	98.1
M	76	3	27	71.7	2.8	25.5
N	41	5	7	77.4	9.4	13.2
O	70	6	30	66	5.7	28.3
P	57	8	40	54.3	7.6	38.1
Q	4	2	100	3.8	1.9	94.3
R	45	24	37	42.5	22.6	34.9
S	60	11	35	56.6	10.3	33.0
T	52	13	41	49.1	12.3	38.7
W	1	3	102	0.9	2.8	96.3
V	0	3	103	0	2.8	97.2
X	0	2	104	0	1.9	98.1

Table 22. Typing of strains from various body sites of the same patient.

GC Strain	Patient	Isolate site	B	C	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	W	V	X	
212	AB	Cervix	+	+	+	0	0	0	+	0	+	+	+	+	+	0	±	+	+	0	0	0
214	AB	Urethra	+	+	+	0	0	0	+	0	+	+	+	+	+	0	±	+	+	0	0	0
203	BC	Cervix	+	+	+	±	±	±	0	0	0	0	0	0	0	0	0	0	0	0	0	
211	BC	Urethra	±	+	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
218	CD	Urethra	+	+	+	0	0	0	+	0	+	+	+	+	+	0	±	+	±	0	0	0
219	CD	Vagina	+	+	+	0	0	0	+	0	+	+	+	±	0	±	+	+	0	0	0	0
287	EF	Cervix	+	+	+	+	+	+	±	0	0	0	0	0	0	0	0	0	0	±	±	
279	EF	Rectum	+	+	+	+	±	±	0	0	0	0	0	0	0	0	0	0	0	0	+	
161	Gd	Cervix	+	+	+	n	0	0	+	n	+	n	+	+	+	0	+	+	+	0	0	0
162	GH	Nasopharynx	+	+	+	n	0	0	+	n	+	n	+	+	+	0	+	+	+	0	0	0
492	CM	Vagina	±	+	+	n	0	0	+	n	+	n	+	+	+	0	+	+	+	0	0	0
493	CM	Urethra	±	+	+	n	0	0	±	n	±	n	±	0	0	±	±	±	0	0	0	0
494	CM	Cervix	+	+	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0	0	0
495	CM	Nasopharynx	±	±	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0	0	0
316	KL	Cervix	+	±	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0	0	0
317	KL	Joint	+	+	+	n	0	0	+	n	+	n	+	+	+	0	+	+	+	0	0	0
342	CM	Rectum	+	+	+	n	0	0	+	n	+	n	+	+	+	0	+	+	+	0	0	0
343	CM	Cervix	+	+	+	0	0	±	0	±	0	±	+	+	+	0	+	+	+	0	0	0
344	CM	Urethra	+	+	+	±	0	±	±	0	+	+	+	+	+	0	+	+	+	0	0	0
345	CM	Vagina	+	+	+	+	0	±	+	0	+	+	+	+	+	0	+	+	+	0	0	0

+ = Killing

0 = No kill

± = Light overgrowth

n = Not tested

Table 23. Typing of single cohort of Ig consort strains.

GC strain	Date isolated	B	C	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	W	V	X
55 - ST	10/18/72	±	+	+	n	0	0	±	n	+	n	+	+	0	+	±	±	0	0	0
85 - ST	11/2/72	+	+	+	0	0	0	±	0	+	±	+	±	0	0	+	±	0	0	0
349 - ST	9/17/73	+	+	+	+	±	+	+	0	+	+	+	+	0	+	+	±	0	±	0
342 - CM	9/18/73	+	+	+	+	±	+	+	0	+	+	+	+	0	+	+	±	0	±	0
357 - JT	9/19/73	+	+	+	+	±	+	+	0	+	+	+	+	0	+	+	±	0	±	0
484 - ST	2/12/74	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
492 - CM	2/19/74	+	+	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0	0
527 - ST	3/25/74	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
530 - CM	3/26/74	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
533 - JT	3/27/74	+	+	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0	0
545 - ST	4/19/74	+	+	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0	0
550 - CM	4/23/74	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
553 - JT	4/25/74	+	+	+	n	0	0	0	n	0	n	0	0	0	0	0	0	0	0	0

+ = Killing

0 = No kill

± = Light overgrowth

n = Not tested

Table 24. Typing of consort strains.

GC strain	Date isolated	B	C	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	W	V	X
195 - JP	6/15/73	+	+	+	0	0	0	n	0	±	0	n	0	0	0	±	0	0	0	0
211 - JA	6/23/73	±	±	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
179 - NZ	6/7/73	0	±	+	n	0	0	0	n	±	n	0	0	0	0	0	0	0	0	0
298 - RO	8/7/73	±	±	+	n	0	0	±	n	±	n	±	±	0	±	±	±	0	0	0
287 - ED	8/2/73	+	+	+	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	+
279 - BH	7/26/73	+	+	+	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	+
180 - WE	6/14/73	+	+	+	n	0	0	+	n	+	n	+	+	0	±	±	±	0	0	0
182 - TC	6/14/73	+	+	+	n	0	0	+	n	+	n	+	+	0	+	±	±	0	0	0
202 - TC	6/26/73	+	+	+	0	0	0	0	±	+	±	+	+	0	+	+	+	0	0	0
165 - LS	5/9/73	±	+	+	n	0	0	+	n	+	n	+	+	0	+	+	+	0	0	0
173 - AA	6/5/73	±	+	+	n	0	0	+	n	+	n	+	+	0	+	+	+	0	0	0
152 - CP	7/31/72	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
154 - AP	8/31/72	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129 - WD	5/15/72	+	+	+	±	±	+	±	0	+	+	+	+	0	+	+	+	+	±	±
130 - MD	5/16/72	+	+	+	±	±	+	0	0	0	0	0	0	0	0	0	0	0	0	0
161 - TR	5/1/73	±	+	+	n	0	0	+	n	+	n	+	+	0	+	+	+	0	0	0
158 - BL	5/1/73	±	+	+	n	0	0	±	n	+	n	±	±	0	+	±	±	0	0	0
210 - OC	6/19/73	+	+	+	0	0	0	+	0	+	+	+	+	±	+	±	±	0	0	0
200 - SD	6/26/73	+	+	+	0	0	0	±	0	+	±	+	±	0	±	±	±	0	0	0
368 - SD	10/17/73	±	+	+	n	0	0	±	n	±	n	±	+	0	+	+	+	0	+	0

+ = Killing

0 = No kill

± = Light overgrowth

n = Not tested

Rabbit antisera against each of these organisms was then quantitated for antibody directed against J-5 and Re LPS using a solid phase radio-immune assay. The J-5 antisera contained 88 ng specific antibody/ml and the Re antisera contained 139 ng/ml. The reaction between J-5 antibody at a concentration of 100 ng/ml and J-5 LPS or the reaction between Re antibody (100 ng/ml) and Re LPS was then inhibited by varying concentrations of homologous or heterologous LPS. J-5 LPS at a concentration of 250 ng/ml inhibited 96 percent of the J-5 antibody versus J-5 LPS reaction. Re LPS (250 ng/ml) inhibited 97 percent of the reaction between Re antibody and Re LPS. Lipopolysaccharide from J-5 inhibited 87 percent of this Re anti Re reaction while Re LPS was unable to inhibit the J-5 anti J-5 reaction, confirming that J-5 has determinants missing from the rougher Re mutant. The Re anti Re reaction was inhibited by 250 µg/ml LPS from E. coli 0111 (91% inhibition), S. typhimurium (82%), S. marcescens (45%), P. aeruginosa type 6 (95%), N. meningitidis 981 (69%), N. meningitidis 891 (66%), N. gonorrhoeae 137 (85%), N. gonorrhoeae 78 (72%), N. gonorrhoeae 104 (84%). Therefore, a core determinant found in Re LPS and also located in J-5 LPS core structure is shared by Enterobacteriaceae, such as E. coli, Salmonella, Serratia, as well as P. aeruginosa, N. meningitidis and N. gonorrhoeae.

The J-5 LPS also has antigenic determinants not found in the Re core which are shared with other organisms. For example, J-5 antibody reacted with N. meningitidis 981 LPS. This reaction could be inhibited by N. meningitidis 981 LPS (63%), N. meningitidis 978 LPS (56%), J-5 LPS (52%) but not Re LPS. Antibody raised against P. aeruginosa type 7 reacted with J-5 LPS. This reaction could be inhibited by P. aeruginosa LPS 7 (96%), J-5 LPS (94%) but only 37% by Re LPS.

The finding that common antigenic determinants in the core structures of LPS from Enterobacteriaceae are also found in LPS from P. aeruginosa, N. meningitidis and N. gonorrhoeae significantly extends the range of prophylactic and therapeutic possibilities for this approach to the control of gram negative bacterial infections.

#### Summary and Conclusions.

Group C vaccine was shown to retain its immunogenicity after storage for up to 5.8 years. Procedures for isolating meningococcal group Y capsular polysaccharide for testing as a candidate vaccine have been developed. Polysaccharides isolated from P. aeruginosa were characterized chemically and immunologically and found to provide protection in a mouse model. The presence of antigenic determinants in the lipopolysaccharide (LPS) of P. aeruginosa, N. meningitidis and N. gonorrhoeae that cross react with the rough LPS of the E. coli J5 and Re mutants was demonstrated. A solid phase radioimmunoassay (SPRIA) has been used to quantitate antibody to the E. coli J5 LPS in immune human sera, thus allowing standardization of passive transfer clinical trials and evaluation of active immunization. The SPRIA was shown to be clinically useful in measuring the antibody response of patients with streptococcal and staphylococcal

infections to cell wall teichoic acids. Inhibition of attachment of N. gonorrhoeae to epithelial cells by specific IgA and IgG antibodies in genital secretions was demonstrated. The killing of gonococci by normal human sera was found to be primarily due to IgM antibodies directed against the LPS antigens. The serum sensitivity of gonococcal strains was shown to be independent of the clinical state of the patient from whom the organism was isolated. Sensitivity of gonococci to P. aeruginosa pyocins was found to be a feasible approach to gonococcal typing. The gonococcal capsule was shown to be antiphagocytic and, therefore, of functional significance.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168 Bacterial diseases of military importance

Literature Cited.

References:

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Publication:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
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75 07 01	U	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
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(U) Leishmaniasis (U) Latin America (U) Epidemiology (U) Chemotherapy							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish 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) Seroepidemiologic surveys and field studies were used to determine extent of visceral leishmaniasis, a disease of military importance in other areas, in a newly discovered focus in Honduras. New <u>in vitro</u> and rodent model systems were used for testing antileishmanial drugs to replace unsatisfactory current therapy. Oxidative metabolism measurement techniques were applied to antileishmanial drug studies.</p> <p>25. (U) 75 07 - 76 06. Closure of this laboratory and transfer of functions to WRAIR required early termination of projects. A pattern of widespread but low intensity distribution was found in seroepidemiological study of visceral leishmaniasis. Isolates from 2 house rats are the first from a reservoir in Central America, and first record of a rodent host in the New World. A known vector species, <u>Lutzomyia longipalpis</u>, was found to be the most prevalent sandfly in Honduras and was infected experimentally. The <u>in vitro</u> system preliminarily reported last year was shown to be valuable for anti-leishmanial drug studies, after a major problem in producing axenic amastigotes was overcome. The <u>Mystromys</u> model of cutaneous infection was confirmed to be useable for drug evaluation and was found to parallel human infections with recurrent lesions after antimony therapy. Oxygen uptake studies provided basic information on <u>L. braziliensis</u> and demonstrated inhibitory action by known antileishmanial compounds. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 75 - 30 June 76.</p>							

\* Available to contractors upon originator's approval

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Project            3A161102B71Q : 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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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 these constitute a current military medical problem. Because of the lack of a completely effective drug to treat leishmaniasis, a major effort continued to be directed toward development of in vitro models for screening compounds for antileishmanial activity and animal models for testing drug efficacy against infection.

Progress

I. Leishmaniasis

1. Epidemiology of Visceral Leishmaniasis in Central America

A. History and Background

Visceral leishmaniasis is primarily a disease of the Old World, but over the years sporadic cases have been reported from widely separated areas in at least 7 countries of South America.<sup>1</sup> The clinical picture is very similar to that seen in the Mediterranean area, with acute infection and mortality occurring predominantly in young children. The widespread, but diffuse, distribution, of the parasite indicates that the causative parasite had not been introduced

in the recent era, but that it was a long established New World entity. Upon this basis it was considered to be distinct from Leishmania donovani and designated Leishmania chagasi.<sup>2</sup> Epidemiologic investigations established that the major nonhuman vertebrate host was a wild canine, and that the principal vector was Lutzomyia longipalpis.<sup>3</sup>

Visceral leishmaniasis from Central America has been little known, since almost all references have appeared in local journals. However, confirmed cases have been known from Mexico, El Salvador and Guatemala for many years. In Mexico 1 adult and 3 children have been diagnosed in the states of Guerrero and Puebla,<sup>4</sup> and in Guatemala, five cases, all in young children are known from an arid area of the Department of El Progreso.<sup>5</sup> In El Salvador, between the years 1950-54, four cases were recognized in children from 3 months to 3 years of age, of which 3 were fatal.<sup>6</sup> All originated in the mountainous border area in the southwest limits of the country. In each of these countries discovery of the autochthonous cases stimulated epidemiologic investigations, but none of these revealed additional cases, and none shed any light on reservoir hosts. The phlebotomine Lutzomyia longipalpis, known to be a vector in South America, was found to be the predominant sandfly throughout the range of distribution of known cases of disease and was therefore presumed to be the vector.

In 1974, this laboratory was consulted in connection with the first case of kala azar recognized in Honduras<sup>7</sup> and later an invitation of the Ministry of Health to study the problem was accepted when 2 young adult male patients were hospitalized with a confirmed diagnosis. Field teams visited areas of presumed transmission on several occasions in 1975-76, and in collaboration with Dr. Ramon Custodio, and with the support of the Division of Epidemiology, Ministry of Health, parasitological and entomological field studies were conducted. At the invitation of the Central American Research Station (CARS), Bureau of Tropical Diseases, Center for Disease Control, USPHS-DHEW, and with the concurrence of the Ministry of Health, a staff member visited El Salvador. In collaboration with Dr. Guillermo Sancho, Hospital Benjamin Bloom and Dr. Carl N. Muchnick, CARS Epidemiologist, a study was made to assess the extent of the disease distribution in that country.

#### B. Seroepidemiological Studies

To determine the extent and distribution of the infection in the 2 countries, and to pinpoint foci of transmission for studies on vectors and reservoirs, and seroepidemiological survey was conducted using the indirect fluorescent antibody test (IFAT) with amastigote

antigen developed in this laboratory.<sup>8</sup> Preliminary studies established that cross reactions permitted the use of readily available L. braziliensis amastigote antigen. However, this is a one-way cross, since sera from cutaneous cases do not cross react at the same level with L. chagasi antigen (Table 1-1). Positive reactors were verified with homologous antigen. A total of 1,089 sera from Honduras and 2,226 from El Salvador from a 1967-68 INCAP survey were available in the MARU serum bank and were tested.

In El Salvador, positive serologic reactors were encountered in approximately 30% of the localities sampled (16 of 30). However, the positivity rate was low, only one locality exceeded 4% positives, and the majority were <2% (Table 1-2). However, none of the communities where cases had occurred were included in the samples from these Departments in the INCAP survey. This pattern of diffuse distribution and low intensity is consistent with the scattered and sporadic cases and verifies the low level of transmission. These similarities to the epidemiologic pattern in South America suggest that in Central America the principal reservoir is also a wild, rather than a domestic, animal, and that the common man-biting sandflies rarely become infected. In Honduras the percentage of positive localities was somewhat higher, 19 of 29 localities (65%) and 3 of 4 military units had positive reactors. However, the intensity of infection was significantly higher, with 15 of 19 localities >2% and 7 localities and 3 military units exceeded 4% (Table 1-3).

#### C. Surveys in Areas of Suspected Transmission

Attempts were made to obtain population samples from areas with active transmission. One locality in El Salvador and 4 in Honduras were selected for study. The Salvador community was listed as the home of the last of 5 cases in children diagnosed by Dr. Sancho; Canton San Gregorio, near Sesuntepeque in the District of Cabañas. Filter paper blood specimens were collected from 260 persons, but only one positive reactor was encountered. Although this community is in the known endemic zone and close to other communities where cases occurred, it was subsequently learned that the child actually lived in another village across the border in Honduras, and that the family had claimed to be from San Gregorio to obtain medical treatment they might not receive as Salvadoreans. In Honduras the 4 study areas were chosen because they were at, or near, sites of transmission of recent cases. The first site visit to Las Playas revealed a suspect case which was subsequently reported by Nuernberger et al.<sup>9</sup> The 3 localities where cases occurred varied greatly in prevalence of antibody from 0 in Paraiso, 4% in Tefideros to 21% in Las Playas. It is of interest that six communities closely adjoining to Las Playas showed no positive reactors (Table 1-4). The fourth study area of Villa San Francisco

and 3 adjoining communities was not known to have had any cases, but was selected because the 3 adjoining communities had the same altitude as Las Playas and ecologic similarities. Two of the 3 adjoining communities had a significant antibody prevalence, La Mesa 8% and El Hato 6%, as had been suspected. However, the 8% prevalence encountered among inhabitants of the much lower and drier town of Villa San Francisco was entirely a surprise. The Ministry of Health physician who sees patients on weekly visits to the Health Center there has been alerted to the possibility of encountering active kala azar.

#### D. Reservoir Studies

Although canines were prime suspects as reservoirs of human disease, extreme difficulties were encountered in sampling dogs and a total of only 12 dogs from the Las Playas area of Honduras was tested in the IFAT and 3 by blood cultures. All were negative. Laboratory studies of the course of infection in dogs with a recent human isolate of L. chagasi from Honduras were conducted in 2 experiments. The first consisted of a series of 4 mixed breed dogs, 1 adult female, 1 juvenile (4 months) and 2 adult males. All animals were inoculated i.p. with  $1.04 \times 10^8$  P-3 promastigotes of strain 1164, housed in the laboratory, and fed commercially prepared feed. Blood was collected weekly, serum was separated, and cultures on blood agar made from whole blood and buffy coat. Animals were killed on the following schedule post-inoculation: dog #3 at 2 weeks, dog #2 at one month, dog #1 at 2 months, and dog #4 at 3 months. At necropsy, all animals were bled, and cultures inoculated from whole blood, buffy coat, liver spleen, kidney and bone marrow. Impression smears and histopathological sections were made of these organs. Only one animal, dog #2, was shown to be infected by positive cultures of bone marrow taken at necropsy one month after inoculation. In the second experiment, 5 puppies, whelped by dog #4, were inoculated at 2 weeks of age with P-4 promastigotes of strain 1164. Two animals received  $5.8 \times 10^7$  organisms by intracardiac inoculation, one dog received this inoculum i.p. and 2 dogs were inoculated with approximately  $1 \times 10^7$  subcutaneously on the nose. All animals were bled and cultures taken as previously described at 4, 5, 9, 10, 11, and 12 months post inoculation. Six months post inoculation 2 of the 5 dogs exhibited palpable and visible popliteal lymph nodes, and needle aspirates were made for culture. During the following 6 months, 2 more dogs developed palpable nodes, and cultures were made. At one year post inoculation, all dogs were sacrificed and the same procedures as in experiment #1 were followed at necropsy. Again, infection could be demonstrated only in one dog by a single culture. This was obtained from a needle aspirate of the popliteal node of a dog taken at 6 months. The failure to demonstrate infection in 7 of 9 dogs evidently is an indication of the inadequacy of the methods used,



since appearance of lymphadenopathy of popliteal nodes of 4 of 5 pups, and of serum antibodies detectable by IFAT in 3 of 3 dogs of experiment #1 which were tested, suggests active infection did occur. However, this low level of infection suggests that they would have been an unlikely source of infection for sandflies.

A series of rodents was inoculated to study susceptibility and course of infection. Fifteen laboratory mice of the University of Montana inbred strain reported to be susceptible to L. donovani infection<sup>10</sup> were inoculated i.p. and sacrificed at intervals between 4 and 8 months. Liver and spleen tissue was cultured and histopathological specimens prepared. Culture tubes of 1 animal were contaminated, and 12 of the remaining 14 cultures were positive. Hamsters were inoculated many times, including use as inoculum infectivity controls, and were invariably susceptible, with death usually occurring at about 4 months. Three hamsters were shown to be infective for sandflies in the vector studies. Because of the complete susceptibility of this cricetid, another species of this family, the African white-tailed rat, Myiostomys albicaudatus was tested for susceptibility. Losses from undetermined causes reduced the animals that could be tested to one, but it was positive both by culture, and by infectivity to sandflies in the vector studies. The susceptibility of rodents, both murids and cricetids, with long lasting infections and proven ability to infect sandflies, suggests that this group of animals might play a role as natural reservoirs of infection.

At Teñideros, wild animals were trapped in November 1975 and March 1976 in a search for natural reservoirs. A series of 4 gray foxes Urocyon cinereoargenteus, 6 opossums Didelphis marsupialis, 1 rabbit Sylvilagus sp., and 16 incompletely identified rodents, was tested by culture of whole blood, liver and spleen tissue. Of these, four animals, 2 opossums and 2 rats, had cultures positive for hemoflagellates. The 2 isolates from opossums were identified as containing Trypanosoma cruzi when they protected mice from an LD<sub>100</sub> challenge of T. cruzi (Brazil strain), but the possibility that these are mixed cultures has not been ruled out. The 2 isolates from rodents, strains 1314 and 1317, were identified as Leishmania on the basis of promastigote form in culture, and the production of intracellular amastigotes in Vero cells. The rodent hosts of these 2 isolates were identified by Dr. E. Mendez of Gorgas Memorial Laboratory as Rattus rattus on the basis of skull and dentition. In attempts to confirm these isolates as L. chagasi, promastigotes were inoculated into hamsters, guinea pigs, Myiostomys, and Montana strain mice. No infection has been demonstrated in any of these hosts to date, however, histopathological review of organs is pending.

Since Leishmania chagasi is known to occur in this locality, where other species of the genus is known, the isolates must be presumed to be L. chagasi. This represents the first discovery of a

rodent reservoir of human visceral leishmaniasis in the New World. The only other report of rodent infections of a viscerotropic form is of L. donovani from Rattus rattus, Acomys sp., and Arvicanthus niloticus in an area of the Sudan where human kala azar is endemic.<sup>11</sup> It is of interest that with 2 isolates from Arvicanthus, infection in hamsters was also not produced by promastigotes from cultures. The importance of natural infections in rats taken within human dwellings would appear to be great. However, surveys of Rattus rattus from communities where there is more evidence of active human transmission must be accomplished before the true significance as a reservoir can be appraised. The small number of gray foxes sampled cannot rule out the importance of a wild canine reservoir. The coyote, Canis latrans is also common in the area, but none were obtained.

#### E. Phlebotomine Vector Studies

The general distribution of Phlebotomines in Central America is not well documented, Panama being the only country where they have been intensively studied. Knowledge of sandflies in Honduras is principally from 1953-54 collections by Gorgas Memorial Laboratory which recorded 17 species from this country.<sup>12</sup> Collections were made in the present study using light traps and aspirator collections. The study areas were 3 localities in Honduras where confirmed cases of visceral leishmaniasis occurred: 1) Las Playas, Coyolito, La Mesa is an area at an altitude of 1,000-1,500 meters, situated in mountain ranges rising to 2,000 M.; 2) Yuscaran, a village of 1,500 inhabitants at the base of a 1,600 M. mountain; and 3) Tefideros, a village of scattered adobe houses at 700-1,000 M. altitude, approximately 10 kilometers East of Yuscaran. All 3 areas are in central Honduras within a 50 Km radius of the capital. Burros were utilized as bait and attracted primarily Lu. longipalpis. A single Lu. gomezi was aspirated from a burro in Yuscaran, and one engorged Lu. longipalpis was aspirated from a rodent burrow in Yuscaran. All other specimens were collected with standard CDC light traps. Results of these collections are summarized in Table 1-5. Five species were identified, of which Lu. longipalpis, gomezi, evansi, and cruciata are recognized as potential Leishmania vectors. Lu. longipalpis was taken in large numbers from the semi arid and rocky areas of Tefideros. Additional specimens were categorized as belonging to the verrucarum or cruciata series and a few remained unidentified. Two of the species collected, Lutzomyia gomezi (Nitzulescu, 1931) and Lu. chiapanensis (Dampf, 1947), had not previously been recorded from Honduras, and constitute new distribution records. The first record from Central America for Lu. gomezi was from Panama,<sup>13</sup> and this anthropophilic species is considered to be a vector of L. braziliensis. It has been reported also from El Salvador, Nicaragua, and Costa Rica, so the Honduras record is not surprising, although extensive collecting efforts in British Honduras<sup>14</sup> failed to identify this species and it is not known from Guatemala or Mexico. The single specimen collected was aspirated from a burro at dusk in

Table 1-1

IFAT Reactions of Serum of Cutaneous and  
Visceral Leishmaniasis Patients with Amastigote Antigen  
of L. braziliensis and L. chagasi

Type & Origin of Infection		ANTIGEN	
		<u>L.</u> <u>braziliensis</u> (Panama)	<u>L.</u> <u>chagasi</u> (Honduras)
SERA	1. Cutaneous Panama	1:64	1:16
	2. Cutaneous Panama	1:16	Neg
	3. Visceral Honduras	1:512	1:256
	4. Visceral Honduras	1:1024	1:1024
	5. Control Normal	Neg	Neg



Table 1-2

Results of Leishmania Indirect Fluorescent Antibody Tests  
by Locality in Seroepidemiologic Survey of El Salvador

Department	Locality	No. Tested	No. Pos.	% Pos.
Ahuachupán	Concepción de Ataco	62	0	
	San Pedro Puxtla	67	2	2.9
	Metapán	67	1	1.49
Santa Ana	Santiago de la Frontera	61	0	
	Texistepeque	101	1	.99
Sonsonate	Cuisnahuat	58	0	
	Salcoatitán	78	0	
*Chalatenango	La Reina	84	1	1.19
La Libertad	Comasagua	68	3	4.41
	Quetzaltepeque	53	1	1.88
San Salvador	San Salvador	217	3	1.38
	Ayutuxtepeque	56	0	
	Nejapa	63	1	1.58
	Panchimalco	61	0	
Cuscatlán	Monte de San Juan	47	0	
	Oratorio de Concepción	67	0	
	Santa Cruz Analquito	83	0	
La Paz	Cuyultitán	70	1	1.43
	San Juan Talpa	98	0	
	Santiago Nonualco	46	1	2.17
*Cabañas	Tejutepeque	75	1	1.33
*San Vicente	San Cayetano Istepeque	83	1	1.20
Usulután	Estanzuelas	54	2	3.70
	Tecapán	69	1	1.45
San Miguel	San Rafael Oriente	75	1	1.33
	Uluazapa	79	0	
Morazán	Cacaopera	80	0	
	San Simón	56	0	
La Unión	San José	73	2	2.74
	Yayantique	75	0	
*Known endemic area	TOTAL	2,226	23	

Table 1-3

**Results of Leishmania Indirect Fluorescent Antibody Tests  
by Locality in Seroepidemiologic Survey of Honduras**

Department	Locality	No. Tested	No. Pos.	% Pos.
*Francisco Morazán	Lepaterique	56	-	
	Nueva Armenia	50	-	
	Regimiento de Tegucigalpa	75	4	5.33
	Regimiento de la Fuerza Aerea	75	3	4.0
Cortés	San Pedro Sula	423	4	.95
	Puerto Cortés	64	1	1.56
	San Antonio de Cortés	77	2	2.6
	Guardería-del-Mercado Guamilite	30	-	
	Regimiento de San Pedro Sula	75	5	6.67
Atlántida	El Porvenir	73	-	
Santa Barbara	Arada	78	4	5.13
Yoro	Yoro	96	2	2.08
Copán	Dulce Nombre	68	-	
	Veracruz	74	6	8.1
	Santa Rita	81	4	4.94
Comayagua	Minas de Oro	68	2	2.94
Choluteca	San Marcos de Colón	74	3	4.05
	Concepción de María	80	-	
Olancho	Campamento	94	-	
	La Union	76	-	
	Dulce Nombre	64	1	1.56
*Paraiso	Yuscaran	73	3	4.11
	San Antonio de Flores	96	-	
Valle	Alianza	91	1	1.1
Lempira	Gracias	66	5	7.58
	Lepaera	108	-	

Department	Locality	No. Tested	No. Pos.	% Pos.
Ocotepeque	Marcala	75	2	2.67
	Tutule	72	2	2.78
Intibuca	Jesus de Otoro	82	3	3.66
	Yamaranguila	65	3	4.62
Colon	Tocoa	71	-	
Bahia	Roatan	39	1	2.56
Gracias a Dios	Puerto Lempira	70	2	2.86
		<hr/>	<hr/>	
		TOTAL	2,759	63

\*Known endemic area

Table 1-4

Prevalence of Antibodies to Leishmania in  
Kala Azar Endemic Area in Honduras

Locality	No. Sampled			No. Pos.	% Pos.
	♂	♀	Total		
Entire Country (Incap Survey)	1,308	1,451	2,759	63	2.28
*Las Playas	24	23	47	10	21.0
*Teñideros	47	33	85	3	4.0
*Paraiso	21	39	60	0	
Los Corrales	5	11	16	0	
La Cienega	8	6	14	0	
Los Potreros	3	2	5	0	
La Canada	1	3	4	0	
Cerro Grande	23	17	40	0	
El Sauce	12	21	33	0	
Villa San Francisco	32	86	118	9	8.0
La Mesa	14	23	37	3	8.0
El Hato	13	5	18	1	6.0
Coyolito	4	5	9	0	

\*With known cases

Table 1-5

Phlebotomine Collections from Three Areas of Honduras in July 1975

Sandfly Species	El Teñideros			Las Playas Area*			Yuscaran		
	♂	♀	Total	♂	♀	Total	♂	♀	Total
<u>L. longipalpis</u>	112	15	127				9	3	12
<u>L. chiapanensis</u>	1	0	1						
<u>L. cruciata</u>				2	12	14	0	1	1
<u>L. evansi</u>	1	0	1	3	1	4			
<u>L. gomezi</u>							0	1	1
Series Verrucarum	2	0	2						
Series Cruciata	2	1	3						
Unknown			7			4			0
TOTAL	118	16	141	5	13	22	9	5	14

\*Includes El Coyolito and La Mesa

Table 1-6

Lutzomyia longipalpis Collected at El Teñideros, Honduras  
in January 1976

Collection	Date	No. Collected		% of Coll.		Total
		Males	Females	Males	Females	Collection
1	3 Jan	623	119	83.9	16.1	742
2	5 Jan	1,254	145	89.6	10.4	1,399
3	6 Jan	<u>655</u>	<u>45</u>	<u>93.5</u>	<u>6.5</u>	<u>700</u>
Total		2,532	309	89.5	10.5	2,841

Yuscaran. Lu. chiapanensis, described from Mexico, has also been reported from El Salvador, Costa Rica and Panama, but its importance in disease transmission is unknown. The single male specimens was taken in a light trap collection in Teñideros. Identification of both species was confirmed by David Young, University of Florida.

Lutzomyia longipalpis is a known vector of visceral leishmaniasis in Brazil<sup>5</sup> and is the presumed vector in Central America, since its distribution coincides with that of the disease. Special collections to obtain this species for laboratory experiments were made in Teñideros in January 1976. Burros were used for bait, and collections were obtained on 4 successive evenings (Table 1-6). It was observed that sandflies were most numerous in areas where burros were contained for the night. Organic detritus from straw and feces humidified by urine provided a favorable milieu for the juvenile stages. From 1800-2100 hrs, sandflies were numerous. They were initially seen on hooves and lower legs of the burro, but later could be found all over the animal. Few sandflies were found on the collectors. Collections consisted primarily of males; a total of 83%, or a ratio of 9.2 to 1 males to females was encountered in the 2,841 specimens separated as to sex.

## 2. Development of Antileishmanial Drug Screening Systems

### A. In vitro Systems

#### (1) Axenic Amastigote System

The use of axenic amastigotes of Leishmania braziliensis offered promise as a screening test for antileishmanial activity, but differences were encountered in various lots of fetal calf serum (FCS) which compromised the system (Annual Report 1974). This difficulty was circumvented by acquiring a large-volume lot of FCS which possessed satisfactory growth and conversion characteristics. This permitted experiments to standardize the screening system using the 8 compounds previously tested, and to test 2 additional compounds. As previously reported, all testing was done against a standard strain #1077 of Leishmania braziliensis. Standard drug dilutions of 1.0, 10 and 100 µg/ml were used whenever practicable, and organisms exposed for 48 and 96 hours in liquid medium at 34°C. Drug effect was determined by recovering exposed organisms for back cultures at 25°C and determining number of promastigotes produced at 4, 6, and 8 days. The clinically active pentavalent antimonial, methylglucamine antimoniate (Glucantime<sup>®</sup>), served as the reference drug. The LD<sub>100</sub> for the reference drug was confirmed to be 6,000 µg/ml. Typical results for compounds exhibiting antileishmanial effect and no effect are

demonstrated in the examples in Table 2-1. Graphic representation of effects of some compounds tested on the axenic amastigotes is presented in Figures 1-1 to 1-3.

As previously reported, this screening system demonstrated antileishmanial activity that correlated with known activity in other tests; Lampit, a diamidine compound, and Primaquine, all of which have been reported to have some effect in clinical trials, and Astiban, an antimonial compound, predictably demonstrated marked antileishmanial activity in this screening test. The 8 aminoquinoline AG 75499 (Fig. 1-2) demonstrated marked activity as it did in the University of Georgia screen with a L. donovani/hamster model. The two antifols, ZM 86024 and ZN 29917, which have demonstrated antimalarial properties in several systems were also active and the latter was the most active of any compound tested. This particular group of antimalarials would appear to merit testing in an animal model. In view of the fact that the most active antileishmanial drug known, amphotericin B, is an antimycotic agent, it is of interest that the antimycotic agent Nystatin in this series showed no activity.

## (2) Tissue Culture Systems

Earlier attempts to use a tissue culture/Leishmania braziliensis system for drug screening were dropped because of difficulties in assessing drug effects by microscopic examination of stained slides (Annual Report 1973). The convenience of evaluating drug effect by back culture in the axenic amastigote system suggested the application of this technique to the tissue culture system to permit further evaluation. Accordingly, trials were made by exposing both intracellular parasites and amastigotes freed from the host cells. In the extracellular experiments, amastigotes were harvested 7 days after infection of tissue cultures by the method used in this laboratory.<sup>8</sup> A concentration of  $1.0 \times 10^6$  amastigotes/ml was resuspended in T.C. medium 199 + 1% FCS (2 ml total) and exposed to various concentrations of Glucantime and the 8-aminoquinoline (AG 75499) at 34°C for 48 and 96 hours. At the duration of the proper drug exposure intervals, 0.1 ml samples were removed and added to Offut's blood agar media (agar + 15% defibrinated rabbit blood) to which 0.4 ml of Locke's solution had been added. These screw-cap culture tubes with organisms were then placed at 4, 6, and 8 days post 24°C incubation and the resulting promastigotes counted with the aid of a hemocytometer. Results are presented in Table 2-2. These preliminary results indicate that antileishmanial drug effect can be demonstrated in this system, but that at least quantitative differences exist between this and the axenic amastigote system, since the well established LD<sub>100</sub> level for

Glucantime was not lethal for Vero cell-produced amastigotes after 48 hours exposure.

The trials for drug exposure of intracellular amastigotes utilized large volume/parasite numbers because the experiments were designed to permit monitoring of  $O_2$  uptake which requires large numbers of organisms. Tissue cultures in 16 oz. bottles were used, with Glucantime in 10, 100 and 1,000  $\mu\text{g/ml}$  concentrations, and exposures of from 24 to 96 hours. After exposure, the drug overlay was discarded, the monolayers washed and the parasites harvested, counted and an aliquot inoculated into blood agar tubes for incubation at  $24^\circ\text{C}$ . Control parasites not exposed to drug were similarly treated. The cultures were examined at 2, 4, and 6 days, and motile promastigotes counted by aid of a hemocytometer. The results were uniformly negative with no drug effect detectable at the concentrations tested, including the known  $LD_{100}$  for Glucantime in the axenic amastigote system. It is likely that the host cell wall constitutes a barrier to the drug which has a strong protective effect over the relatively short exposure periods of these experiments. It has been demonstrated with isotope-tagged drug that cell walls of Vero cells are impervious to Metranidazole.<sup>15</sup> Exposure over periods of 6 to 8 days are feasible with the Vero cell system and would appear to be of interest. Such a system might prove to have a place as a secondary screening system for those drugs which exert their effects indirectly upon the parasite by altering the metabolism of the host cell.

#### B. Mystromys Model

The African white-tailed rat, Mystromys albicaudatus, has been shown to be exceptionally susceptible to infection with L. braziliensis which produces open lesions similar to those of human infection. (Ann. Report 1974). To confirm the preliminary results reported last year, and to evaluate this model as a tool for experimental treatment, two additional series of animals were inoculated with the standard strain (1077A) of L. braziliensis panamensis, and treatment with Glucantime approximating human treatment regimens was given at 60 and 90 days after infection. A total of 26 animals was inoculated, and 24 of 26, or 92.3%, developed discrete lesions. Treatment with 0.1 ml (=10 mg) Glucantime i.m. daily for 10 days was given in each hind limb on alternate days. In the group treated at 60 days, 5 of 6 animals demonstrated an immediate healing response and uneventful recovery while the lesions of the untreated controls continued to enlarge (Figure 1-4). Of 10 Mystromys treated 90 days post-infection 7 were apparently successfully treated, while 3 showed temporary improvement with reduction in lesion size, followed by recurrent progression (Figure 1-5). The delayed closure of lesions from the



16th to 18th week as indicated in the graph, was due to a single animal with an exceptionally large lesion which had a secondary bacterial infection which persisted for more than a week after the others had healed. One animal, A2253, a treatment failure at 60 days, was retreated at 90 days with little or no improvement of the lesion. When it died from unknown causes 10 days after completion of the second series, positive cultures were obtained from skin at the border of the lesion, indicating that the second treatment was also unsuccessful. The complete failure of the antimony compound in this one animal is of special interest because of the similarity to such occurrences in human disease, and gives a clue that the cause lies with the patient and is not due to parasite characteristics, such as inherent antimony resistance.

Some animals exhibited an aberrant course of infection which varied from the usual pattern of papule formation approximately 10-21 days post-infection, followed by ulceration and progressive increase of lesion size. Two animals exhibited no response to inoculation, one of these was reinoculated 30 days later, and again developed no discrete lesion. Self healing of an open lesion after 2 months occurred in 2 animals, with reactivation of the lesion after 4 months in 1 animals, and after 12 months in the other. These responses are suggestive of some of the unusual courses seen in human infection. A few animals have been maintained over a year post-infection, but no indication of mucous tissue involvement have yet been seen.

Table 2-1

Examples of Compounds With and Without Antileishmanial  
Activity After 48 and 96 Hours Exposure in the  
Axenic Amastigote Screening System

Drug  Concentration	48 HOURS EXPOSURE			96 HOURS EXPOSURE		
	No. of Motile Promastigotes/ml in Back Cultures					
	4 days	6 days	8 days	4 days	6 days	8 days
0.0 µg/m	$5.5 \times 10^5$	$3.3 \times 10^6$	$1.0 \times 10^7$	$5.5 \times 10^5$	$3.3 \times 10^6$	$1.3 \times 10^7$
Controls	$3.8 \times 10^6$	$1.5 \times 10^7$	$1.2 \times 10^7$	$4.6 \times 10^5$	$2.6 \times 10^6$	$1.2 \times 10^7$
Glucantime	0*	0	0	0	0	0
LD <sub>100</sub>						
AH 16970						
100 µg/ml	$1.0 \times 10^5$	$1.8 \times 10^6$	$4.0 \times 10^6$	$6.9 \times 10^5$	$4.5 \times 10^6$	$1.9 \times 10^7$
10 µg/ml	$4.5 \times 10^5$	$2.2 \times 10^6$	$1.0 \times 10^7$	$6.5 \times 10^5$	$3.4 \times 10^6$	$1.4 \times 10^7$
1 µg/ml	$9.0 \times 10^5$	$2.2 \times 10^6$	$5.4 \times 10^6$	$7.1 \times 10^5$	$3.7 \times 10^6$	$1.5 \times 10^7$
ZM 80228						
100 µg/ml	0	0	0	0	0	0
10 µg/ml	$5.0 \times 10^4$	$2.5 \times 10^5$	$1.2 \times 10^6$	0	0	0
1 µg/ml	$2.0 \times 10^5$	$9.5 \times 10^5$	$6.2 \times 10^6$	$6.9 \times 10^6$	$1.8 \times 10^9$	$8.7 \times 10^6$

\*  $< 1.0 \times 10^3$ , therefore not countable

Table 2-2

Effect of 48 and 96 Hour Exposure to Drugs  
on Leishmania braziliensis Amastigotes  
Harvested from Tissue Culture

Drug Concentration	48 HOURS EXPOSURE No. of Motile Promastigotes/ml in Back Cultures			96 HOURS EXPOSURE No. of Motile Promastigotes/ml in Back Cultures		
	4 days	6 days	8 days	4 days	6 days	8 days
0.0 µg/ml	$2.4 \times 10^5$	$3.0 \times 10^6$	$1.3 \times 10^6$	$< 1.0 \times 10^3$	$3.0 \times 10^5$	$4.8 \times 10^6$
Controls	$4.9 \times 10^5$	$7.5 \times 10^6$	$2.8 \times 10^7$	$< 1.0 \times 10^3$	$2.8 \times 10^5$	$5.4 \times 10^6$
Glucantime						
100 µg/ml	$5.0 \times 10^5$	$8.2 \times 10^6$	$1.2 \times 10^7$	$1.0 \times 10^4$	0	0
1 µg/ml	$4.3 \times 10^5$	$7.7 \times 10^6$	$1.3 \times 10^7$	$2.0 \times 10^4$	$1.9 \times 10^5$	$3.4 \times 10^6$
AG 75499						
100 µg/ml	0*	0	0	0	0	0
10 µg/ml	0	0	0	0	0	0
1 µg/ml	$2.0 \times 10^5$	$2.6 \times 10^6$	$1.2 \times 10^7$	0	0	0
Glucantime						
6000 µg/ml**	$2.0 \times 10^4$	$6.8 \times 10^5$	$7.2 \times 10^6$	0	0	0

\* $< 1.0 \times 10^3$ , therefore not countable

\*\*Known LD 100 of Glucantime in axenic system

FIGURES 1-1 THRU 1-3  
 EXAMPLES OF COMPOUNDS WITH ANTILEISHMANIAL ACTIVITY AFTER  
 96 HOURS EXPOSURE IN THE AXENIC AMASTIGOTE SCREENING SYSTEM

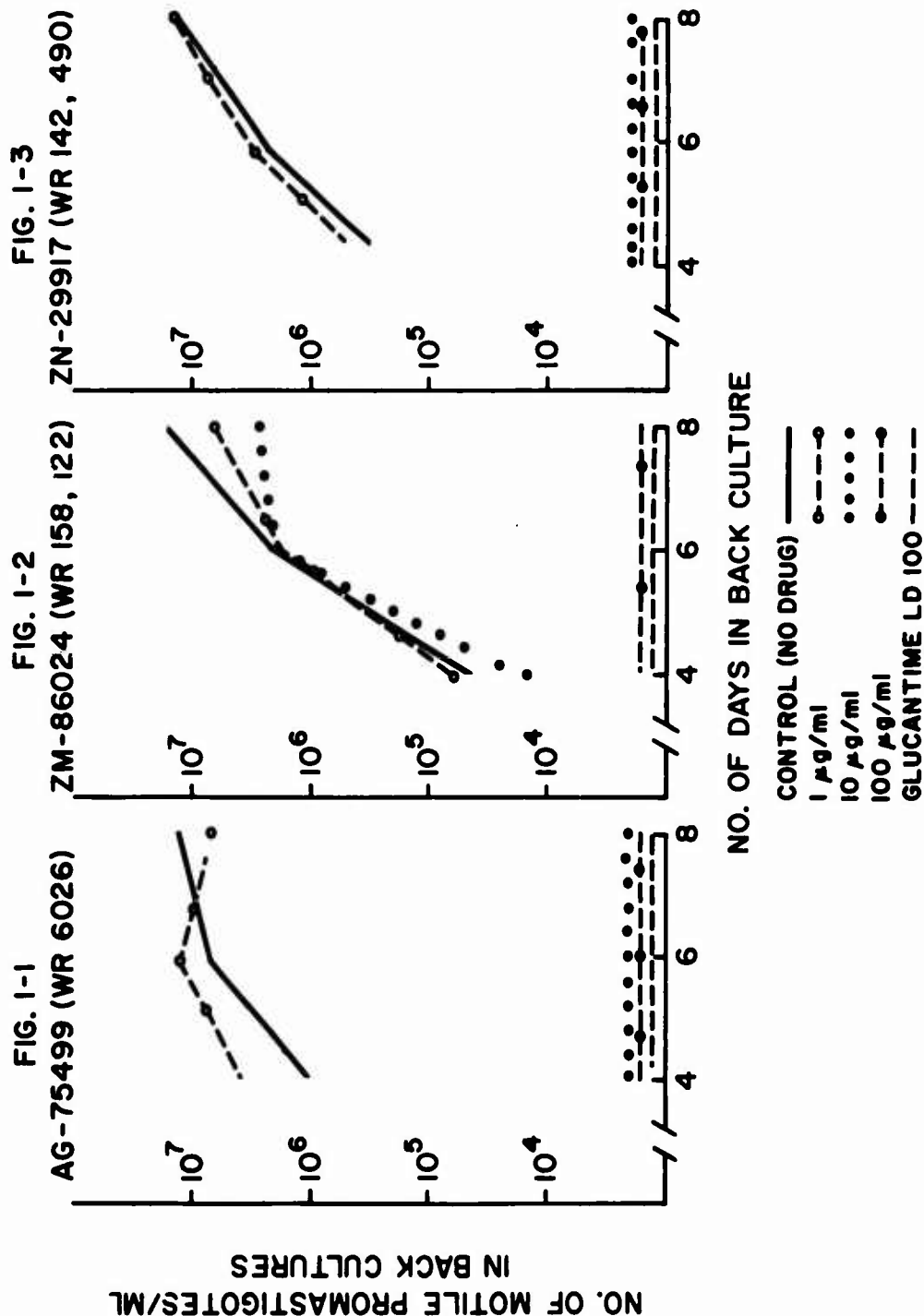


Figure 1-4

Effect of Methylglucamine Antimoniate on Size of  
Cutaneous Lesions of Mystromys albicaudatus when Treated 60 Days  
After Experimental Infection with Leishmania braziliensis

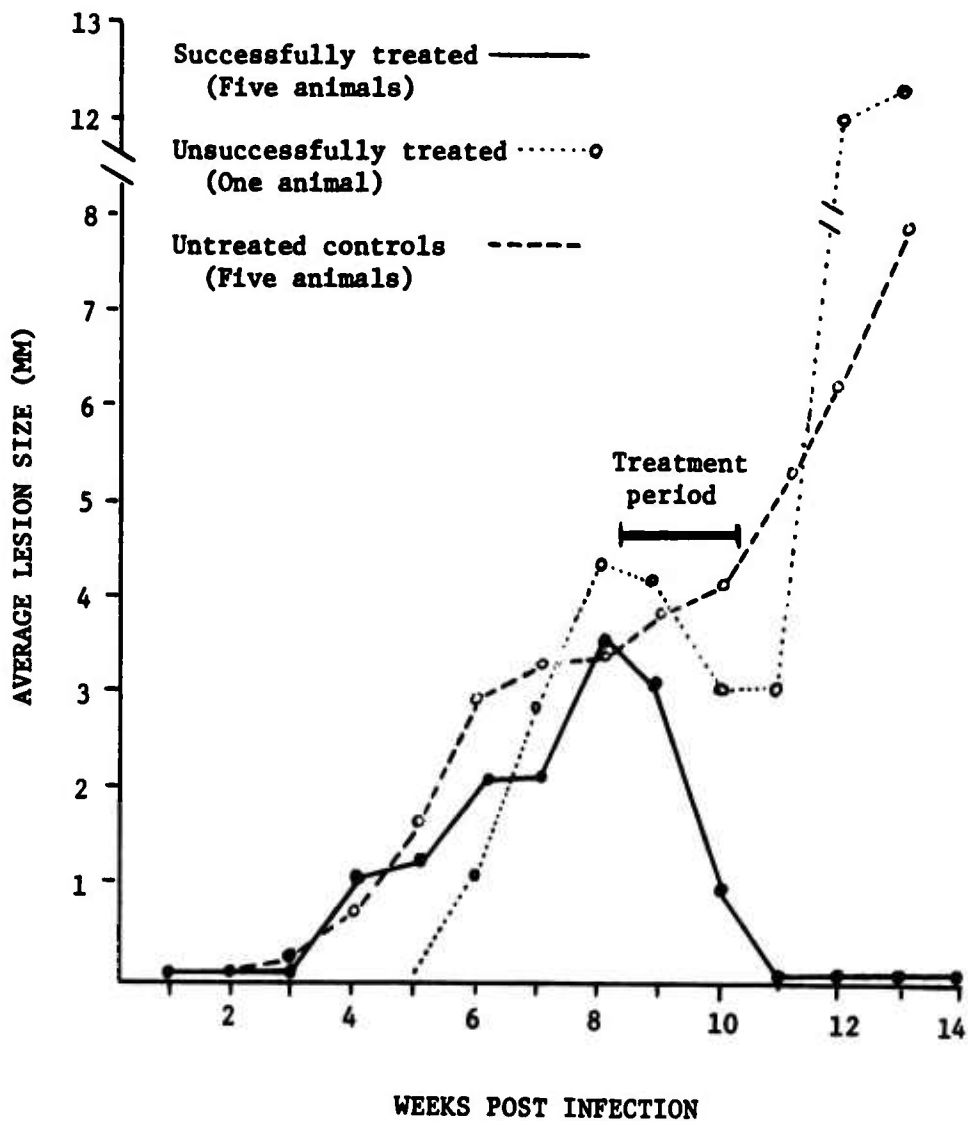
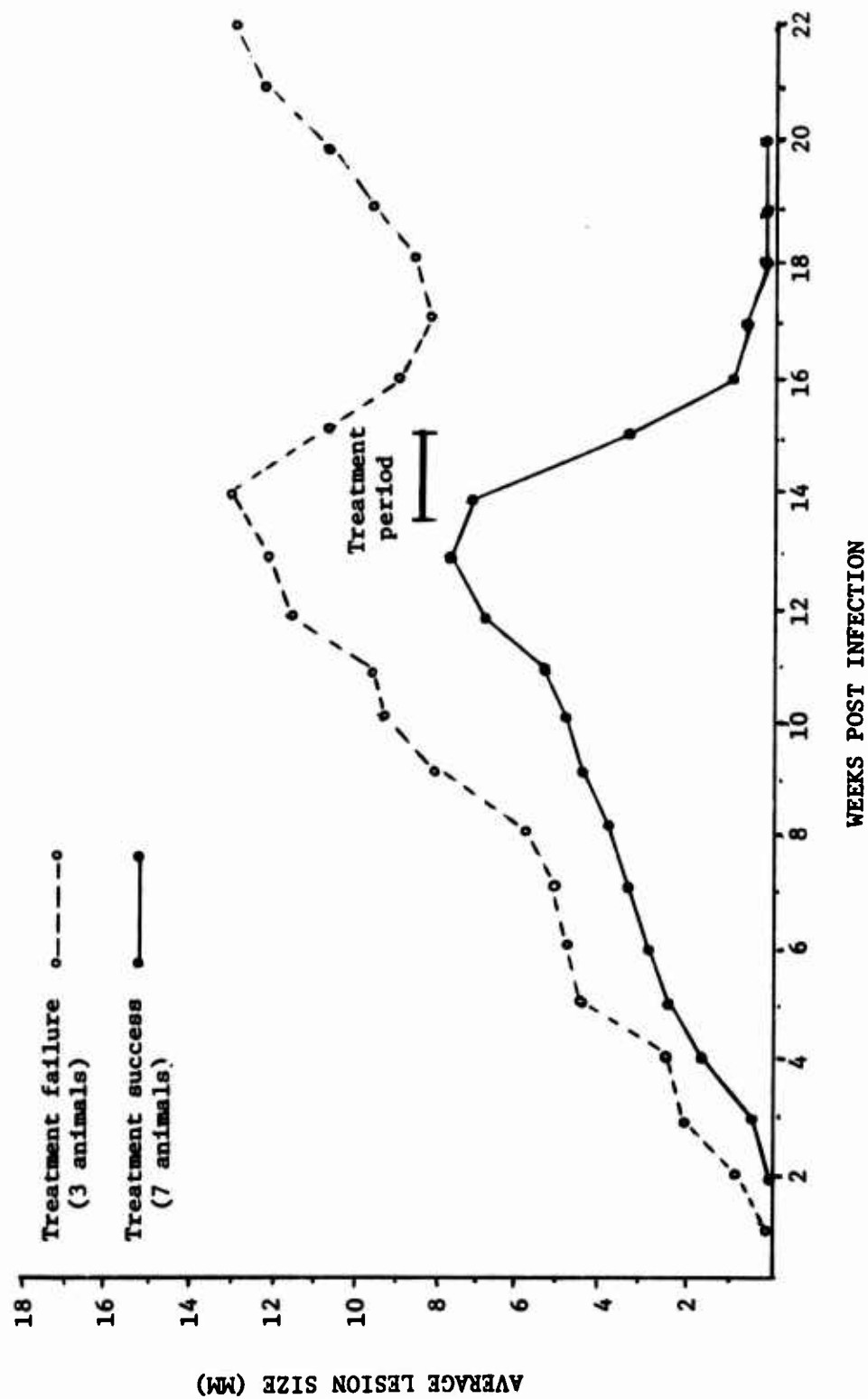


Figure 1-5  
 Effect of Methylglucamine Antimoniate on Size of Cutaneous Lesions of *Myiostomys albicaudatus*  
 When Treated 90 Days After Experimental Infection With *Leishmania braziliensis*



### 3. Biochemical Studies

#### A. Oxygen Utilization Studies: Introduction

Although biochemical investigations were severely limited by the non-arrival of a UV recording spectrophotometer, much progress and valuable information was obtained using the polarographic oxygen monitor (Yellow Springs Instrument Co.) purchased at the end of the last FY. This instrument provided the means of measuring oxygen utilization rates (respiration) of Leishmania in a rapid and reproducible manner.

A number of investigators <sup>16-18</sup> have studied the respiration of various hemoflagellates. Most of these studies were laborious and involved the use of a Warburg apparatus which required long incubation times of up to several hours. Additionally, these investigations primarily utilized the in vitro culture form of the organism, analogous to the form in the invertebrate host, rather than the form found in the vertebrate host. This distinction is important, in that it has been shown repeatedly over the years that there are significant differences between these forms as demonstrated in biochemical as well as morphologic parameters.

With the exception of one study by Adler and Ashbel <sup>20</sup>, all early Leishmania respiration rates were determined using culture (promastigote) forms. In 1967 Janovy <sup>17</sup> and 1968 Simpson <sup>16</sup> measured oxygen utilization patterns of Leishmania donovani amastigotes. The main problems with these studies were the same as those encountered by Adler and Ashbel in 1940; namely that the amastigote preparations were grossly contaminated by host tissue which itself had an oxygen uptake. The availability in this laboratory of large numbers of amastigotes harvested from tissue culture provided an unusual opportunity for study of this form of the parasite.

The advantage of this system is that essentially pure preparations of Leishmania amastigotes were used for all observations. An additional advantage of the oxygen monitor was the rapid measurements (2-5 min.) which allowed short incubation times. However, because this instrument employs a constant temperature bath and stirring apparatus, Leishmania preparations could be maintained under very stable conditions for extended periods of time.

The laboratory standard strain (L. braziliensis panamensis #1077A) isolated from a cutaneous lesion of a U.S. infantryman in the Canal Zone, was used throughout the study. The data obtained represent the first oxygen utilization measurements for the amastigote form of Leishmania braziliensis.

## B. Basic Metabolic Studies: Quantitation of O<sub>2</sub> Uptake Rates

In vitro cultured Vero cells served as host cells for the reproduction and mass production of the intracellular amastigote form of the parasite. This technique was described by Walton et al.<sup>8</sup> Initial experiments showed that there was frequent contamination of amastigote preparations with disrupted Vero cell material. While this contamination would be negligible in some experimental systems, it proved unacceptably high for oxygen utilization studies, since the contaminant cellular material was quantitatively quite variable and was itself responsible for some O<sub>2</sub> consumption.

An acceptable purification technique was derived after testing a variety of column chromatography materials. The DEAE cellulose technique of Lanham and Godfrey<sup>19</sup> was efficient in removing all Vero cell contaminating material (Table 3-1), yet despite numerous attempts using eluting buffers of various ionic strengths and pH, unacceptably large numbers of amastigotes were retained in the column bed.

Gel filtration of Vero-derived amastigote preparations was found to be a highly satisfactory technique. Efficient separations were obtained using Sephadex G-50 Fine (particle size 20-80 $\mu$ ). Concentrated suspensions of freshly harvested amastigotes (1-5 x 10<sup>8</sup> cells/ml) were passed over 3cm (height) gel beds in standard one centimeter glass columns. While the unfiltered harvest contained from 2 - 8% whole Vero cells in addition to varying amounts of cellular debris, washed column filtered material showed that the nuclear contamination rate never exceeded 0.4%. This level did not appear to affect oxygen studies. The recovery rate of amastigotes consistently averaged approximately 90%.

To detect any possible deleterious effects of gel filtration on amastigote suspensions, O<sub>2</sub> consumption of once-filtered cells was compared with that of twice-filtered cells. No significant difference could be detected. Gel filtered amastigotes were utilized for all oxygen studies unless otherwise noted.

Early studies showed that precision of O<sub>2</sub> uptake rates between experiment was relatively poor compared to within-experiment variations. Therefore, all comparative effect studies were carried out on the same day, using the same amastigote preparation, in the same experiment.

In a series of 10 experiments, the oxygen utilization rate of L. braziliensis amastigotes at 34°C in phosphate buffered saline with glucose (PBS) was found to be 1.11 ( $\pm$  .33)  $\mu$  Moles O<sub>2</sub>/10<sup>8</sup> cells/hr. These determinations were made at 60 to 70 minutes after initial release from tissue culture host cells.



These data can be compared to those of earlier investigators; however, such comparisons are limited because of the use of different Leishmania species, life-cycle forms, and/or experimental conditions. For example, data presented by Janovy (1967) for Leishmania donovani amastigotes derived from hamster spleen can be recalculated so that the respiratory rate was approximating  $0.15 \mu \text{ Moles}/10^8 \text{ cells/hr}$  after one hour. Although the temperature is not mentioned, these are presumed to be room temperature incubations.

Promastigotes of L. braziliensis were also studied with respect to basal  $\text{O}_2$  utilization rates. Preparative procedures for these forms were not identical or as extensive as for amastigotes and therefore are not exactly comparable. Nevertheless, it was found that PBS incubated promastigotes had an  $\text{O}_2$  uptake rate of  $3.15 (\pm .63) \mu \text{ Moles } \text{O}_2/10^8 \text{ cells/hr}$ .

Figure 2-1 depicts the decrease in  $\text{O}_2$  utilization rates with increasing time of incubation in PBS at  $34^\circ\text{C}$ . This temperature was chosen for all amastigote oxygen studies since it is the temperature at which amastigotes are grown in the host Vero cells. Time zero ( $T_0$ ) in this figure actually represents the beginning time for oxygen measurements which, in this typical experiment, is 67 minutes after initial release of amastigotes from host Vero cells. If it is assumed that there is a linear fall off in  $\text{O}_2$  utilization rate, at least initially as this figure suggests, the data can be subjected to standard linear regression analysis. Such calculation reveals that the slope of this line is  $- 0.0049$  and the Y intercept ( $T_0$ ) =  $1.4 \mu \text{ Moles}/10^8 \text{ cells/hr}$ . Still assuming a linear relationship, it can be calculated that at the time of host cell release ( $T_0-67$ ) the  $\text{O}_2$  uptake rate would be  $1.73 \mu \text{ Moles}/10^8 \text{ cells/hr}$ .

Certainly the decreasing rate of  $\text{O}_2$  utilization indicated in Figure 3-1 is to be expected. Primarily it can be attributed to the artificial isolated system in which depletion of energy sources and build up of toxic end-products can effect change. It should not be overlooked, however, that the decline, in part, could result from ongoing biochemical processes similar to those described by Janovy 17 and Simpson<sup>16</sup>. Both of these investigators found that there was an initial decline in  $\text{O}_2$  consumption of L. donovani amastigotes followed by increasing rates concomitant with metamorphosis from amastigote to promastigote forms. In the present studies, however, it was found by microscopic examination of the contents of amastigote containing flasks incubated for 3 hours at  $34^\circ\text{C}$  that no apparent morphological transformation was taking place.

Figure 2-2 shows the effect of varying pH on the  $O_2$  uptake rates of L. braziliensis amastigotes. The rate differences between pH 5, 6, and 7 are not considered significant, however, the depressant effect of PBS adjusted to pH 8.0 is apparent.

#### C. Antileishmanial Drugs and Other Chemicals as Inhibitors of Oxygen Utilization

The oxygen electrode has proved a valuable instrument for the detection of drug effects on Leishmania. In a variety of experimental designs, use of the oxygen monitor allows one to detect the effect of certain drugs and antimetabolites on the rate of oxygen utilization. Quantitative and qualitative data are obtained rapidly and accurately. In most cases, the kinetics of drug action are easily visualized.

An excellent example is the experiment depicted in Figure 2-3 which is a drawing of an actual recorder tracing. In this case, a well equilibrated amastigote suspension (2.5 ml) is respiring  $O_2$  at a rapid rate. At approximately To-24, 0.1 ml of a saline solution of WRAIR compound AG 75499 is injected through an injection port. The control rate is indicated by the dashed line. The effect of the drug is apparent almost immediately, and respiration is completely abolished within 20 minutes. The effects of other drugs are varied in this system. Potassium cyanide ( $10^{-5}$  M), for example, a well known, potent inhibitor of  $O_2$  consumption in all cells utilizing the typical mammalian cytochrome system, abolishes respiration even more rapidly than AG 75499. In contrast, an inhibitory effect of the well known antileishmanial drug, Glucantime, cannot be visualized in short term (0-120 min.) experiments of this type. As will be described below, this does not mean that Glucantime is without inhibitory action.

It is apparent from Figure 2-3 that the degree of drug inhibition is directly related to time. Such kinetics are important in that they suggest the mechanism of drug action. For example, the rapid and immediate inhibition by  $CN^-$  or  $Cu^{++}$  ions suggests direct interference with key metabolic sequences such as inhibition of cytochrome oxidase ( $CN^-$ ) or membrane transport ( $Cu^{++}$ ). Longer term inhibition effects may be by less direct means, such as interference with DNA synthesis. These hypotheses can be explored by other methods.

The action of Glucantime on the oxygen utilization of L. braziliensis was studied in some detail. This pentavalent antimonial of known clinical value is used as the reference drug in all in vitro and animal model studies in this laboratory. In these experiments,

six 16 oz. bottles, each containing a monolayer of Vero cells, were inoculated with L. braziliensis. At day 2, 3, 4, or 5 PI, depending upon the experiment, the culture media was changed in all bottles. Glucantime was added to three bottles while the other three served as controls. On day 6 PI, parasites were harvested as follows: the liquid culture media was poured out and the infected Vero cell monolayer was rinsed twice by gently swirling approximately 2 ml of veronal buffered saline (VBS) over the monolayer to remove any extracellular parasites. Immediately after the final rinse, 10 mls of VBS were added to each bottle. The culture bottles were placed in an incubator for 15 min. at 37°C. Upon removal, each bottle was vigorously shaken for 15 secs. The combination of VBS and mechanical shaking effects the liberation of amastigotes from infected cells while the majority of uninfected Vero cells remains attached to the bottle wall. The parasite suspensions were pooled for each group, centrifuged, rinsed 1 time in PBS, column filtered as described previously, and, finally, resuspended in PBS. The rates of O<sub>2</sub> uptake between drug treated and control amastigotes were compared directly on the oxygen monitor.

Figure 2-4 shows the effect on the respiration of L. braziliensis amastigotes by two concentrations of Glucantime for varying treatment periods. The percent inhibition was determined by comparing the O<sub>2</sub> utilization rates of drug-treated with control samples. It is immediately obvious that the inhibition effects are dose and time related. After a treatment period of 48 hours, Glucantime at a concentration of 1,000 µg/ml effects a 68% inhibition, while little or no inhibition could be detected at 100 µg/ml. However, after 72 hours the effects of 100 µg/ml Glucantime become apparent in this system. These data clearly demonstrate the antileishmanial action of Glucantime, but the mode of action of this drug remains obscure.

The effect of three well known anti-Protozoan drugs and Glucantime is shown in Table 3-2. Although Tryparsamide was not effective in 48 hours, it is possible that longer exposures would result in inhibition.

To determine if exposure of infected Vero cells to the drug effects a reduction of the number of parasites produced, pooled amastigotes from Glucantime treated and control harvests were counted. The data is presented in Table 3-3. The results clearly show that the number of parasites produced is reduced by increasing time of exposure. The concentration effect (100 µg/ml versus 1,000 µg/ml Glucantime) is less clear, however. This relationship appears to merit further investigation. Nevertheless, these observations suggest that Glucantime interferes with parasite reproduction.

Table 3-1

Column Chromatography Separation of Amastigotes  
from Contaminating Host Cell Material

Column Material	Buffer	Host Cell Contaminants	Percent Recovery of Amastigotes
DEAE cellulose	PBS*	None	< 35%
DEAE cellulose	TBS*	None	< 25%
Sephadex G-25 part. size: 100-300 $\mu$	PBS	< 1% Vero nuclei	> 80%
Sephadex G-100 part. size: 100-300 $\mu$	PBS	< 1% Vero nuclei	> 80%
Sephadex G-50 part. size: 20-80 $\mu$	PBS	0.24 Vero nuclei/ 100 amastigotes	89%

\*PBS: NaCl            8.0 g/l  
            $\text{KH}_2\text{PO}_4$         2.72 g/l  
           Glucose        1.8 g/l            final pH adjusted to 7.3

\*TBS: NaCl            8.0 g/l  
           Tris Hcl        3.43 g/l  
           Tris base       0.4 g/l  
           Glucose        1.8 g/l            final pH = 7.3

Table 3-2

Inhibitory Effect of Some Anti-Protozoan Drugs  
on O<sub>2</sub> Utilization Rates by Amastigotes of L. braziliensis

Drug	Concentration	Length of Treatment	Percent Inhibition
Sulfadiazine	100 µg/ml	72 hrs	11%
Tryparsamide	100 µg/ml	48 hrs	0
Lampit	100 µg/ml	72 hrs	98%
Glucantime	100 µg/ml	72 hrs	20%

Table 3-3

Effect of Glucantime on Amastigotes Harvested  
from Vero cells

[Glucantime]	Length of Treatment	Amastigotes Harvested as % of Controls
1000 µg/ml	24 hrs	57%
1000 µg/ml	48 hrs	56%
100 µg/ml	72 hrs	81%
100 µg/ml	96 hrs	60%

Table 3-4

Comparative Effects of Glucantime (1000  $\mu\text{g/ml}$  for 72 hrs)  
Treatment on  $\text{O}_2$  Uptake by Amastigotes  
and Promastigotes of L. braziliensis

Cell Type	$\text{O}_2$ Utilization Rate	% Inhibition
Amastigotes	Treated - $0.2\mu$ Moles/ $10^8$ cells/hr Control - $1.4\mu$ Moles/ $10^8$ cells/hr	85%
Promastigotes	Treated - $3.2\mu$ Moles/ $10^8$ cells/hr Control - $3.0\mu$ Moles/ $10^8$ cells/hr	No inhibition

Figure 2-1  
Decrease in  $O_2$  Uptake of L. braziliensis Amastigotes

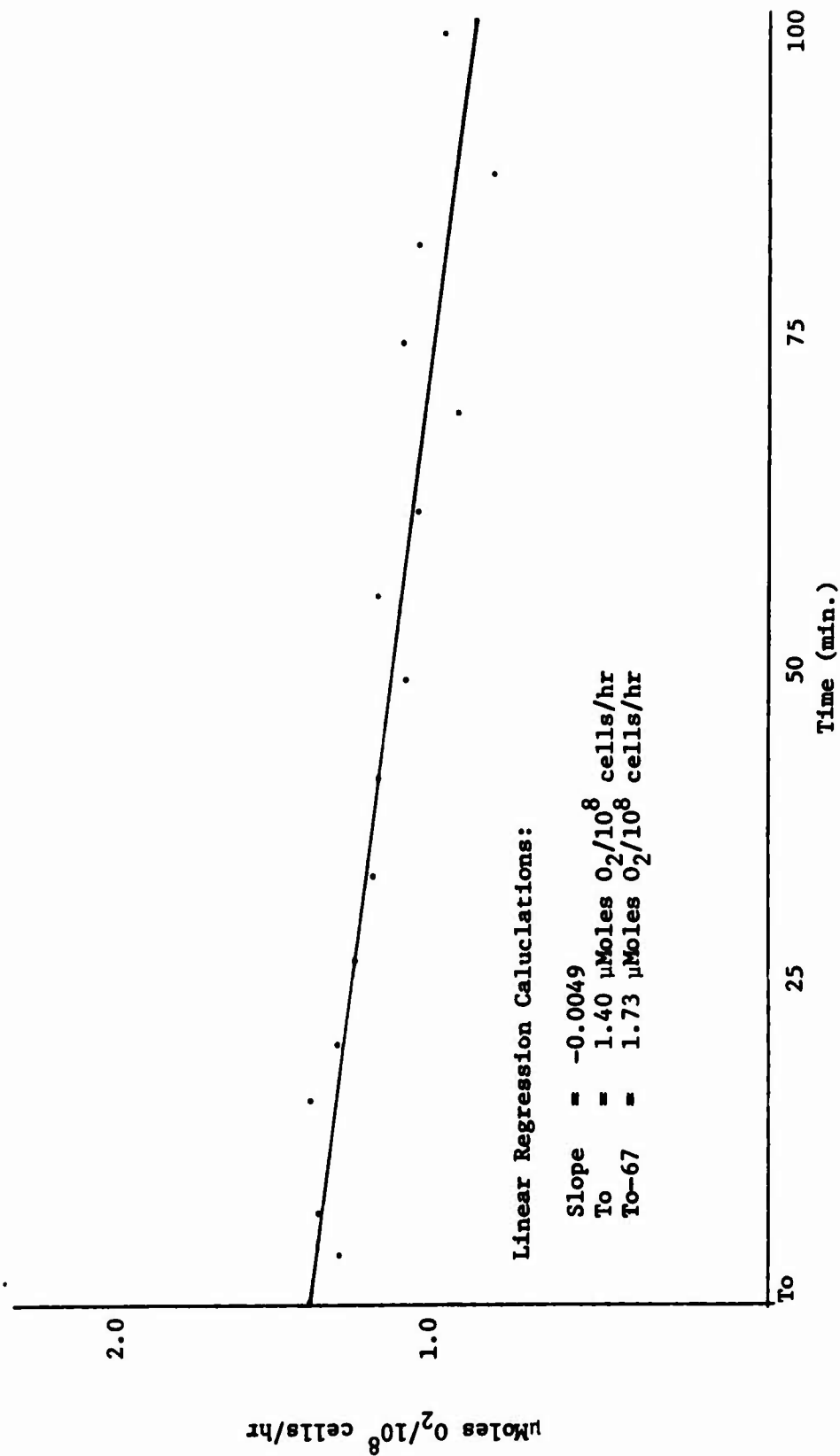


Figure 2-2  
Effect of pH on  $O_2$  Consumption of L. braziliensis Amastigotes

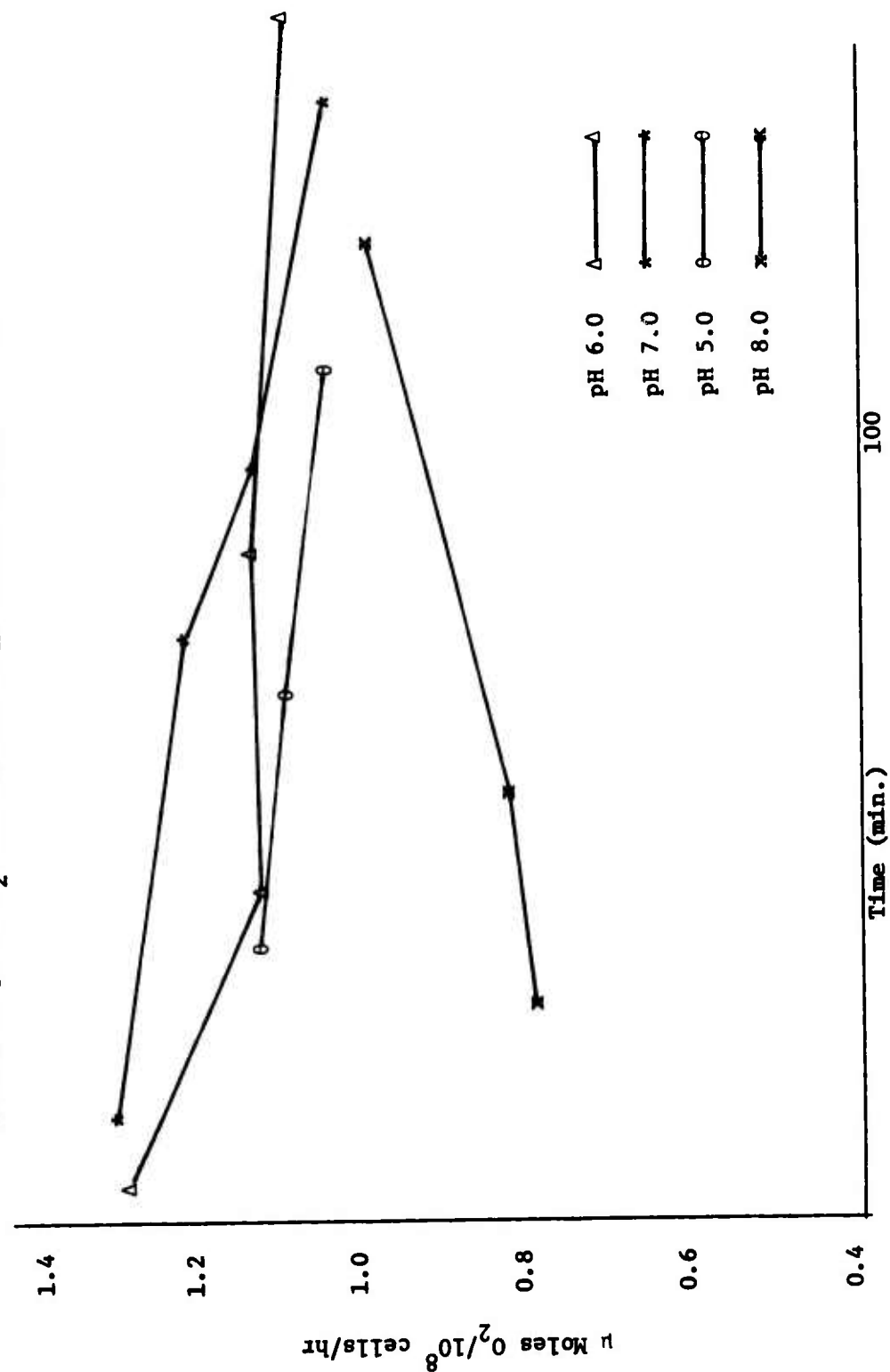




Figure 2-3  
Effect of Added inhibitory Drug on the  $O_2$  Utilization of L. braziliensis Amastigotes

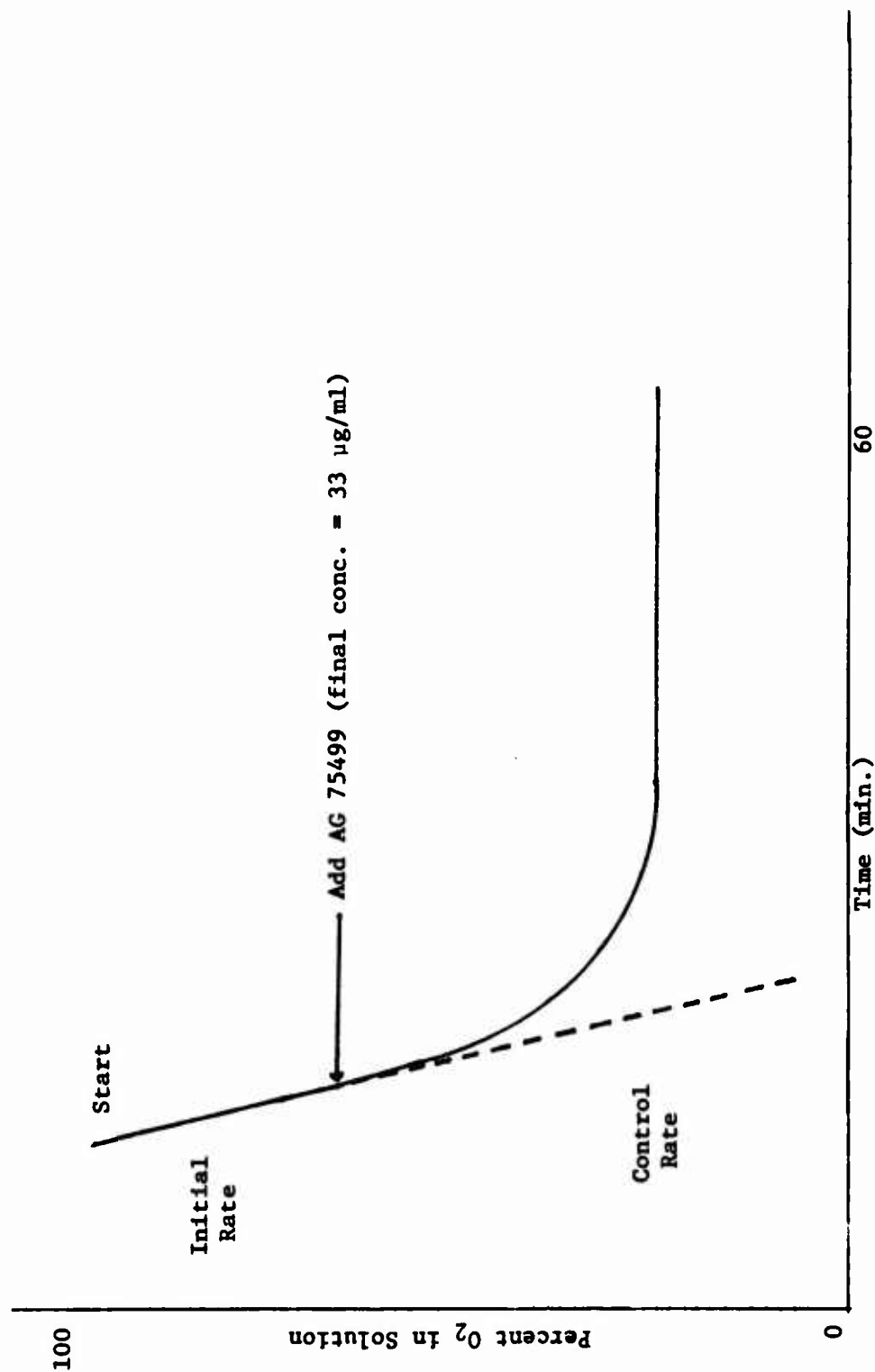
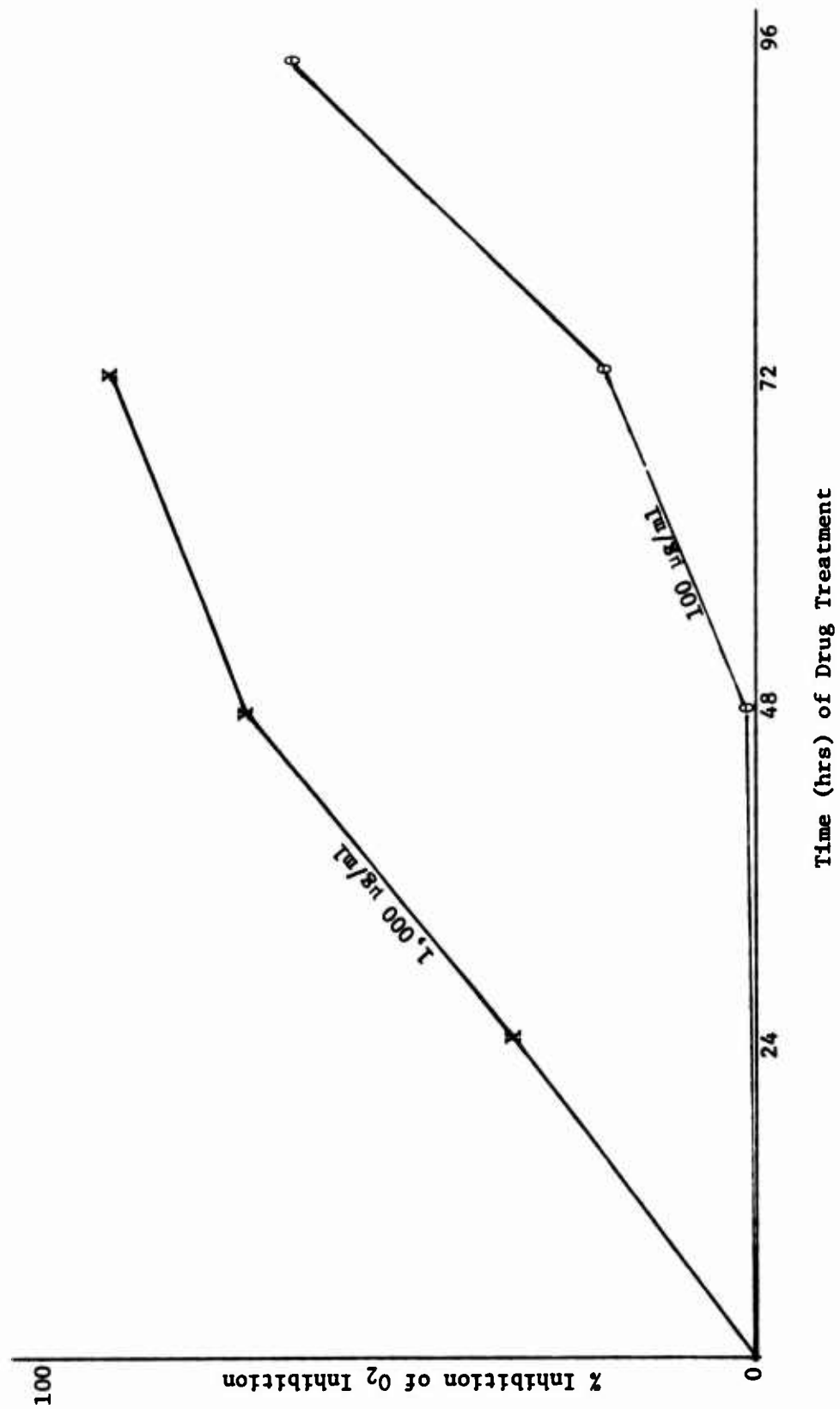


Figure 2-4  
Glucantime Inhibition of Oxygen Utilization by *L. braziliensis* Amastigotes



The comparative effects of Glucantime on the oxygen utilization of L. braziliensis promastigotes and amastigotes is shown in Table 3-4. The difference between the two life-cycle stages is rather dramatic. Amastigote  $O_2$  utilization was inhibited by 85% after 72 hours of exposure to Glucantime (1,000  $\mu\text{g/ml}$ ) while a similar concentration and exposure period effected no detectable inhibition of the promastigotes.

These preliminary polarographic oxygen monitor studies indicate that there are significant differences between amastigote and promastigote forms of Leishmania braziliensis with regard to the effect of Glucantime on  $O_2$  utilization. Further, they also demonstrated that utilization of the oxygen monitor allows the study of basic metabolic patterns and provides a method by which the effect of antileishmanial drugs can be demonstrated. It is apparent that other techniques and instrumentation are required to evaluate fully the implications of these data, but the potential for such studies and the practical application of the polarographic oxygen monitor have been clearly demonstrated.

#### 4. Vector Studies

##### A. Development of Leishmania spp. in Panamanian Sandflies

The taxonomy of the genus Leishmania has undergone less development, and is in a greater state of confusion than is found in any other group of parasites which are a major cause of human disease. One of the parameters which has been suggested for classification is the behavior and location in the gut of the sandfly vector. Although the role of phlebotomines as vectors of leishmaniasis is well accepted, the relationship between various species of sandflies and their Leishmania infections is little known. In the current studies, 3 Leishmania isolates were used: Leishmania braziliensis (strain 1128) isolated from a mucous lesion of an infection acquired in eastern Peru, L. chagasi (strains 1166 and 1167) isolated from bone marrow aspirates of kala azar patients in Honduras, and L. mexicana (strain 1156) isolated from an autochthonous case from Texas. Experimentally infected hamsters were used as a source of infection for the sandflies in all cases. Three anthropophilic species; Lutzomyia gomezi, Lu. trapidoi, and Lu. panamensis were used. Engorged laboratory reared sandflies which had fed on an infected hamster were held in plaster-lined vials for 3-4 days and maintained on boiled raisins. Dissection procedures were essentially those of Hertig and McConnell<sup>21</sup>, except that  $\text{CO}_2$  rather than cold was the anesthetic, and the initial cut was made directly behind the thorax rather than severing the head. This variation left the esophagus esophageal valve and diverticulum intact, which facilitates observation of the flagellates. The susceptibility of each species of sandfly to the species of Leishmania is shown in

Table 4-1. In spite of failure to infect Lu. panamensis, L. braziliensis had an overall average of 54.8% infectivity. Lu. panamensis exhibited the lowest susceptibility to infection with all 3 species of parasite. The very much higher infection rates with L. mexicana are probably related to the very much higher concentration of amastigotes in the hamster. Sandfly infections have been characterized as occurring in hindgut, midgut, or foregut. The foregut includes the esophagus, esophageal diverticulum, pharynx, buccal cavity and stylets. Midgut infections are contained in the stomach and cardia. Hindgut infections include the hind triangle, rectal ampulla, and malpighian tubules. Table 4-2 indicates the location of flagellates of each of the 3 species. Promastigotes of L. braziliensis were found primarily in the hindgut, 100% of all infected flies having flagellates in this location. However, an average of 53% of infected flies also had flagellates in the midgut with this infection. With L. chagasi, midgut infections predominated, although 53% of infected flies also had infections in the foregut. L. mexicana presented a picture of primarily midgut infections, but with large numbers also found in both foregut and hindgut locations, also. Some of this "spilling" is probably due to the heavier infections resulting from the much heavier inoculum in the infective meal with this parasite.

Characteristically, sandflies harboring infections from L. braziliensis contained rounded or ovoid shaped flagellates in medium to heavy numbers in the hind triangle. Whereas, with L. chagasi, long spindle-like flagellates could be found with smaller oval promastigotes in rosettes located in the cardia and esophagus, but infections were medium to heavy in the cardia. Leishmania mexicana presented the most consistent data. Large numbers of slender and very active flagellates were observed in the stomach, cardia, and esophagus of the fly. Promastigotes of L. mexicana were observed in all three body areas in twenty-six percent of the sandflies as compared to only 6.6 percent with L. chagasi. In addition, combined hindgut/midgut infections were three times more common in L. mexicana than with L. chagasi.

The variety of growth patterns observed in sandflies does not provide any real answer as to how it influences transmission of the parasite by the invertebrate host. It does, possibly, contribute further information required to differentiate strains of Leishmania that may appear equal by other means of comparison.

#### B. Liquid Nitrogen Cryopreservation of Leishmania in Sandflies

Cryopreservation has been applied to numerous species of protozoa in the laboratory. The methodology involves crystallizing cell fluids

extracellularly, thereby reducing or eliminating internal structural damage to the cell. This technique to insure extracellular crystallization which depends on lowering temperatures slowly<sup>22</sup> was initially applied to the preservation of protozoa by Coggeshall<sup>23</sup>, who successfully preserved the erythrocytic stages of Plasmodium knowlesi and P. inui, at  $-76^{\circ}\text{C}$ . Furthermore, Ward<sup>24</sup> and Shute and Maryon<sup>25</sup> preserved whole insects and upon dissecting mosquito salivary glands recovered viable sporozoites. Fluch<sup>26</sup> recovered Trypanosoma cruzi from experimentally infected triatomines after freezing to  $-79^{\circ}\text{C}$ . These parasites also retained their infectivity to mice. Diamond<sup>27</sup> provides a comprehensive list of 44 species comprising 9 families of protozoa that have been preserved by various methods of freezing. Liquid nitrogen, as a coolant, was used by Weathersby and McCall<sup>28</sup> to preserve P. gallinaceum sporozoites in Aedes aegypti. Minter and Goldbloed<sup>29</sup> recently reported studies designed to determine the effectiveness of liquid nitrogen as a field expedient for preserving naturally infected haematophagous vectors of trypanosomid flagellates. The present study was designed to evaluate liquid nitrogen as a coolant for preserving known leishmanial parasites in sandflies infected in the laboratory.

Two species of sandflies, Lutzomyia gomezi and L. sanguinarius, both anthrophilic and zoophilic, were collected and transported to the laboratory and given the opportunity to feed on a hamster infected with Leishmania mexicana. Previous studies with these two species of sandflies have shown that both maintain midgut infections with L. mexicana. Engorged sandflies were held for 72 hours, a control group of flies was dissected, and the rest preserved in liquid nitrogen. Sandflies to be frozen were lightly anaesthetized with  $\text{CO}_2$  and placed in 2.5 ml plastic ampules with screwcap tops. Approximately 2 ml of 10% dimethyl sulphoxide (DMSO) in phosphate-buffered saline (PBS) was added to the vial as a protectant. It was then shaken lightly to "wet" each specimen. One or two ampules, containing 8-10 sandflies each, were placed inside a styrofoam cylinder 5 1/2 inches long and 1 1/4 inches in diameter. A 10 liter liquid nitrogen container was filled with coolant until a steady state vapor phase to the top of the container was maintained. Filling to a gross weight of 32 lbs was found to be a rapid and convenient method to determine this level. Ampules were placed within the styrofoam cylinder, sealed with adhesive paper tape, and the cylinder suspended in the vapor phase of the coolant. Preliminary experiments with a thermistor probe inside the cylinder in place of the ampules indicated that the cooling rate was  $2^{\circ}\text{C}$  per minute and the time to reach  $-40^{\circ}\text{C}$  was determined. At the proper time, the ampules were removed from the cylinder, placed in nylon stockings and lowered into the liquid phase for storage. Ampules removed from coolant were thawed for 2-3 minutes in lukewarm water. Sandflies were then dissected and examined for motile promastigotes.

Table 4-1

Susceptibility of Panamanian Sandflies to Infection  
After Bloodmeals from Hamsters Infected with 3 Species of Leishmania

Sandfly Species	Leishmanial species								
	L. braziliensis			L. donovani			L. mexicana		
	Number		%	Number		%	Number		%
	Diss.	Pos.	Pos.	Diss.	Pos.	Pos.	Diss.	Pos.	Pos.
<u>L. trapidoi</u>	21	10	47.6	30	8	26.6	45	38	84.4
<u>L. gomezi</u>	10	7	70.0	12	3	25.0	11	9	81.8
<u>L. panamensis</u>	-	-	-	18	4	22.8	5	2	40.0
Total	31	17	54.8	60	15	25.0	61	49	80.3

Table 4-2

Location of Leishmania spp. in Panamanian Sandflies

	Sandfly Species	Pos. Flies	Foregut		Midgut		Hindgut		All Areas	
			No.	%	No.	%	No.	%	No.	%
L. braziliensis	<u>L. trapidoi</u>	10	0	0	7	70.0	10	100	0	0
	<u>L. gomezi</u>	7	1	14.2	2	28.5	7	100	1	14.2
	Total	17	1	5.8	9	52.9	17	100	1	5.8
L. chagasi	<u>L. trapidoi</u>	8	4	50.0	8	100.0	1	12.5	0	0
	<u>L. gomezi</u>	3	3	100.0	2	66.6	1	33.3	1	33.3
	<u>L. panamensis</u>	4	1	25.0	4	100.0	0	0	0	0
	Total	15	8	53.3	14	93.3	2	13.3	1	6.6
L. mexicana	<u>L. trapidoi</u>	38	19	50.0	36	94.7	24	63.1	10	26.3
	<u>L. gomezi</u>	9	6	66.6	9	100.0	2	22.2	2	22.2
	<u>L. panamensis</u>	2	1	50.0	2	100.0	1	50.0	1	50.0
	Total	49	26	53.0	47	95.9	27	55.1	13	26.5

To verify the infectivity as well as motility of the parasites after preservation, 4 hamsters were inoculated subcutaneously at the base of the tail with promastigotes from individual flies. Infection was confirmed by culture of needle aspirates in blood agar medium.

High infection rates from the infected blood meals were found in the control groups. Seventy-two hours following engorgement, 97% of Lu. gomezi and 100% of Lu. sanguinarius harbored promastigotes (Table 4-3). The combined figure was 98%. Infections were normally moderate to heavy, and located primarily in the stomach and cardia.

Table 4-3

Effect of Liquid Nitrogen Freezing on  
Detection of Leishmania mexicana in Sandflies

Sandfly Species	Control Group			Frozen Group		
	Number Flies		%	Number Flies		%
	Diss.	Pos.	Pos.	Diss.	Pos.	Pos.
<u>L. gomezi</u>	108	105	97	133	67	50.4
<u>L. sang.</u>	<u>13</u>	<u>13</u>	<u>100</u>	<u>42</u>	<u>21</u>	<u>50.0</u>
	121	118	98	175	88	50.2

In comparison, 50% of the sandflies from liquid nitrogen harbored live promastigotes. The infection rates in the 2 species were essentially equal, therefore the data for both vectors can be combined. The period of preservation was for 5-11 days. The length of time sandflies were preserved should not influence viability or infectivity of the promastigotes, since damage and mortality occur during the critical temperature drop. However, each batch was evaluated separately to determine if differences occur between freeze-downs. These results are presented in Table 4-4. There was little variation among the batches in regard to percentage of flies without flagellates (16.4 - 28.5%) but there was considerable variation in regard to presence of dead or non-motile parasites (0 - 50%), with an average of 30%. In the test for infectivity, 2 of 4 hamsters inoculated with preserved organisms from individual flies have been shown to be infected as of this writing (Table 4-5). Hamster A2286 had a noticeable

Table 4-4

Effect of Liquid Nitrogen Freezing on the  
Viability of Leishmania mexicana in Sandflies

Batch	No. Days Frozen	No. Flies		Percentage of Flies		
		Diss.	Pos.	Pos.	Neg.*	Dead**
1	5	67	45	67.2	16.4	16.4
2	6	8	2	25.0	25.0	50.0
3	7	34	13	38.2	26.5	35.3
4	10	59	23	39.0	16.9	44.1
5	11	7	5	71.5	28.5	0.0
		<u>175</u>	<u>88</u>	<u>50.2</u>	<u>19.4</u>	<u>30.4</u>

\* No parasites

\*\* Parasites dead or moribund

Table 4-5

Effect of Liquid Nitrogen Freezing on  
Leishmania mexicana in Sandflies on Infectivity to Hamsters

Hamster No.	Date Inoc.	Site	Results
2284	2/2/76	nose	
2285	2/2/76	tail	Pos. culture 14/5/76
2286	3/2/76	tail	Pos. culture 15/4/76



lesion approximately 1 month post-inoculation, and positive cultures were obtained one week after making a needle aspirate. Positive cultures were obtained from hamster A2285, 102 days after inoculation.

The distinctly adverse effect of cryopreservation demonstrated, (50% infectivity vs 98% of non-frozen controls) indicates that this technique could result in missing many light infections when searching for natural infections in field collections. Only rarely did all parasites in the frozen flies survive the process. Frequently, morphologically intact organisms were observed which were non-motile, and occasionally obvious cell wall damage had occurred and the flagellates were hardly recognizable. In a comparative study, Minter and Goldbloed<sup>29</sup> froze naturally infected sandflies in DMSO and PBS to  $-40^{\circ}\text{C}$  and stored them at  $-196^{\circ}\text{C}$  for 5-15 weeks and found 2.3-10.3% infected with flagellates as compared with 12.1-13.3% in an unfrozen control group. Further experimentation with other protectant agents and cooling regimens might significantly improve the efficacy of this method for field studies.

## II. Other Parasitic Infections

### 1. Coccidiosis

An unsporulated oocyst resembling that of Isospora felis was found in a fecal sample of a sick ocelot, Felis pardalis, housed at the Air Force Survival School zoo, Albrook Air Force Station, Canal Zone. Upon sporulation, approximately 100,000 of these oocysts were given to each of two ocelots and to one domestic cat via gavage. Daily fecal examinations on these animals through 18 days post inoculation failed to disclose a patent infection. At this point, four MARU mice were fed approximately 26,000 oocysts each. Fifteen days post inoculation, two of these mice were fed to a house cat. After a prepatent period of 5 days, this animal began passing oocysts on the 6th day post feeding, and continued for 4 additional days. These oocysts provided enough material for additional animal experimentation. The number and types of animals subjected to exposure to this parasite are presented in Table 5-1.

The eleven felines, 5 cats, 5 ocelots, and the jaguarundi that were fed previously infected mice have all developed patent infections. The prepatent period for the cat ranged from 5 - 8 days (6.5 mean) with the patent period ranging from 5 - 7 days (6.5) in length. The prepatent period in the 5 ocelots was 6 - 8 days (7) with a patent period ranging from 6 to 13 days (8.3). The three felines, one cat and two ocelots, which were fed approximately 100,000 sporulated oocysts each,

failed to develop patent infections. These 3 animals, however, all passed oocysts within the 6 - 9 day patency range after feeding on mice that had received oocysts 15 or more days previously. It is interesting that a prior exposure to these animals with oocysts not only does not result in a patent infection, but also does not appear to make them resistant to a later challenge to "bradyzoites" from rodents. This feature also demonstrates that this parasite does in fact require an intermediate host to complete its life cycle. Tissue stages have been observed in experimentally infected mice, but a more comprehensive study of this and further material will be necessary to determine what life-cycle stages they represent. Gametocytes have been found in the intestinal epithelium of 2 laboratory reared kittens, but no schizonts or merozoites were seen in either. However, both were sacrificed at seven days P.I. and were oocyst positive at that time.

This organism is very similar in appearance to a parasite described as a murine sarcocystis<sup>30</sup> while this study was in progress. It is of interest that the forms they originally encountered were the tissue stages in a mouse, hence their title identified it as a murine Sarcocystis, while the organism described here was discovered as an isosporan-like oocyst in the feces of an ocelot. This was sufficiently distinct from any oocyst described from felines that it could have been validly described as a new species of Isospora from the ocelot. This emphasizes the inadequacy of the traditional reliance on morphology and measurements of one life-cycle stage as taxonomic criteria in this group of parasites.

## 2. Amphimerus Life Cycle Studies

In 1970 an operculated trematode egg was found in the stool of a Cuna Indian food handler, and in 1972 he was again examined and found to still be passing eggs. The parasite was identified as Amphimerus guayaquilensis, and constituted the first report of a human liver fluke from Panama, and only the third from the Western Hemisphere (Annual Report, 1973). The parasite had previously been reported from Panama in the cat and common opossum.

A literature review revealed that the taxonomy of this group is uncertain, and the validity of the generic name Amphimerus has been disputed, early authors considered this parasite as belonging to Clonorchis and another synonymized it with Opisthorchis. The life cycle of the genus is little known. The metacercaria of an avian species was found in the muscle of a minnow Notropis deliciosus stramineus in Minnesota, and it is known that the second intermediate host of A. pseudofelineus is a sucker, Catostomus commersoni. Consultation established that neither of these genera occur in Panama, but that there were several fish which fill the same ecologic niche.

Seining of small streams near Arraijan, where naturally infected cats were known, produced 3 major types of fish, as well as decapods. Accordingly, 4 laboratory reared cats were obtained, and each fed the fresh catch of one of these groups. Cat #1 received fish from the characid superfamily, which included "sabalo" and "sardinas". Cat #2 was fed the cichlids, which include the perch, sunfish and "peacock bass". Cat #3 received the silurid catfish, and #4, shrimps, prawns, and crayfish.

Cat #1 which was fed on 8 and 10 January, 13 February and 3 March, 1975 first produced eggs of Amphimerus on 4 June 75, and was found to harbor several hundred A. guayaquilensis in the gall bladder and throughout the larger bile ducts. None of the cats fed the other 3 groups became positive. Unfortunately, the infected meals for cat #1 included several genera of fishes, which came from more than one locality.

In an attempt to define the naturally infected intermediate hosts, a second series of experiments was conducted. The fish from group #1 which are large enough to serve as food for humans received special attention, and a site was selected on the Rio Cocoli, which is in the same water shed as the town of Arraijan. However, these plans had to be abandoned due to an oil spill which killed all fish, and the Rio Cardenas, a stream on the east bank of the canal was selected as the collecting site. Upon termination of these experiments, 9 of 11 laboratory reared cats were found to be harboring adult A. guayaquilensis. However, errors in feeding schedules were discovered for 3 cats, and the data was rejected as being compromised. The following fish were found to be naturally infected intermediats hosts.

Astyanax ruberrimus, known locally as the "sardina", or spot-tailed minnow. Piabucina panamensis, a small trout-like fish living in swift, rocky streams, and Brycon petrosus, the "sabalo", the largest of the 3 fish. One cat, which was positive 45 days after feeding had received only one fish, a P. panamensis. This fish had been continuously exposed for 3 months to cercaria from snails sharing the same small aquarium.

A large semi-aquatic snail Pomacea paludosa Say, 1929, was the only snail found in the streams where fish were collected. Three of eleven snails from the Rio Cocoli were found to be shedding oculate lophocercous cercaria of the type characteristic of the family Opisthorchidae. These cercaria repeatedly disappeared from a small container when exposed to both P. panamensis and A. ruberrimus and presumably penetrated these fish. These data indicate that these species constitute the intermediate hosts in the life cycle of Amphimerus guayaquilensis in Panama. However, final review of all material has not yet been completed.

Table 5-1  
Experimental Infection of Felines and Raccoons with  
Oocysts of Intestinal Coccidian of the Ocelot

Host	Inoculum	No. Pos./ No. Inoc.	Days	
			Prepatent Period range (mean)	Patent Period range (mean)
<u>Felis catus</u> House cat	100,000 oocysts	0/1	-	-
	Mice previously inoculated	5/5*	5-8 (6.5)	5-7 (6.5)
<u>F. pardalis</u> Ocelot	100,000 oocysts	0/2	-	-
	Mice previously inoculated	5/5*	6-8 (7)	6-13 (8.3)
<u>F. yagouaroundi</u> Jaguarundi	Mice previously inoculated	1/1	6	7
<u>Procyon lotor</u> Raccoon	Mice previously inoculated	0/4	-	-

\*Number includes 1 animal previously administered oocysts with negative results.

Project        3A161102B71Q : COMMUNICABLE DISEASES AND IMMUNOLOGY  
Task                00 : Communicable Diseases and Immunology  
Work Unit            169 : Field Studies of Leishmaniasis and Other  
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24. (U) Conventional microbiological and chemical techniques are used. New procedures are developed as needed.							
25. (U) 75 07 - 76 06 This is a final report on research conducted on the intermediary metabolism of the Mycoplasmatales and Pseudomonads. The work unit "Zoonotic Diseases of Military Importance" was terminated as being not unique to the Division of Veterinary Resources, and redundant to the overall WRAIR mission. Glyoxalate was found to prolong the longevity of U. ureaplasma, particularly between pH 6.4-7.2. Glyoxylate inhibited the production of urease in cultures of U. urealyticum, but did not inhibit the enzymatic activity of urease once it was formed. Malate synthase and isocitrate lyase activity, the latter probably directly inhibited by glyoxylate, were present in all mycoplasma tested. Nitrate and nitrite reductases were shown for rough and smooth strains of P. pseudomallei. Smooth strain 165 does not show nitrite reduction in ordinary laboratory testing. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

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

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Zoonotic Diseases of Military Importance

Investigators:

Principal: LTC T. J. Keefe, VC

Associate: M. Rogul, Ph.D., J. J. Brendle, B.S., C. Osmola,  
SP4

Description:

During March, 1976, the Division of Veterinary Medicine was reorganized and its work unit "Zoonotic Diseases of Military Importance" was discontinued as being redundant to the overall mission of the WRAIR. Within this work unit, research was being conducted on the intermediary metabolism of the Mycoplasmatales and Pseudomonads. This research is outside the scope of Divisional mission statements and objectives as currently defined, and consequently has been discontinued. This is a final report on the intermediary metabolism of the Mycoplasmatales and Pseudomonads under the work unit Zoonotic Diseases of Military Importance. Microbial metabolism and physiology will be investigated as necessary to fulfill research and diagnostic requirements under the two new work units: (1) Diseases of the Military Dog, and (2) Health Care and Management of Laboratory Animals.

1. Nutritional requirements of the Mycoplasmatales.

Ureaplasma urealyticum is an obligate parasite of the urogenital tract of a number of mammals, including man. The organism is suspected of causing a variety of diseases including infertility, non-specific urethritis, and bladder stone production. The commensal and pathogenic properties of this organism appear in part to be due to their urease activity and other physiological properties. A specific type of parasitism may depend on molecular competition between the host's Krebs cycle and the microorganism's glyoxylate cycle. The presence of a glyoxylate by-pass was suspected and found in U. urealyticum and a number of Mycoplasma sp. and Acholeplasma sp. Nutritional and growth response patterns of U. urealyticum 960 indicated that glyoxalate prolonged the longevity of this organism but did not allow an increase in growth as indicated by color-changing-unit (CCU) assays. This affect was in marked contrast to the response of Acholeplasma laidlawii, Mycoplasma pneumoniae, M. fermentans, M. arthritidis, and M. hominis. These latter strains were immediately inhibited by 1% glyoxalate (WRAIR Annual Report

1975). The growth response was studied in greater detail in U. urealyticum.

The affect of pH on growth patterns and glyoxylate toxicity was investigated. Our studies indicated that U. urealyticum growth was suppressed and longevity prolonged the most on 1% glyoxalate-urea media adjusted between pH 6.4 and 7.2 (Figure 1). We also found that the stationary phase of urea broth cultures was prolonged by either 1% L-histidine or 1% imidazole additions to these broths and the rise in culture pH was retarded. The implication was that these substances buffered the medium and decreased toxicity due to the pH rise.

The affect of glyoxylate on prolonging the growth of U. urealyticum was examined by exposing cultures to glyoxylate at predetermined times during the growth cycle. Five flasks containing 100 ml of urea broth pH6 were inoculated with U. urealyticum 960. Glyoxylate was added to one flask to a final concentration of 1% immediate at the time of inoculation. Thereafter, the same amount of glyoxylate was added to a different flask at 10, 14, and 21 hours (Figure 2). It can be seen that up to 10 hours longevity was prolonged by glyoxylate. At 14 hours and after, the cultures were apparently committed to the decline phase. Glyoxylate did not increase their longevity. When Figure 2 is compared to Figure 1, it can be seen that pH adjusted urea broths containing 1% glyoxylate maintained their original pH throughout their culture incubation. This observation suggests that glyoxylate might have inhibited the production of urease or any urease enzymatic activity in these static growth cultures. However, in Figure 2, it can be deduced that it was the production of urease that was inhibited by glyoxylate because the zero time introduction of glyoxylate kept the pH at 6 and slightly below. By contrast, it is apparent from the pH of the culture to which glyoxylate was introduced ten hours after inoculation, that glyoxylate did not greatly reduce the steady progression of pH rise in the culture. Consequently, it was obvious that glyoxylate did not decrease urease activity, only its production.

An attempt was made to identify enzymes of the glyoxylate by-pass. Since glyoxylate is not an end product of urease activity, it must be assumed that any end product inhibition would occur elsewhere. The very likely areas for the study would be the enzyme allantoinase in purine degradation or isocitrate lyase of the glyoxylate by-pass. For technical reasons, the isocitrate lyase enzyme was easier to investigate. During these studies, strains of A. laidlawii A, M. hominis PG 21, M. arthritidis PG6, and M. pneumoniae Mac were all grown in Edwards-Hayflick media containing 0.5% acetate. U.

urealyticum 960 was grown in urea broth containing 0.5% acetate. All cultures were harvested during their late logarithmic or early stationary growth phase. The cultures were centrifuged at 8,000 rpm in a GSA rotor 1 hour at 30 C. The cells were concentrated from 300 to 500 fold in deionized water. Each concentrate was then frozen and thawed at least twice and centrifuged at 5,000 rpm in a SS-34 rotor at 5 C for 30 min. The resulting supernatant fluids and sediment were separated and stored in the vapor phase of liquid nitrogen refrigerators. Enzyme assays for isocitrate lyase and isocitrate dehydrogenase were first conducted spectrophotometrically as described by Muller et al<sup>1</sup> and Charles<sup>2</sup>. No isocitrate lyase or isocitrate dehydrogenase NAD or NADP enzymatic activity was observed in any of the supernatant or sediment portions which were assayed spectrophotometrically. Polarographic studies of U. urealyticum 960 were thwarted because of a high concentration of peroxide. However, when isocitrate lyase assays were performed by discontinuous electrophoresis in<sub>3</sub> polyacrylamide gels according to the methods of Reeves and Volk<sup>3</sup>, isocitrate lyase activity was found in all the supernatant fluid extracts of the mycoplasmas tested (Table 1). In fact, U. urealyticum was found to have three isoenzymes, M. hominis and M. arthritidis had two each and M. pneumoniae and A. laidlawii each had one band of enzymatic isocitrate lyase activity.

Thus, it was obvious that glyoxylate had great potential as an enzyme inhibitor of isocitrate lyase in all of the mycoplasma strains tested. Since we had already found isocitrate lyase, it was of interest to determine whether a complete glyoxylate by-pass existed in these organisms. The second enzyme of the by-pass is malate synthase. This enzyme was not assessable by the continuous electrophoresis method of Volk et al<sup>4</sup>, so we resorted to a continuous electrophoretic method employing the Gradipore molecular sieving polyacrylamide gel. On the basis of molecular sieving, U. urealyticum, M. hominis, M. arthritidis, M. pneumoniae Mac and A. laidlawii all have malate synthase activity (Table 1).

In summary, glyoxylate was found to prolong the longevity of U. ureaplasma cultures. The prolongation was optimal between pH 6.4 and 7.2. It was found that glyoxylate inhibited the production of urease in cultures of U. urealyticum, but did not inhibit the enzymatic activity of urease once it was formed. Since we have also demonstrated isocitrate lyase in all of the mycoplasmas tested and isoenzymes of this type in some, it is highly probable that glyoxylate directly inhibited isocitrate lyase enzymatic activity and perhaps its production. Malate synthase activity has also been found in these mycoplasmas.

2. 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. Normal clinical laboratory biochemical tests indicate that strain 7815 reduced nitrate past nitrite and produces a gas, while strain 165 reduces nitrate to nitrite and produces no gas. Previous studies (WRAIR Annual Report 1975) indicated that both strains were able to utilize nitrate and nitrite as sole nitrogen sources. This was contrary to what was expected.

Whole cell extracts of both strains were assayed for the presence of both nitrate reductase and nitrite reductase. Both strains were grown in broth cultures of Stanier's Medium with glycerol (10g/l) as a carbon source and  $\text{KNO}_3$  (10g/l) as a nitrogen source. Forty-eight hour old cultures were harvested by centrifugation, and whole cell extracts were prepared as previously reported (WRAIR Annual Report 1974). Attempts to detect nitrate and nitrite reductase activity spectrophotometrically were unsuccessful since both strains contained NAD(P)H oxidase which could not be poisoned with quipazine. An assay not utilizing NAD(P)H measurements was chosen, and the enzyme assays were performed. Both strains showed reductase activity; however, strain 165 had approximately twice the enzymatic activity of strain 7815 (Table 2). There appeared to be strain differences in the rate of nitrate or nitrite utilization over the 60 minute period under observation. During anaerobic incubation with strain 165 as contrasted with aerobic incubation, it appeared that nitrate reduction was reduced and nitrite reduction was enhanced. This difference may be due to differential functioning of the reductases, since in bacterial systems nitrate can be utilized as a source of nitrogen, or under anaerobic respiration nitrate can be a terminal electron acceptor.

In summary, both smooth and rough strains of *Pseudomonas pseudomallei* possess a nitrate and a nitrite reductase; however, because of probable differences in the activity of these enzymes, smooth strain 165 does not show nitrite reduction in ordinary laboratory testing.

Table 1: Demonstration of isocitrate lyase and malate synthase in polyacrylamide gels

Strain	Isocitrate lyase			Malate synthase <sup>b</sup>	Acetyl CoA <sup>b</sup> deacylase
	M <sub>R</sub>	Bands <sup>a</sup>			
<u>U. urealyticum</u> 960	.12	.35 .53		+	-
<u>M. hominis</u> PG-21		.36 .53		+	-
<u>M. arthritidis</u> PG 6		.38 .53		+	-
<u>M. pneumoniae</u> Mac		.57		+	-
<u>A. laidlawii</u> A		.51		+	-
<u>P. pseudomallei</u> 7815		N.D.		? <sup>c</sup>	+

<sup>a</sup>. Electrophoresis of isocitrate lyase was performed in 7.5% polyacrylamide gel cylinders. Relative migration (M<sub>R</sub>) bands are the ratio of the enzymes distance from the origin to the distance traveled by brom phenol blue, the tracking dye.

<sup>b</sup>. Electrophoresis of malate synthase was performed in a 4-27% gradient polyacrylamide gel slab.

<sup>c</sup>. Acetyl CoA deacylase precluded the demonstration of any possible malate synthase.

Table 2: Comparison of the enzymatic activity of Pseudomonas pseudomallei strain 7815 and strain 165

Strain #	Incubation conditions	Substrate	Enzyme activity
			mM substrate utilized/mg protein/min
7815	anaerobic	NO <sub>3</sub>	1.80
165	anaerobic	NO <sub>3</sub>	2.89
7815	anaerobic	NO <sub>2</sub>	2.33
165	anaerobic	NO <sub>2</sub>	4.11
7815	aerobic	NO <sub>3</sub>	2.52
165	aerobic	NO <sub>3</sub>	5.69
7815	aerobic	NO <sub>2</sub>	3.15
165	aerobic	NO <sub>2</sub>	6.74

Figure 1

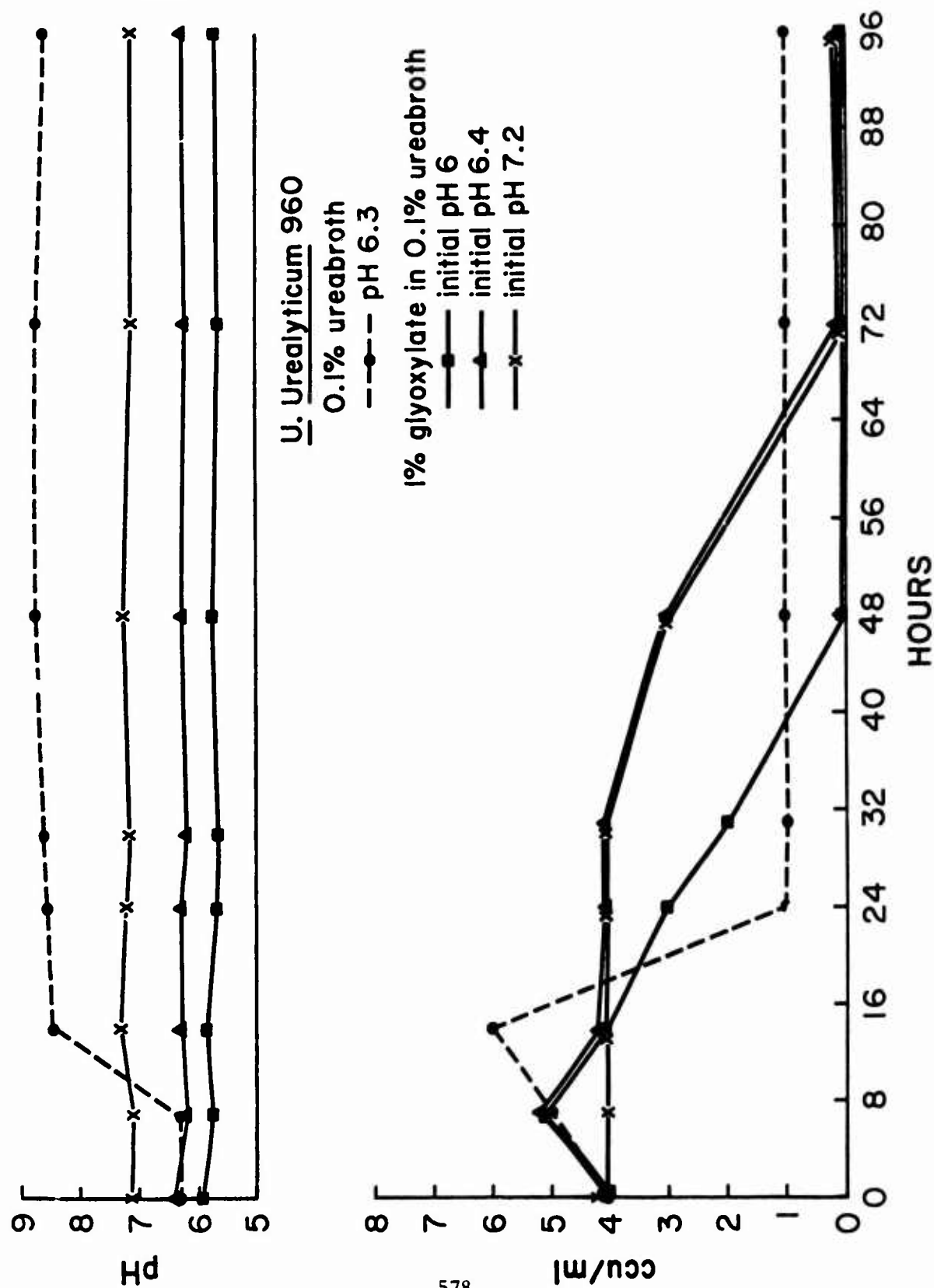
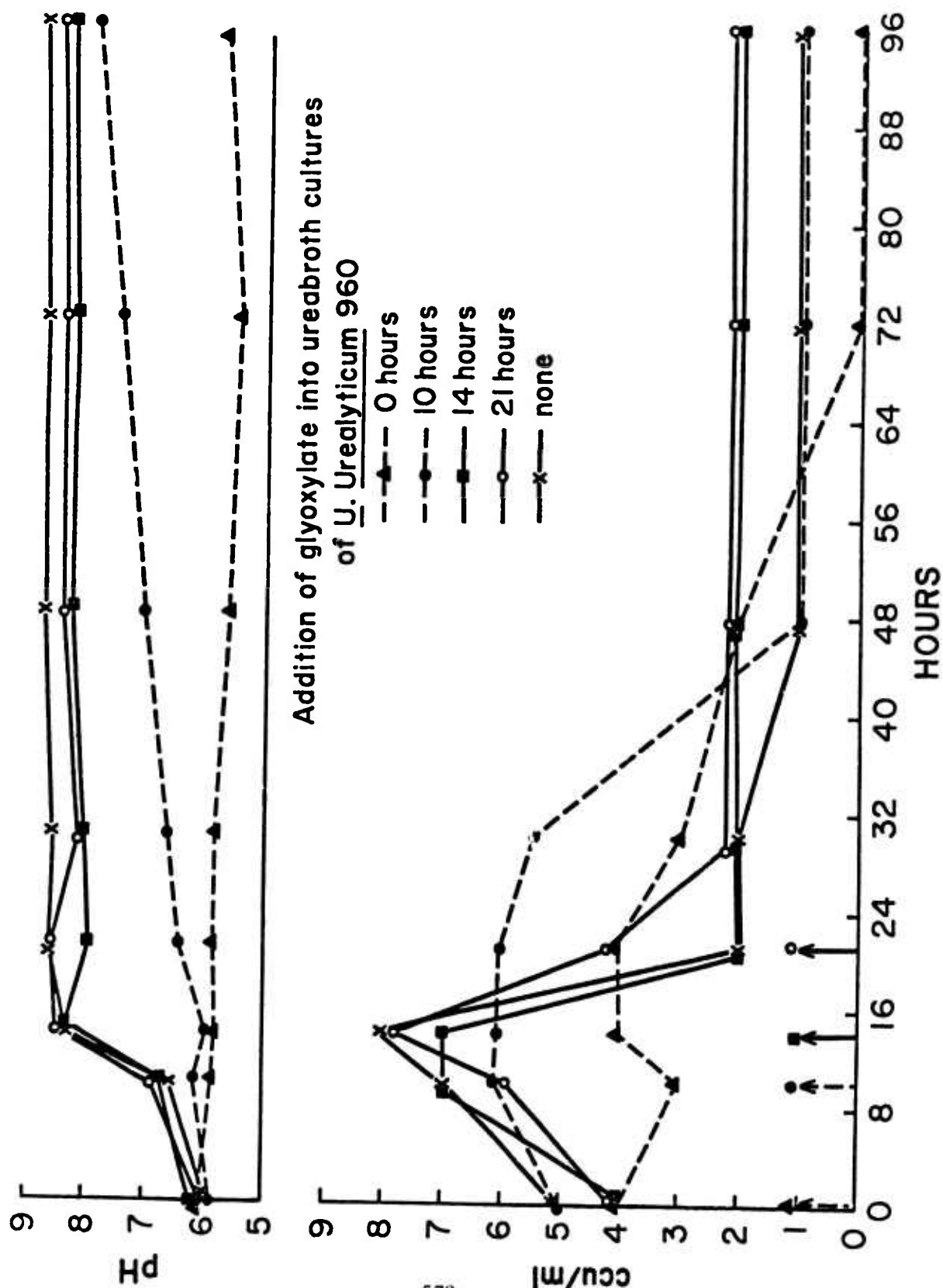




Figure 2





Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Zoonotic Diseases of Military Importance

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
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75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>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.</p> <p>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.</p> <p>25. (U) 75 07 - 76 06 - Experimental studies on the development of new and improved biological products for military use have continued. - 1. Investigations on meningococcal vaccines have been concerned with improvement of production methods for groups A and C polysaccharides, preparation of a protein-polysaccharide complex group B antigen, and production of group Y polysaccharide. 2. Work has continued on the use of attenuated strains of the plague bacillus for the preparation of inactivated plague vaccine. 3. Additional studies have been carried out on dengue viruses, types 2 and 3, in cell culture systems suitable for vaccine production. 4. Investigations on the feasibility of preparing freeze-dried and tableted diagnostic antigens and antisera have continued. 5. Studies on the addition of iron to substandard lots of mucin to enhance the virulence of challenge suspensions in mouse potency assays for bacterial vaccines have continued. 6. An investigation on the sublimation of formaldehyde during freeze-drying was initiated. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$96,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>							

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 171 Development of biological products

Investigators.

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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 Vaccines.

During the past year studies directed toward the improvement of the methods employed for the production of the groups A and C meningococcal polysaccharides were continued, by altering the constituents of the culture medium and by utilizing a different purification procedure. Laboratory studies were also carried out on the development of methods for the preparation of a protein-polysaccharide complex antigen derived from the group B meningococcus for possible use in the immunization of man against this type of meningitis. In addition, investigations were initiated on procedures for the production of purified polysaccharides from the Boshard strain (group Y) of Neisseria meningitidis, for use as an immunogen.

a. Previous investigations in this laboratory on the production of group A meningococcal polysaccharide vaccine indicated that the A-4 (M1027) strain of N. meningitidis, grown in either a modified Franz's medium or in the same medium fortified with 0.2% yeast extract dialysate, gave greater yields of polysaccharide than did the previously used A-1 strain under the same conditions. Furthermore, extraction of the crude product with phenol, in place of ethanol and chloroform,

showed promise as a means of simplifying the purification procedure (Annual Report, 1975). During this period additional studies were carried out with the A-4 strain for the purpose of further improving the yield of purified polysaccharide suitable for use as a vaccine for man.

Through a series of experiments designed to define the optimum medium and growth conditions for the A-4 strain, it was determined that the best results were obtained by employing Franz's basal medium fortified with 0.2% yeast extract dialysate in lieu of casamino acids (World Health Organization, 1975), incubated at 37 C for 12 hours, with constant shaking and aeration. The 12 hour meningococcal cultures, in 15 liter batches, were inactivated with Cetavlon, and the sediments collected by Sharples centrifugation were extracted with 1 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . After removal of DNA with a final concentration of 25% ethanol, the crude polysaccharide was precipitated with ethanol and dried. The polysaccharide precipitates were then divided and further purified at a concentration of 20 mg/ml by fractionation with either chloroform or phenol.

During the course of these studies it was found that certain factors in either the production or purification steps were critical, to insure a final product meeting all specifications (Bureau of Biologics, FDA, 1975) and suitable for human use:

- 1) After 12 hours incubation of the culture the final pH must be between 5.2 and 5.4, indicating proper growth of the organism. If this pH value was not achieved it was difficult to purify the available polysaccharide and consequently the yield of purified polysaccharide was considerably reduced.

- 2) To insure a final product with a DNA content of less than 1.0%, as required, it is important to store the initial crude extracts containing 25% ethanol at 5 C for a minimum of 2 hours, followed by centrifugation at 43,500 x g for 40 minutes, thereby obtaining a clear supernatant.

- 3) It was found that, in the fractionation procedures with either chloroform or phenol, the volume of the crude polysaccharide solution processed is critical. As shown in Table I, when the processing of 100 ml (or less) of solution is compared to the processing of 200 ml, more steps are required with the latter volume to obtain a product of acceptable purity (at least 8% phosphorus). However, the yields obtained when fractionating a 200 ml volume are considerably greater than those obtained with a 100 ml volume. In addition, the data also show that the phenol procedure is considerably more efficient than the chloroform procedure for extracting polysaccharide from the crude materials.

TABLE I

Processing of Crude Group A Polysaccharide\* to Purified Products  
Using 100 ml and 200 ml Volumes

Chloroform Fractionation	Volume 100 ml					Volume 200 ml				
	Yield mg/L	Phosphorus %	Protein %	Nucleic Acid %	Yield mg/L	Phosphorus %	Protein %	Nucleic Acid %	Yield mg/L	Nucleic Acid %
Post Chloroform 100,000 x 8, 4 hr 100,000 x 8, 4 hr Opalescence + 100,000 x 8, 4 hr		7.8	1.3	0.86		7.8	1.5	1.7		
		8.3	0.89	0.88		7.6	0.25	1.6		
		---	----	----		7.9	0.17	1.5		
	76	---	----	----	119	8.7	0.17	0.45		
Phenol Fractionation										
	Yield mg/L	Phosphorus %	Protein %	Nucleic Acid %	Yield mg/L	Phosphorus %	Protein %	Nucleic Acid %	Yield mg/L	Nucleic Acid %
Post Phenol 100,000 x 8, 4 hr 100,000 x 8, 4 hr Opalescence + 100,000 x 8, 4 hr		7.6	0.61	0.78		6.7	0.58	1.1		
		8.3	0.45	0.69		7.3	0.33	1.1		
		---	----	----		7.8	0.22	1.1		
	152	---	----	----	218	8.2	0.15	0.23		

\* Concentration = 20 mg/ml.

These studies have demonstrated that, by utilization of the yeast dialysate supplemented medium and the phenol fractionation procedure, the yield of purified group A polysaccharide is increased to 218 mg/L, a 13+ fold increase over the yield obtained in this laboratory in 1969 (Berman. et al., 1970) when work with the meningococcal polysaccharide immunogens was in its initial stages.

Because of the above results, it was of interest to apply the improved medium and purification procedure to the production of group C meningococcal polysaccharide. Consequently, the C-11 strain of N. meningitidis was grown in the Franz's basal medium fortified with 0.2% yeast extract dialysate and 12 hour cultures were processed as described above. The results are recorded in Table II.

TABLE II  
Processing of Group C Meningococcal Polysaccharide from a  
Culture Grown in Yeast Extract Dialysate Medium\*

Stage of Procedure	Yield gm/L	Protein %	Nucleic Acid %	Sialic Acid %
Sharples Sediment	4.0			
Crude Precipitate	0.154	24.5	4.9	49.5
Chloroform Extraction	0.018	1.6	2.3	79.0
3rd Alcohol Treatment	0.010	1.2	1.4	84.5
High Speed Centrifugation	0.006	0.98	0.67	81.3
Phenol Extraction	0.056	4.3	1.0	65.0
3rd Alcohol Treatment	0.030	1.3	0.60	80.1
High Speed Centrifugation	0.025	0.98	0.82	82.9

\* Time of Incubation - 12 hours  
Culture Fluid, pH 5.4

As indicated by the yield of Sharples sediment, the growth of the C-11 strain in the yeast extract dialysate was satisfactory. Fractionation of the crude extract with chloroform resulted in a yield

considerably less than the 0.016 gms per liter usually obtained from cultures grown in Franz's medium using the same fractionation procedures (Berman, et al., 1970). However, fractionation of the crude extracts with phenol gave a considerably greater yield of final purified polysaccharide, indicating that this procedure is worthy of further investigation.

b. Initially, studies on the development of a vaccine from the group B meningococcus were directed toward the production of a purified polysaccharide similar to the products that proved successful with groups A and C meningococci. However, since the group B polysaccharide failed to exhibit immunogenicity in volunteers (Wyle, et al., 1972), the emphasis was shifted to the preparation from the group B strain of various protein fractions suitable for human use. These in turn failed to induce the production of bactericidal antibodies in volunteers (Annual Report, 1973, Work Unit 181). Current efforts, therefore, are directed toward producing a vaccine that will consist of a complex of the polysaccharide and protein components of the microorganisms.

Accordingly, cultures of the B-11 strain of N. meningitidis were grown in a modification of Franz's medium in which the casamino acid component is increased 3-fold and the dextrose concentration reduced to 1/5 the original (Annual Report, 1971). This medium permits control of the pH during the growth of the cultures and minimizes the exposure of the polysaccharides and proteins to the more acid conditions usually obtained with Franz's medium. Several methods for inactivation and purification are being explored in an effort to produce a combined protein-polysaccharide antigen suitable for human use.

c. An experimental study on the development of techniques for the large-scale production and purification of polysaccharides derived from cultures of a group Y strain (Boshard, 80-Y) of N. meningitidis was initiated in cooperation with the Department of Bacterial Diseases, WRAIR.

The 80-Y strain was grown in 15 liter volumes in three different media; Modified Franz's, Modified Franz's with the phosphate component increased 4-fold, and Franz's basal medium fortified with yeast extract dialysate in place of casamino acids. The three cultures were incubated for 16 hours at 36 C and were then inactivated and precipitated with Cetavlon. The resulting precipitates were collected by centrifugation and the final pH of the cultures and the sediment weights are shown in Table III.

TABLE III

The Growth of the 80-Y Strain in Three  
Different Media

<u>Medium</u>	<u>Final pH</u>	<u>Yield of Precipitate (gms/L)</u>
Modified Franz's	5.3	3.0
Modified Franz's with 4X PO <sub>4</sub>	6.3	4.6
Yeast Extract Dialysate	5.3	4.6

Visually the group Y organism appeared to grow well in all of the media and this was confirmed by the adequate yields of precipitate obtained after centrifugation. The final pH of the culture was higher in the medium with four times the phosphate content than in the other two cultures, exposing the polysaccharide to a lesser degree of acidity and thus would be less likely to adversely affect molecular size. The precipitates from each of the three media are being processed by several extraction and purification techniques, to determine the optimum procedures for obtaining purified group Y polysaccharide suitable for evaluation of immunogenicity in man.

## 2. Plague Vaccine.

Work has continued on a collaborative study with the Department of Hazardous Microorganisms, WRAIR, to determine whether an attenuated strain of Yersinia pestis, inactivated with formalin, could replace the highly hazardous virulent strain currently used in the preparation of the U. S. Army plague vaccine.

Each of the attenuated strains, EV-76S and A1122, was grown on Bacto Blood Agar Base (Difco) Agar at 37 C for 72 hours and the resulting organisms were harvested in saline. The harvest pools of each strain were divided into four aliquots and different concentrations of formalin (0.0%, 0.05%, 0.1% and 0.2%) were added. The aliquots were held at room temperature, with frequent shaking, and samples were withdrawn at designated time periods to determine rates of inactivation. The results are summarized in Table IV.



TABLE IV

Formalin Inactivation of *Yersinia pestis* (Strains  
EV-76S and A1122) Harvests Grown at 37 C

<u>Formalin</u> (%)	<u>EV-76S</u>			<u>A1122</u>		
	<u>Inactivation Time (Hrs)</u>					
	<u>3</u>	<u>6</u>	<u>24</u>	<u>3</u>	<u>6</u>	<u>24</u>
0.0	+	+	+	+	+	+
0.05	+	+	-*	+	+	-
0.1	+	+	-	+	+	-
0.2	+	-	-	+	-	-

\* No apparent growth on agar.

The harvest pools from both attenuated strains were completely inactivated within 24 hours by the addition of 0.05% formalin. Nitrogen assays on these two inactivated 24 hour preparations yielded equal results, but indicated that they contained approximately three times the nitrogen present in the standard U. S. Army plague vaccine as is shown in Table V.

TABLE V

Nitrogen Assays on Inactivated Plague Preparations

<u>Preparation</u>	<u>Nitrogen</u> mg/ml
EV-76S	0.964
A1122	1.03
U. S. Army Vaccine	0.36

These preparations have been submitted to the Department of Hazardous Microorganisms, WRAIR, for evaluation in animals. The antibody responses and protective activities of the attenuated vaccines will be compared with those of the standard vaccine on an equivalent nitrogen basis.

### 3. Dengue Virus Vaccines.

During the past year additional studies were carried out on the development of a dengue virus type 2 (DEN-2) vaccine for human use.

Attempts were also made to adapt dengue virus type 3 (DEN-3) to grow in WI-38 cells. In addition, studies were continued on the development of a plaquing assay for dengue viruses in FRhL and WI-38 cells.

a. In a previous experiment in which there were some unavoidable fluctuations in the temperatures of some of the incubators, DEN-2 (strain PR 159) appeared to replicate optimally in DBS-FR<sub>h</sub>L-2 cells at a temperature of incubation of 33 C (Annual Report, 1975). This experiment was repeated at a subsequent time during which the incubators were under better control. Confluent monolayers of DBS-FR<sub>h</sub>L-2 cells in 75 cm<sup>2</sup> flasks were washed with 25 ml of Hank's Balanced Salt Solution (HBSS) and then inoculated with DEN-2 seed material (Annual Report, 1974) at a multiplicity of infection (MOI) of 1. Virus was adsorbed at the various temperatures for 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 with antibiotics but no serum additives), the flasks were incubated at the different incubation temperatures. At 0, 1, 2, 3, 4, 5, 6 and 7 days post-inoculation, 0.3 ml samples from each of two flasks were removed, 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 VI.

TABLE VI  
Growth Curves of Dengue Virus Type 2 in DBS-FRHL-2 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					
1	5.4X10 <sup>2</sup> *	5.6X10 <sup>2</sup>	5.5X10 <sup>2</sup>	5.0X10 <sup>2</sup>	4.6X10 <sup>2</sup>
2	5.7X10 <sup>2</sup>	5.7X10 <sup>3</sup>	3.0X10 <sup>4</sup>	1.1X10 <sup>5</sup>	1.3X10 <sup>5</sup>
3	2.5X10 <sup>4</sup>	1.6X10 <sup>5</sup>	2.1X10 <sup>5</sup>	1.8X10 <sup>5</sup>	1.0X10 <sup>5</sup>
4	2.5X10 <sup>5</sup>	6.7X10 <sup>5</sup>	4.3X10 <sup>5</sup>	3.0X10 <sup>5</sup>	1.0X10 <sup>5</sup>
5	3.0X10 <sup>5</sup>	1.5X10 <sup>6</sup>	1.1X10 <sup>6</sup>	1.0X10 <sup>6</sup>	3.6X10 <sup>5</sup>
6	3.2X10 <sup>5</sup>	8.2X10 <sup>5</sup>	3.2X10 <sup>5</sup>	1.0X10 <sup>6</sup>	7.2X10 <sup>5</sup>
7	4.0X10 <sup>5</sup>	5.9X10 <sup>5</sup>	5.7X10 <sup>5</sup>	1.4X10 <sup>6</sup>	7.5X10 <sup>5</sup>
	1.3X10 <sup>5</sup>	3.2X10 <sup>5</sup>	2.1X10 <sup>5</sup>	7.6X10 <sup>5</sup>	3.1X10 <sup>5</sup>

\* PFU/0.2 ml

From the above results, it appears that there are basically no differences in the titers obtained at the incubation temperatures of 31, 33 and 35 C. Peak titers were reached on day 4 post-inoculation at these three temperatures. However, maximal titers persisted for several days at the incubation temperature of 35 C. On this basis, 35 C was selected as the optimal incubation temperature.

Since FBS was omitted from the maintenance medium (MM), it was of interest to determine the effect, if any, on the yields of intracellular and extracellular virus. The growth curve procedure as previously described was used except that 2 oz glass prescription bottles were utilized. Also, one set of bottles was refed with MM containing 2% FBS whereas the other set of bottles was refed with MM without any serum additives. Two bottles from each set were processed on a daily basis, starting from day 0 to day 7 post-inoculation, by the following procedure. The fluids from the infected monolayer cultures were harvested and centrifuged at 2000 RPM for 10 minutes. A sample of the supernatant was mixed with an equal volume of inactivated FBS and then dispensed into ampules. The ampules were sealed and stored at -70 C for later testing. The same infected monolayer cultures were now washed thoroughly with HBSS and then received the original volume of MM. After three cycles of freezing and thawing, a sample was taken using the same procedure as above. The results of the assays for virus titer are shown in the following table.

TABLE VII

Comparison of the Effect of FBS and No Serum Additive in the Maintenance Medium on the Yield of Fluid Virus and Cell-Associated Virus in DBS-FRhL-2 Cell Cultures

	<u>Serum (FBS)</u>		<u>No Serum Additive</u>	
	<u>Fluid Virus</u>	<u>Cell-Associated Virus</u>	<u>Fluid Virus</u>	<u>Cell-Associated Virus</u>
Day 0	1.1X10 <sup>2</sup> *	1.3X10 <sup>3</sup>	<10	6.9X10 <sup>2</sup>
1	2.4X10 <sup>4</sup>	1.0X10 <sup>5</sup>	1.4X10 <sup>4</sup>	3.0X10 <sup>4</sup>
2	4.3X10 <sup>5</sup>	4.8X10 <sup>5</sup>	6.7X10 <sup>4</sup>	8.6X10 <sup>4</sup>
3	1.4X10 <sup>6</sup>	1.0X10 <sup>6</sup>	1.4X10 <sup>5</sup>	1.3X10 <sup>5</sup>
4	2.0X10 <sup>6</sup>	1.1X10 <sup>6</sup>	1.6X10 <sup>5</sup>	2.4X10 <sup>5</sup>
5	4.4X10 <sup>6</sup>	9.9X10 <sup>5</sup>	7.4X10 <sup>5</sup>	6.0X10 <sup>5</sup>
6	5.2X10 <sup>6</sup>	1.0X10 <sup>6</sup>	3.9X10 <sup>5</sup>	2.3X10 <sup>5</sup>
7	3.4X10 <sup>6</sup>	8.5X10 <sup>5</sup>	3.0X10 <sup>5</sup>	5.0X10 <sup>4</sup>

\* PFU/0.2 ml

As can be seen from the above table, slightly higher titers were obtained in the fluids of the infected cultures, especially when FBS is added to the MM. On the basis of these results, as well as for practical reasons, only the infected culture fluids will be harvested for use in the development of a vaccine.

Growth curves were conducted to determine the effect of age of the DBS-FR<sub>h</sub>L-2 cell cultures at the time of inoculation on the yield of virus in the infected fluids. The same procedure as previously described was used except that a series of DBS-FR<sub>h</sub>L-2 monolayer cultures from 1 through 4 days old, all of about equal cell numbers except for the 1 day old monolayers, were used. The results are presented in the following table.

TABLE VIII

Growth Curves of Dengue Virus Type 2 in Different Age Cultures of DBS-FR<sub>h</sub>L-2 Cells

	<u>Age of Culture at Time of Inoculation</u>			
	<u>1 Day</u>	<u>2 Day</u>	<u>3 Day</u>	<u>4 Day</u>
Day 0	2.6X10 <sup>2</sup> *	1.6X10 <sup>3</sup>	1.4X10 <sup>3</sup>	1.9X10 <sup>3</sup>
1	2.0X10 <sup>5</sup>	1.8X10 <sup>5</sup>	1.4X10 <sup>5</sup>	9.4X10 <sup>4</sup>
2	4.9X10 <sup>5</sup>	8.0X10 <sup>5</sup>	2.3X10 <sup>5</sup>	2.0X10 <sup>5</sup>
3	2.2X10 <sup>5</sup>	3.8X10 <sup>5</sup>	4.2X10 <sup>5</sup>	2.9X10 <sup>5</sup>
4	2.0X10 <sup>5</sup>	1.6X10 <sup>5</sup>	4.5X10 <sup>5</sup>	2.5X10 <sup>5</sup>
5	1.2X10 <sup>5</sup>	2.4X10 <sup>5</sup>	4.6X10 <sup>5</sup>	4.0X10 <sup>5</sup>

\* PFU/0.2 ml

The results appear to indicate that 2 day old monolayers yielded slightly higher titers of virus. However, this experiment will be repeated since there was a loss in titer in some of the seed ampules that were used for the inoculation of the DBS-FR<sub>h</sub>L-2 cell cultures.

To determine the relationship between the number of virus particles in the inoculum and optimal time of harvest, growth curves were obtained with three different concentrations of virus particles, using the procedure previously described. The results are given in Table IX.

TABLE IX

Effect of Viral Concentration of Inoculum on Growth  
Curves of Dengue Virus Type 2

	<u>Multiplicity of Infection (MOI)</u>		
	<u>1</u>	<u>0.1</u>	<u>0.01</u>
Day 0	1.4X10 <sup>3</sup> *	1.1X10 <sup>2</sup>	2.0X10 <sup>1</sup>
1	1.6X10 <sup>5</sup>	1.2X10 <sup>4</sup>	1.7X10 <sup>3</sup>
2	6.3X10 <sup>5</sup>	1.1X10 <sup>5</sup>	2.3X10 <sup>4</sup>
3	9.4X10 <sup>5</sup>	1.5X10 <sup>5</sup>	8.0X10 <sup>4</sup>
4	1.5X10 <sup>6</sup>	5.7X10 <sup>5</sup>	1.3X10 <sup>5</sup>
5	1.3X10 <sup>6</sup>	7.0X10 <sup>5</sup>	2.5X10 <sup>5</sup>
6	1.3X10 <sup>6</sup>	1.3X10 <sup>6</sup>	3.1X10 <sup>5</sup>
7	1.5X10 <sup>6</sup>	2.2X10 <sup>6</sup>	1.2X10 <sup>6</sup>

\* PFU/0.2 ml

The maximal titer obtained was approximately the same, regardless of the number of virus particles in the seed inoculum. However, with an MOI of 1 the maximal titer was reached on day 4 post-inoculation as compared with day 6 and 7 post-inoculation for the other concentrations of virus particles. Presently the virus seed is being concentrated in order to determine the effect of higher MOI on virus yield.

The following experiment was done in order to observe the temporal sequence of inactivation of DEN-2 at 22 C. Culture fluids from infected cell monolayers were collected, centrifuged at 2000 RPM for 10 minutes and the supernatant passed through a 0.20  $\mu$ m filter. This filtered material was divided into five portions. Four portions received 2% Human Serum Albumen (HSA) and different concentrations of formalin (0.025%, 0.05%, 0.1% and 0.2%) were added. The fifth portion had a concentration of 0.05% formalin but no HSA was added to the infected culture fluid. The inactivating fluids were held at room temperature and sampled at 6, 24, 48 and 72 hours. Inactivation of the virus was measured by plaquing in LLC-MK<sub>2</sub> cell monolayers. The results are summarized in the following table.

TABLE X

Formalin Inactivation of Dengue Virus  
Type 2 at 22 C

<u>Formalin (%)</u>	<u>Inactivation Time</u>			
	<u>6 Hrs</u>	<u>24 Hrs</u>	<u>48 Hrs</u>	<u>72 Hrs</u>
0.05 (No HSA)	+	+	-	-
0.025 (+ 2% HSA)	+	+	+	+
0.05 (+ 2% HSA)	+	+	-	-
0.1 (+ 2% HSA)	+	+	-	-
0.2 (+ 2% HSA)	+	-**	-	-

\* Plaques observed in LLC-MK<sub>2</sub> cell monolayers.

\*\* Partial destruction of monolayers at undilute due to formalin.

A concentration of 0.05% formalin, with or without 2% HSA, inactivated the virus at 48 hours. Since inactivation at 22 C was accomplished within a satisfactory time period, both of these inactivated preparations will be tested for immunogenicity in animals. If the inactivated preparation containing HSA is as immunogenic as the preparation without HSA, this will be the method of choice since HSA is a good stabilizer for freeze drying.

DEN-2 was previously shown to replicate in WI-38 cells if the virus had been previously passaged in DBS-FRHL-2 cells (Annual Report, 1974). Two more passages were made in WI-38 cells to bring the total to six passages. The results of these two passages are presented in the following table.

TABLE XI

Passages of Dengue Virus Type 2 in WI-38 Cells

<u>Passage</u>	<u>Inoculum (0.5 ml)</u>	<u>Day of Harvest</u>	<u>Titer (PFU/0.2 ml)</u>
5	1.8X10 <sup>5</sup> PFU	4	1.3X10 <sup>5</sup>
6	1.6X10 <sup>5</sup> PFU	4	1.2X10 <sup>5</sup>

The titers of these two passages were the same as that of passage 4, indicating that adaptation of DEN-2 in WI-38 cells was fairly rapid.

It was also of interest to determine whether attenuation had taken place during the passage of the virus in WI-38 cells. Therefore, passage 3 and passage 6 materials were compared in terms of mouse neuro-virulence. The results are shown in Table XII.

TABLE XII

Inoculation of Suckling Mice with DEN-2 from WI-38 Cells

<u>Dilution</u>	<u>Passage 3</u>	<u>Passage 6</u>
Undil.	14/14*	14/14
10 <sup>-1</sup>	14/14	14/14
10 <sup>-2</sup>	14/14	14/14
10 <sup>-3</sup>	13/14	12/14
10 <sup>-4</sup>	9/14	8/14
10 <sup>-5</sup>	1/14	0/14

Each mouse was inoculated IC with 0.02 ml.

\* No. of deaths/total inoculated.

From the above results, it appears that attenuation for suckling mice has not taken place since the virulence of the passage 6 material is identical to that of the passage 3 material.

b. Studies were initiated to determine whether dengue 3 virus (DEN-3) could be adapted to WI-38 cells by applying the same passage procedures as previously described for DEN-2 (Annual Report, 1974). Confluent monolayers of WI-38 cells in 25 cm<sup>2</sup> flasks were inoculated with 0.25 ml of a 1:5 dilution of the DEN-3 infected human serum in MM. Table XIII shows the results of the passage of DEN-3 in WI-38 cells.

TABLE XIII

Passage of Dengue Virus Type 3 (CH 53489) in WI-38 Cells

Inoculum:	Infected Human Serum	9.5X10 <sup>2</sup> PFU*
	WI-38/1/Day 6**	0
	WI-38/1/Day 10**	0
	WI-38/1/Day 16**	0
	WI-38/1/Day 22**	0
	WI-38/1/Day 30	0

\* Volume of inoculum was 0.25 ml; harvest titers in terms of PFU/0.2 ml.

\*\* Culture fluids harvested and cell sheets refed with MEM + 2% FBS.



As can be seen from the above table, WI-38 cells appeared to be refractory to DEN-3 infected human serum.

In another attempt to adapt DEN-3 to WI-38 cells, DEN-3 which had previously undergone four passages in primary African green monkey kidney cells (obtained from K. Eckels, Department of Hazardous Microorganisms, WRAIR) was used as the inoculum. In the course of the passage work, however, a distinctive type of CPE occurred by day 28 post-inoculation. The CPE started as a single focal area which eventually affected the entire cell sheet. On the basis of cytological observation of infected WI-38 cells, CF test, microplaque formation in WI-38 cells and virus-cell association, the distinctive CPE appeared to be due to a cytomegalo-virus contaminant. The contaminant was then isolated in WI-38 cells from the same lot of primary African green monkey kidney cells that was used for the original passages of DEN-3.

Since DEN-3 was previously shown to replicate in DBS-FRHL-2 cells (Annual Report, 1974), passage of the virus was initiated in certified DBS-FRHL-2 cells using the same procedure as previously described. Confluent monolayers of DBS-FRHL-2 cells in 25 cm<sup>2</sup> flasks were inoculated with 0.2 ml of a 1:10 dilution of the infected human serum in MM. Table XIV shows the results of the first passage of DEN-3.

TABLE XIV

Passage of DEN-3 Virus (CH 53489) in Certified DBS-FRHL-2 Cells

Inoculum: Infected Human Serum:  $3.8 \times 10^2$  PFU\*

<u>Flask A</u>		<u>Flask B</u>	
	<u>Titer</u>		<u>Titer</u>
FRhL/1/Day 4**	$4.0 \times 10^0$	FRhL/1/Day 7**	$2.0 \times 10^0$
FRhL/1/Day 10**	$7.0 \times 10^0$	FRhL/1/Day 14**	$2.5 \times 10^1$
FRhL/1/Day 17**	$1.1 \times 10^1$	FRhL/1/Day 21**	$9.0 \times 10^1$
FRhL/1/Day 24**	$5.0 \times 10^0$	FRhL/1/Day 28**	$3.0 \times 10^1$
FRhL/1/Day 31	$1.4 \times 10^2$	FRhL/1/Day 35	$2.2 \times 10^3$

\* Volume of inoculum was 0.2 ml; harvest titers in terms of PFU/0.2 ml.

\*\* Culture fluids harvested and cell sheets refed with MEM + 2% FBS.

Further passages will be made in certified DBS-FRHL-2 cells.

c. Studies were also continued on the development of a plaquing assay for dengue viruses in DBS-FR<sub>h</sub>L-2 cells. Further investigations with various plaquing media and procedures did not increase the plaquing efficiency when compared to the LLC-MK<sub>2</sub> plaquing system. However, the distinctiveness of the plaques was improved and the rapid deterioration of DBS-FR<sub>h</sub>L-2 cells after the addition of neutral red was slightly delayed by using the following somewhat laborious technique. 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 10% FBS, 0.5% agarose and antibiotics. The flasks were incubated at 35 C for 6 days and then received 4 ml of a second overlay containing the same components as the first overlay medium. The flasks were reincubated at 35 C for an additional 3 days and then received 4 ml of a third overlay consisting of 0.5% agarose and neutral red at a final dilution of 1:10,000 in EBSS solution. The flasks were incubated at room temperature overnight before plaques were counted. The titer of the DEN-2 material was the same ( $1.3 \times 10^5$  PFU/0.2 ml as previously reported (Annual Report, 1975)). However, the viable DBS-FR<sub>h</sub>L-2 cells were able to adsorb the neutral red dye more efficiently thus making the nonviable cells, that is the plaques, more discernible. For some reason the monolayers were also slightly more stable after the addition of neutral red.

Studies on a plaquing assay for dengue viruses in WI-38 cells were also initiated. The same procedures as described above were used for the inoculation of WI-38 cells and the flasks also received the same first overlay medium except that the volume was increased to 10 ml. After incubation at 35 C for 9 days, the flasks 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 titer of the DEN-2 material was approximately  $1.0 \times 10^5$  PFU/0.2 ml. However, the plaquing system in WI-38 cells as in DBS-FR<sub>h</sub>L-2 cells, is not as sensitive as in the LLC-MK<sub>2</sub> plaquing system, with a difference in titer of approximately one log. Currently, plaquing isolation techniques are being conducted in the two cell strains.

#### 4. Diagnostic Antigens and Antisera.

Experimental studies were continued during this period on the feasibility of preparing freeze-dried and tableted bacterial antigens and antisera for use in diagnostic tests for the febrile diseases. This work was initiated last year in an attempt to provide stable compact diagnostic materials, permitting shipment and storage of multiple samples in one container, thus conserving space and reducing costs.

Pilot studies with the somatic ("O") antigen of Salmonella typhi, strain 0901, indicated that it is possible to obtain freeze-dried preparations of the antigen which, upon the addition of the proper volume of water, readily reconstitute into homogeneous bacterial suspensions suitable for use in the standard agglutination test for the detection of febrile antibodies in acute and convalescent sera (Annual Report, 1975). When stored in the freeze-dried state the antigen retained its agglutinating capacity for prolonged periods of time, even under adverse storage temperatures.

During this period investigations were extended to 6 of the 9 antigens which are standard items distributed by the WRAIR through the Biological Depot, Walter Reed Army Medical Center, to military laboratories around the world. With each antigen, fluid, freeze-dried and tableted preparations were made, and samples of each were tested quantitatively for agglutinability in homologous antisera by the standard tube-type agglutination test. The results are recorded in the following table.

TABLE XV

Agglutination Titers of Fluid, Freeze-Dried  
and Tableted Febrile Antigen Preparations

<u>Antigen</u>	<u>Fluid</u>	<u>Freeze-Dried</u>	<u>Tableted</u>
<u>S. typhi</u> "O"	2560	1280	2560
<u>S. typhi</u> "H"	2560	640	1280
<u>S. paratyphi</u> "H"	320	160	320
<u>S. schottmulleri</u> "H"	1280	1280	640
<u>Proteus</u> OX-K	320	160	320
<u>P. tularensis</u>	1280	320	640

For each of the antigens, the titers obtained for the fluid, freeze-dried and tableted preparations are plus or minus one dilution, and are therefore not significantly different. Samples of each preparation have been placed in storage at 4 C, 37 C, 45 C and 56 C, and periodically will be removed for stability evaluation.

Although many of the diagnostic antisera are already available in the freeze-dried form from commercial sources, it was of interest to determine the effect, if any, of compression of the dried antisera into tablets. Consequently tablets were prepared from dried preparations of Salmonella "O" polyvalent, Salmonella Vi and Vibrio comma "Ogawa" antisera, and the agglutinating activity of these tableted materials were compared with the dried material from which they were prepared in

the standard slide agglutination test. The results are given in the table below.

TABLE XVI

Agglutinating Activity of Freeze-Dried and Tableted Diagnostic Antisera

<u>Antiserum</u>	<u>Freeze-Dried</u>	<u>Tableted</u>	<u>Saline Control</u>
<u>Salmonella</u> "O" Polyvalent	4+	4+	-
<u>Salmonella</u> "Vi"	4+	4+	-
<u>Vibrio comma</u> "Ogawa"	4+	4+	-

The results demonstrate that compression of the freeze-dried powders into tablets did not reduce the agglutinating activities of the antisera tested. Therefore, tableted antisera, packaged as multiple units in one container, would be satisfactory for use in the slide agglutination test for identification of the bacterial cause of febrile disease.

5. Vaccine Potency Assays.

During the past year additional experimental studies have been performed in an attempt to determine whether the addition of iron to substandard lots of gastric mucin would sufficiently enhance the virulence of challenge organisms suspended in the mucin to permit use of the preparation in mouse potency assays for the evaluation of bacterial vaccines.

The studies during this period were limited to the effect of mucin and iron combinations on the enhancement of the virulence of Salmonella typhi, strain Ty2, since the U. S. Food & 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 (Code of Federal Regulations, 1975).

Previous investigations have shown that, in testing several different lots of mucin with the addition of varying concentrations of iron in the form of ferric ammonium citrate, an enhancing effect of iron on virulence could be demonstrated (Annual Report, 1975). A 5- to 100-fold reduction in LD<sub>50</sub> values was obtained in each of the virulence titrations performed. The results also demonstrated that the various mucin preparations require different amounts of added iron to reduce the LD<sub>50</sub> value to an acceptable level.

The favorable results of the virulence titrations led next to an investigation of the pathogenicity-promoting activity of mucin supplemented with iron in challenge suspensions for use in the mouse protection test for the evaluation of typhoid vaccines. The efficacy of the U. S. standard typhoid vaccine, Lot #6A, was determined by challenging previously immunized mice with the Ty2 strain of S. typhi suspended in 5% mucin alone and in 5% mucin supplemented with iron. The results, presented in Table XVII, show no significant change in the ED<sub>50</sub> value of vaccine Lot #6A, when the challenge suspension was prepared in mucin supplemented with iron in lieu of mucin alone.

TABLE XVII

Mouse Protection Activity of U. S. Standard Vaccine #6A  
Challenged with S. typhi Strain Ty2 Suspended in  
Mucin and Mucin Supplemented with Iron

Type of Challenge	LD <sub>50</sub> (orgs.)	Vaccine	ED <sub>50</sub> (ml)	Standard Deviation	Relative Potency
5% Mucin*	350	Lot #6A	0.000393	78-129%	1.00
5% Mucin + Fe <sup>+++</sup> (10 mg/kg body wgt)	12	Lot #6A	0.000419	74-134%	1.07

\* Mucin Lot #96181

Thus, the addition of iron to the mucin challenge suspension, while effectively enhancing the virulence of the infecting organism, does not appear to interfere with the test for determining the potency of typhoid vaccine.

It should be noted, however, that although there was a considerable reduction in the LD<sub>50</sub> value of the challenge suspension upon the addition of 10 mg Fe<sup>+++</sup>/kg mouse body weight, the resulting LD<sub>50</sub> of 12 organisms does not quite satisfy the FDA requirement of "no more than 10 colony forming units." It is believed that this concentration of Fe<sup>+++</sup>, which was selected on the basis of earlier experiments, could in any given assay yield borderline results (10 ± 3 organisms). Consequently, the concentration of iron added to the mucin for future tests was increased to 15 mg Fe<sup>+++</sup>/kg mouse body weight.

A subsequent experiment compared standard typhoid vaccine, Lot #6A, with a commercially prepared typhoid vaccine in a mouse potency assay involving 2 challenges. One challenge contained a mucin lot that required the addition of iron to be acceptable and the second utilized a mucin lot which in earlier studies did not require the addition of iron to be effective. The results of this experiment are presented in Table XVIII.

TABLE XVIII

Comparison between Mouse Protection Activity of U. S. Standard Typhoid Vaccine #6A  
with a Commercial Typhoid Vaccine Challenged with *S. typhi* Strain Ty2  
Suspended in Mucin and in Mucin Supplemented with Fe<sup>+++</sup>

<u>Type of Challenge</u>	<u>LD50 (orgs.)</u>	<u>Vaccine</u>	<u>ED50 (ml)</u>	<u>Standard Deviation</u>	<u>Relative Potency</u>
Mucin Lot #96181 + Fe <sup>+++</sup> (15 mg/kg body wgt)	6.0	Lot #6A	0.000637	72-139%	1.00
		Lot #25806	0.000492	62-161%	1.25
Mucin Lot #135190	3.0	Lot #6A	0.0011	82-121%	1.0
		Lot #25806	0.0010	83-120%	1.1

Although there was a 2-fold difference between the ED<sub>50</sub> values obtained with the 2 challenge suspensions, the relative potencies of the 2 vaccines were identical. In this experiment the LD<sub>50</sub> values of 6.0 organisms for the mucin challenge supplemented with iron and 3.0 organisms for the challenge using mucin alone, were well within the limits of 10 organisms or less as prescribed by the FDA regulations. These results, therefore, demonstrate that iron may be added to sub-standard lots of mucin preparations to obtain satisfactory results in typhoid mouse protection tests.

Additional mouse potency assays for typhoid vaccines will be performed utilizing three different mucin lots, two of which require the addition of iron to be acceptable for use in challenge preparations, in order to confirm the data obtained thus far. The usefulness of iron-supplemented mucin in the preparation of challenge suspensions for the mouse potency assays for cholera and meningococcal vaccines will also be evaluated.

#### 6. Sublimation of Formaldehyde during Freeze-Drying.

Formaldehyde solution is frequently used to inactivate bacterial, rickettsial and viral suspensions during the preparation of vaccines. The presence of some residual formaldehyde in the final product of a fluid preparation is generally considered to be an advantage because of its preservative action. However, during the freeze-drying of biologicals the presence of "free formalin" can have a deleterious effect on the antigen(s). Consequently, the formaldehyde is generally neutralized with sodium bisulfite prior to freeze-drying (Annual Report, 1972).

Since the undesirable effect on the antigen(s) is believed to be due to the concentration of the formaldehyde during freeze-drying, an investigation of the sublimation of formaldehyde in shell-frozen and plug-frozen aqueous preparations was carried out by measuring the residual formaldehyde periodically during the freeze-drying procedure.

Formaldehyde solution was diluted with distilled water to give a 0.2% formalin solution. The actual formaldehyde content was determined by assay. Ten ml aliquots were dispensed into 20 ml vials. Samples were shell-frozen in a dry ice-ethanol bath or plug-frozen in a -60 C freezer. Twelve vials of each group were freeze-dried on a 12 port "pig" at room temperature and 50  $\mu$ m pressure. The vials were removed at periodic intervals, the volume of solution remaining in each vial after warming to room temperature was measured, and the fluid was assayed for free formaldehyde content. The dry samples were taken up in 10 ml of distilled water. Table XIX shows the results obtained.

TABLE XIX

Comparison of Shell-Frozen and Plug-Frozen Freeze-Dried  
Formaldehyde-Water Solution

Time Dried Hrs	Volume ml		mg/ml	Free Formaldehyde		% Loss	
	Shell	Plug		Shell	Plug	Shell	Plug
0.5	8.0	8.6	0.692	0.629	20.3	22.3	22.3
1.0	6.0	7.6	0.681	0.651	41.2	28.9	28.9
1.5	3.8	6.8	0.655	0.740	64.2	27.8	27.8
2.0	2.0	6.0	0.570	0.696	83.6	39.9	39.9
2.5	1.2	5.5	0.840	0.744	85.5	41.2	41.2
3.25	0.8	---	1.333	---	84.6	---	---
4.0	---	3.7	---	1.036	---	44.8	44.8
4.5	Dry	3.4	---	1.055	---	48.4	48.4
6.5	---	2.2	---	1.221	---	61.3	61.3
>8.0	---	Dry	---	---	---	---	---



It can be seen that the sublimation of formaldehyde was greater in the shell samples (85%) than in the plug samples (61%). Furthermore, during freeze-drying the formaldehyde tended to concentrate much sooner in the plug samples than in the shell samples.

Particles of white powder were observed in the bottles taken to dryness. It was determined that this material, which did not assay for free formaldehyde, was paraformaldehyde. Since methanol is used to stabilize commercial formaldehyde solution, it was felt that the formation of paraformaldehyde during freeze-drying might be prevented by employing a higher concentration of methanol. The results obtained using increasing concentrations of methanol in 5% formalin-water solution are shown in Table XX.

TABLE XX  
Effect of Methanol Concentration on the Sublimation of  
Formaldehyde in Freeze-Dried Plug and Shell-Frozen  
Samples of 5% Formalin-Water Solutions

Methanol %	Formaldehyde Sublimed %		Paraformaldehyde mg	
	Plug	Shell	Plug	Shell
0.0	69.2	83.2	48.5	20.8
1.0	79.2	85.0	33.3	20.3
2.5	82.8	88.0	27.3	19.6
5.0	81.6	90.5	29.3	15.1
10.0	83.1	96.4	26.4	5.6
15.0	83.2	98.1	26.4	3.0

With the freeze-dried plug samples the addition of methanol in the 2.5% to 15% range did not appear to have any effect, either on the sublimation of formaldehyde or on the formation of paraformaldehyde. However, with the shell samples, the sublimation of formaldehyde increased with the increased concentration of methanol and the formation of the polymer decreased significantly at the 10% and 15% levels of methanol. By extrapolation it was determined that 1% methanol added to a 0.2% formalin-water solution would be optimum for complete sublimation of formaldehyde without polymerization in shell-frozen samples. The results obtained are shown in Table XXI.

TABLE XXI

Free Formaldehyde in Shell-Frozen, Freeze-Dried  
Formaldehyde-Methanol-Water Solution

<u>Sample</u>	<u>Time Dried Hrs</u>	<u>Volume ml</u>	<u>mg/ml</u>	<u>Free Formaldehyde mg/vial</u>	<u>% loss</u>
Initial	0	10.0	0.636	6.36	0
Shell- 1	0.50	7.4	0.378	2.80	56.0
- 2	0.75	6.0	0.348	2.09	67.1
- 3	1.00	5.6	0.296	1.66	73.9
- 4	1.25	4.7	0.274	1.29	79.4
- 5	1.50	4.6	0.215	0.99	84.4
- 6	1.75	3.4	0.244	0.83	87.0
- 7	2.00	2.5	0.263	0.66	89.6
- 8	2.25	2.1	0.192	0.40	93.7
- 9	2.50	1.8	0.133	0.24	96.2
-10	2.75	0.8	0.370	0.30	95.3
-11	3.00	0.6	0.370	0.22	96.5
-12	4.50	Dry	Non-Detectable		100.0

Although there was a concentration effect of formaldehyde in vials 10 and 11, it was only to half of the concentration of the starting material. Here, with additional methanol, 96.5% of the formaldehyde sublimed as compared to 84.6% without the extra alcohol.

It seems apparent that vaccines and other antigen solutions containing formaldehyde cannot be freeze-dried without first neutralizing with sodium bisulfite. An alternative method may be by the addition of methanol, if the formaldehyde-methanol does not denature the protein. Further studies are planned to investigate the use of methanol in formalized tissue culture media, with and without added protein such as serum albumin.

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>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMMRY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTR <sup>a</sup>	9. SPECIFIC DATA CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM
75 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	3A161102B710		00		172	
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C. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Immunological Mechanisms in Microbial Infections							
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010100 Microbiology 003400 Clinical Medicine							
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23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Infectious Diseases; (U) Immunity; (U) Antibodies;							
(U) Complement Fixation; (U) Radioimmunoassay; (U) Serodiagnosis							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>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.</p> <p>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.</p> <p>25 (U) 75 07-7606 Hepatitis antigen has been shown to depend on disulfide bonds for structural and antigenic integrity. Cholesterol has been found to activate the complement system in conjunction with another human serum constituent. Vitamin A inhibits antibody and complement mediated damage to model membranes. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1975- 30 June 76. Support in the amount of \$70,000 from FY 77 funds is programmed for the period 1 Jul- 30 Sep 76.</p>							

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 172 Immunological mechanisms in microbial infections

Investigators: LTC Carl Alving, MC, M.D.; John Barbaro, Ph.D.; Roberta Richards, Ph.D.; LTC Kenneth W. Hedlund, MC, M.D.; D.T.O. Wong, Ph.D.

Assistants : SP5 Katharine Urban, M.S., B.A.; SP5 Richard O'Guin, B.S.; SP6 Barry Ellis, B.S.; SP5 Adel A. Guirguis, M.S.

1. Hepatitis

A. The role of disulfide bonds in maintaining the structural integrity and serologic recognition of Hepatitis B core antigen (HBcAg).

Objectives. While there is an ever increasing body of evidence attesting to the clinical significance of antibodies to HBcAg little is known about the biochemistry of HBcAg itself. The present work elucidates the role of disulfide bonds in maintaining the structural integrity of HBcAg.

Description. Disrupted cell nuclei of hepatocytes obtained from the livers of two immunosuppressed and experimentally infected chimpanzees were used as a source of HBcAg.

The livers removed at necropsy were stored at  $-70^{\circ}\text{C}$ . When needed, aliquots were thawed and homogenized in a Waring blender and made up in a 20% hypotonic saline solution; this homogenate was clarified by low speed centrifugation and layered onto a continuous 10% to 45% cesium chloride gradient.

After centrifugation at 43,000 rpm for 16 hrs at  $4^{\circ}\text{C}$  in an SW 50.1 rotor, fractions were collected from the bottom of each gradient tube in approximately 0.2ml aliquots. The specific gravities of the resultant fractions were determined by using an optical refractometer. Each fraction was assayed for HBcAg by means of a commercial radio immunoassay (Austria II). HBcAg was assayed for by means of a solid phase micro-titer assay as previously described by Purcell. The HBcAg when injected into animals produced homologous antibodies which did not cross react with normal chimp or human liver or serum proteins. HBcAg obtained by this isopycnic gradient centrifugation banded in fractions with specific gravities from 1.26 grams/ml. to 1.34 grams/ml. Two to three such  $\text{CsCl}_2$  gradient runs were usually required to obtain purified HBcAg. The richest peaks (1.30 grams/ml) were chosen for the following experiments. Amino acid analysis of acid hydrolysed purified HBcAg revealed the presence of cysteic acid and therefore offered presumptive evidence that cysteine was in the original HBcAg. This finding suggested the first set of experiments.

Aliquots of purified core containing 0.1mg/ml of protein by Lowry assay were equilibrated with 0.55M tris (Hydroxymethylaminomethane) buffer, pH 8.0 and run through a variety of reduction and alkylation treatments. Table 1 displays the data obtained.

Table 1. Effect of reduction and alkylation upon HBcAg.

	HBcAg Treated With	Radioimmuno- assay CPM
1	No Treatment	6415 $\pm$ 150
2	0.1M Dithiothreitol (DTT) alone	6373 $\pm$ 119
3	0.2M Dimethylcysteine (Penicillamine) alone	6424 $\pm$ 110
4	0.2M 2-mercaptoethanol (2-ME) alone	6291 $\pm$ 121
5	Iodoacetamide (IA) alone	6350 $\pm$ 29
6	0.1M DTT + IA	342 $\pm$ 35
7	0.2M penicillamine + IA	340 $\pm$ 24
8	0.2M 2ME + IA	300 $\pm$ 40
9	Control	323 $\pm$ 35

To evaluate the dynamics of what is going on the next experiments were done. Identical aliquots of purified HBcAg were equilibrated with 0.55 m Tris buffer and subjected to reduction with 0.1M DTT for time periods of 2 hours, 1 hour, 30 minutes and 15 minutes at 25°C, at the end of which time approximately 6 microcuries of <sup>14</sup>carbon labelled iodoacetamide was added to reaction mixtures and employed as a primary alkylating agent for 1 hour at 40°C in the dark.

At the end of one hour a large excess of cold unlabelled IA was added. One hour later the individual aliquots were layered upon pre-formed CsCl<sub>2</sub> gradients made as previously described and centrifuged for 16 hours at 43,000 rpms at 4°C. At the completion of this isopycnic ultracentrifugation the individually treated aliquots were fractionated as previously described. Untreated HBcAg incubated with <sup>14</sup>C labelled IA and <sup>14</sup>carbon labelled IA alone served as the baseline controls for the experimentally treated HBcAg aliquots.

When these final reduction and <sup>14</sup>C alkylation products are resubjected to isopycnic ultracentrifugation using identical CsCl<sub>2</sub> gradients there is no <sup>14</sup>C found incorporated in the gradient fractions where the original HBcAg was found (1.34 to 1.26 grams/ml). However two new distinct <sup>14</sup>C peaks are found at densities of 1.15 and 1.11 grams/ml.

Neither of these less dense alkylated components are antigenically recognizable as HBcAg. Longer periods of reduction (1 to 2 hours) prior to alkylation result in an obliteration of these lower density peaks.

Progress and Discussion. Reduction (simply cleaving disulfide bonds) alone, and alkylation alone have no effect upon the serologic recognition as measured by antibody binding. When  $^{14}\text{C}$  labelled iodoacetamide is used as an alkylating marker breakdown products of lower densities can be demonstrated which do not serologically cross react with anti HBc. Thus it appears that the presentation of antigenic markers for HBcAg, and indeed the structural integrity of HBcAg depends upon intact disulfide bonds.

#### B. Constituent Polypeptides of Hepatitis B Core Antigen.

Objectives. The purpose of these experiments is to describe to polypeptide components of HBcAg as a prelude to defining their antigenic properties.

Progress. Hepatitis B core antigen (HBcAg) was purified from the livers of experimentally infected and immunosuppressed chimpanzees by a combination of isopycnic banding and rate sedimentation on cesium chloride gradients. The purified HBcAg when injected into animals produced homologous antibodies but no antibodies against normal liver components as measured by radioimmunoassay. Previous work by the authors has shown that the structural integrity of HBcAg depends upon intact disulfide bonds.

When purified HBcAg is disrupted with 2ME and SDS and analysed by sodium dodecyl sulfate - 11% polyacrylamide gel electrophoresis four constituent polypeptides are demonstrated. The molecular weights for these polypeptides obtained by their relative migration against known standards were 68,000, 58,000, 56,000 and 32,000.

Discussion. This represents the first report on the constituent polypeptides of HBcAg and will serve as a starting point in an investigation of their antigenic properties.

### II Lipid Immunochemistry

#### A. Liposome Spin Immunoassay: A New Sensitive Method For Detecting Lipid Substances in Aqueous Media

A new sensitive immunoassay procedure is described for 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



pmol of aqueous Forssman hapten was detected, and the sensitivity could be increased further.

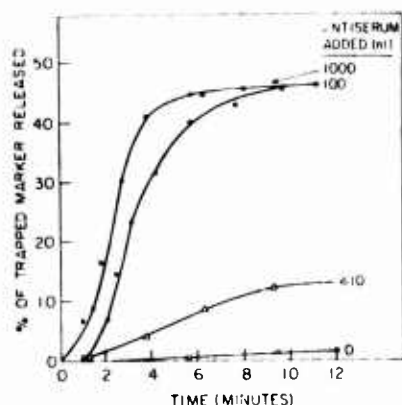


Fig. 1. Antibody requirement and kinetics of spin label release. The data were corrected for baseline spin label observed in the absence of antibody or complement.

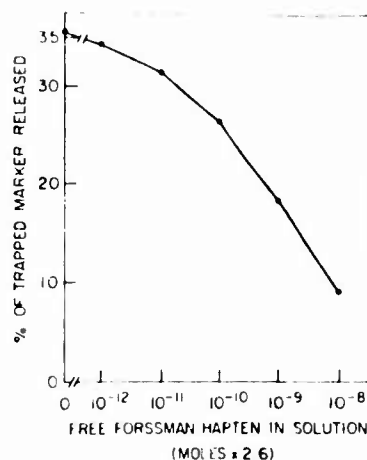
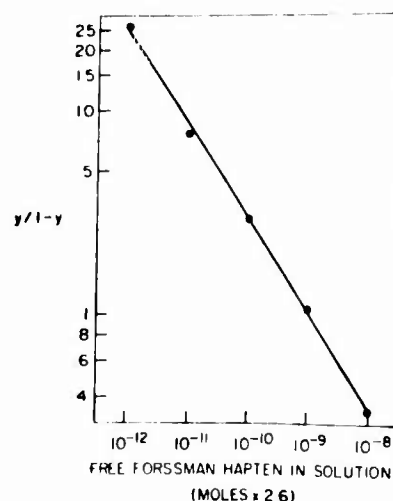


Fig. 2. Inhibition by fluid phase hapten of spin label release. The data were corrected for baseline spin label observed in the absence of antibody or complement.

Fig. 3. Alternate method of illustrating the fluid phase hapten inhibition.  $y$  = Fraction of spin label release (see Results).



#### B. Cholesterol-Dependent Nonimmune Human Complement Activation Resulting in Damage to Liposomes.

Liposomes containing varying concentrations of cholesterol (CHOL) were prepared in order to investigate the role of CHOL in complement (C) -mediated immune damage. The liposomes were comprised of dipalmitoyllecithin, CHOL, dicetyl phosphate, and either contained or lacked galactocerebroside as antigen. At high concentrations of liposomal CHOL each of the preparations (either containing or lacking galactocerebroside) released trapped glucose spontaneously in C in the absence of antiserum.

This did not occur at low concentrations of CHOL. This effect was observed in the presence of human C from 8 of 12 individuals studied, but was not found with guinea pig C. It was eliminated after inactivation of C by a) preheating the serum at 56° for 30 min, b) addition of Mg<sub>2</sub>EDTA, or c) preabsorbing the serum with immune complexes (egg albumin-anti egg albumin). Furthermore, preabsorbing the serum with high CHOL liposomes caused a drop in the hemolytic C titer, whereas low CHOL liposomes did not affect the hemolytic titer. Heated serum from a "reactive" individual caused the appearance of about 50% of reactivity in fresh serum either from a "nonreactive" individual or from normal, but not C4 deficient, guinea pig serum. The above evidence suggested that nonimmune glucose release may have been due to classical pathway activation and was caused by a heat-labile serum factor. Incorporation of either galactocerebroside or ceramide into the liposomal membrane caused a marked enhancement of the CHOL-dependent C damage. This was not due to naturally-occurring anti-galactocerebroside antibodies, however, because at low concentrations of CHOL, C-mediated damage was observed only in the presence of rabbit anti-galactocerebroside serum.

We conclude that human C in sera of certain individuals is activated nonspecifically by liposomes containing high concentrations of CHOL. This is a non-immune phenomenon, and occurs in the absence of antigen or antiserum. These results suggest that, above a critical threshold concentration of membrane CHOL, serum C activation and resultant membrane damage may occur spontaneously.

#### C. Vitamin A In Liposomes Inhibition of Complement Binding and Alteration of Membrane Structure

Incorporation of vitamin A aldehyde (retinal) into liposomes had an inhibitory effect on the amount of human complement protein bound in the presence of specific antiserum. The total membrane-bound protein was directly measured on liposomes which were washed after incubation in antiserum and fresh human serum (complement). At every concentration of complement, decreased protein binding was found with liposomes which contained retinal. Binding of the third component of complement (C3) was also measured directly on washed liposomes and was found to be decreased in the presence of retinal.

The diminution in protein binding due to retinal was not caused by differences in the amount of antibody bound and this was shown by two experiments. First, specific antibody protein binding to liposomes was directly measured and was essentially unaffected by retinal. Second, liposomes were prepared from lipid extracts of sheep erythrocytes. These liposomes were used as an immunoabsorbants to remove antisheep erythrocyte antibodies. The immunoabsorbant capacity was the same in both the presence and the absence of retinal. A further conclusion from these experiments was that retinal did not change the number of liposomal glycolipid antigen molecules available for antibody binding and thus presumably did not change the total number of lipid molecules present on

the outer surface of the liposomes.

Retinal did have an effect on the geometric structure of the liposomes. Size distribution measurements were performed in the diameter range of 1-6.35  $\mu\text{m}$  by using an electronic particle size analyzer (Coulter Counter). Liposomes containing retinal were shifted toward smaller sizes and had less total surface area and volume. It was suggested that retinal-containing liposomes may have had a tighter packing of the molecules in the phospholipid bilayer. This effect of retinal on liposomal structure may have been responsible for the observed decreased binding of C3 and total complement protein.

TABLE II

EFFECT OF RETINAL ON C3 BINDING

Liposome composition	Specific C3 binding to liposomes	
	- Retinal	+ Retinal
Sheep Fraction IIa	61.4	44
Dipalmitoyllecithin, cholesterol, dicetyl phosphate and galactocerebroside	61.4	37.5

TABLE III

EFFECT OF RETINAL ON ANTIBODY BINDING

Liposome composition	Specific protein binding to liposomes		Specific antibody adsorption by liposomes ( $\times 10^{12}$ )	
	- Retinal	+ Retinal	- Retinal	+ Retinal
Sheep Fraction IIa	38.1	41.0	8.66	8.66
Dipalmitoyllecithin, cholesterol, dicetyl phosphate and galactocerebroside	43.7	39.6	—	—

### III Protein Immunochemistry

#### A. Preparation of Immunoabsorbent Columns:

Objective. This investigation was undertaken to determine the optimal condition for the preparation of adequate immunoabsorbent columns.

Description. The two most important variables in the preparation of immunoabsorbent columns are the number of cyanogen bromide activated sites and the concentration of protein to be coupled to the gel matrix. Varying amounts of cyanogen bromide was used to activate known amounts of sepharose 4B under identical conditions. These activated gels were then reacted with increasing amounts of protein solutions. The reaction between activated gels and protein was monitored throughout the procedure. The efficiency of the immunoabsorbent column was determined by the amount of recoverable eluted protein.

Progress. The results demonstrate that the coupling of protein can be controlled by limiting the amount of cyanogen bromide used for activation of the sepharose. As can be seen in Table IV as the concentration of cyanogen bromide is increased a greater number of binding sites are generated with a proportionally larger capacity for binding protein. In addition the protein concentration, although not as pronounced, determines the amount of protein coupled to the activated gel. The amount of protein bound increases with increased amounts of protein although, the percentage of protein coupled showed some decrease.

The efficiency of the immunoabsorbent columns was determined by the ratio of the amount of protein eluted with glycine to the amount of protein bound to the activated gel. Table V clearly demonstrates that there is an optimum which yields the maximum recoverable protein per unit amount of coupled protein.

Discussion. The information obtained from this study will enable the preparation of immunoabsorbent columns with minimum risk of loss of valuable material. The technique offers the capabilities for separating material on the basis of their specific biological interaction. The procedure will be used in all studies particularly, trypanosomiasis and malaria where separation dependent upon physical or chemical means are difficult or impossible to achieve.

TABLE IV

Percentage of Protein Bound as a Function of Cyanogen Bromide and Protein Concentration

		MgCN-BR /ml of gel	MgP added /ml activated gel	MgP coupled /ml gel	Protein Coupled %
A	1	40	15.55	14.33	91.5
	2		7.78	7.10	91.3
	3		3.89	3.48	89.4
	4		1.94	1.84	94.7
B	1	20	15.55	9.62	61.9
	2		7.78	5.13	66.0
	3		3.89	2.68	68.9
	4		1.94	1.46	75.1
C	1	10	15.55	3.71	23.9
	2		7.78	2.13	27.4
	3		3.89	1.43	36.9
	4		1.94	0.72	36.8
D	1	1	15.55	0.22	1.4
	2		7.78	0.40	5.2
	3		3.89	0.36	9.3
	4		1.94	0.76	38.9

TABLE V

Relationship Between Percent Bound Protein to the Ratio of Protein Eluted to Protein on Gel

	Conc. of CN-BR for Activation	% Protein Bound	Mg Protein Eluted	Mg Protein ml gel	Ratio Mg P. Eluted mg P. bound
D3	1	9.3	0.09	0.65	0.143
C2	10	27.4	1.72	3.87	0.444
C3	10	36.9	1.45	2.59	0.561
C3	10	36.9	1.41	2.59	0.545
B3	20	68.9	3.14	4.82	0.650
B4	20	75.1	1.83	2.92	0.625
A3	40	89.4	1.73	5.57	0.310
A4	40	94.7	0.93	3.31	0.280

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunity

Work Unit 172 Immunological mechanisms in microbial infections

Publications:

1. Allen, R, Hedlund, K.,: A simple procedure for the removal of non-specific inhibitors of rubella virus hemagglutination, J. Clin. Microbiol. 2, 524-527, 1975.
2. Hedlund, K, Irvin, G, and Allen, R.: Reduction and alkylation of Hepatitis B core antigen, Fed. Proc. 35, 1114, 1976.
3. Wei, R, Alving, C.R., Richards, R.L., and Copeland, E.S.: Liposome spin immunoassay: a new sensitive method for detecting lipid substances in aqueous media, J. Immunol. methods 9, 165-170, 1975.
4. Alving, C.R., Conrad, D.H., Gockerman, J.P., Gibbs, M.B., and Wirtz, G.H.: Vitamin A in liposomes; Inhibition of complement binding and alteration of membrane structure, Biochim. Biophys. Acta. 394, 157-165, 1975.
5. Alving, C.R. and Guirguis, A.A.: Cholesterol-dependent nonimmune human complement activation resulting in damage to liposomes, J. Immunol., 116, 1727, 1976.

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3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8A. ORIGIN INSTR <sup>a</sup>	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
75 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	
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B. CONTRIBUTING							
C. <del>XXXXXXXX</del>	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Vaccine Development in Trypanosomiasis							
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: JOY, Robert J. T., COL				NAME: Wykoff, Dale E., COL			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
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				NAME: Muriithi, I. Dr.			
				NAME: Wellde, Bruce T.			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Kenya; (U) Trypanosomiasis; (U) Vaccine; (U) Africa; (U) Cattle; (U) Immunity							
23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objective of this program is to develop an effective, practical vaccine against African trypanosomiasis, useful to both military and civilian agencies. Related benefits include acquisition of knowledge pertaining to trypanosome immunity, host response and pathology of infection. There is a requirement for these well-conceived studies which should provide a basis for rational development of a vaccine for this disease which would constitute a serious hazard for military personnel operating in the endemic area.</p> <p>24. (U) Experiments conducted at WRAIR and in Kenya have demonstrated that experimental animals can be successfully immunized with irradiated trypanosomes. Rodents, cattle, and monkeys can be rendered completely resistant to a challenging infection of T. rhodesiense. Partial immunity has been achieved against T. congolense.</p> <p>25. (U) 75 07 - 76 06 Continuing studies in Lambwe Valley, Western Kenya, suggest that 15 of 21 human and 5 of 25 bovine trypanosome isolates over a six year period were neutralized by a single antibody indicating that a predominant strain of T. rhodesiense is active in this area. This research is complementary to DAOB 7521, Work Unit 096, entitled Study of African Trypanosomiasis. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$24,000 from FY 7T funds is programmed for the period 1 Jul - 30 Sep 76.</p>							

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

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3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 173 Vaccine development in trypanosomiasis

Investigators:

Principal: Dale E. Wykoff, Ph.D., COL, MSC

Associate: Robert M. Kovatch, LTC, VC; Bruce T. Wellde, DAC

ANTIGENIC RELATIONSHIPS BETWEEN ORGANISMS OF THE TRYPANOSOMA BRUCEI  
SUBGROUP IN THE LAMBWE VALLEY, SOUTH NYANZA, KENYA

PROBLEM AND BACKGROUND: To determine the extent of antigenic variability in trypanosomes of the T. brucei group collected from man and animals in the 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. 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 indistinguishable, their relationship to each other remains questionable. In nearby 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).

PROGRESS: Isolates of the T. rhodesiense parasites were collected from patients at the Homa Bay Hospital on Lake Victoria, western Kenya, by members of the Kenya Medical Department. Blood was injected IP into rats which were then transported to us for study. Two strains of T. rhodesiense from Gambella, Ethiopia, were collected by the US Navy Medical Research Unit, 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 in August 1972. During the past year 8 new isolates from humans have been collected bringing the total to 24 (Table 1). Three other strains of T. rhodesiense were also tested, two from southern Ethiopia and a laboratory strain (Wellcome) which has been isolated in Tanzania and maintained in rodents for many years. These findings are shown in Table 2.

It is noteworthy that 15 of the 21 isolates tested from man were neutralized by the antiserum against LVH-1, indicating a persistence of these similar types since 1970.

We also tested a second antiserum prepared against LVH-1. Its reaction was identical to that of the original antiserum. An antiserum to LVH-9 was also prepared and tested against the various isolates. This antiserum neutralized the homologous trypanosomes (LVH-9) but while antiserum against LVH-1 had no effect on LVH-9, antiserum to LVH-9 neutralized LVH-1. Antiserum (LVH-9) was similar in the pattern of neutralization to anti LVH-1, however, some differences were evident. Isolates LVH-9 and LVH-16 were also neutralized as opposed to the other two antisera. Isolate LVH-13 was not neutralized by this antiserum whereas it was neutralized by both anti LVH-1 antisera.

When isolates of T. brucei from cattle were tested (Table 3) the antiserum showed a strong effect on 5 of 25 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 variant specific. The percentage of cattle parasites which react with the antibody is surprising and indicates that cattle may be a more important aspect in the cycle of the disease.

While it has been shown by Onyango that parasites from cattle can cause infections in man identical to that of T. rhodesiense, we hope to better define the extent of this zoonosis.

TABLE I  
EFFECTS OF ANTISERUM TO TRYPANOSOMA RHODESIENSE ON 24 ISOLATES OF T.  
RHODESIENSE FROM MAN  
(+ INDICATES NEUTRALIZATION)

STRAIN	DATE ISOLATED	DONOR		ANTISERUM		
		AGE	SEX	ANTI LVH-1a	ANTI LVH-1b	ANTI LVH-9
LVH-1	16 Aug 72	52	M	+	+	+
LVH-2	17 Dec 72	37	F	+	+	+
LVH-3	73	?	?	+	-	-
LVH-4	20 May 74	10	F	+	+	+
LVH-5	18 Jul 74	55	M	+	+	+
LVH-6	3 Aug 74	52	F	+	+	+
LVH-7	3 Aug 74	22	F	+	+	+
LVH-8	8 Aug 74	25	M	+	ND	ND
LVH-9	28 Aug 74	41	M	-	-	+
LVH-10	2 Oct 74	50	M	+	+	+
LVH-11	28 Oct 74	28	F	-	-	-
LVH-12	24 Dec 74	50	M	+	+	+
LVH-13	22 Jan 75	30	F	+	+	-
LVH-14	23 Apr 75	42	M	-	-	-
LVH-15	26 Jun 75	60	M	+	+	+
LVH-16	1 Jul 75	42	M	-	-	+
LVH-17	8 Aug 75	38	F	-	-	-
LVH-18	3 Sep 75	58	M	-	-	-
LVH-19	4 Sep 75	21	F	+	+	+
LVH-20	10 Sep 75	25	F	+	+	+
LVH-21	20 Mar 76	30	F	ND	ND	ND
LVH-22	29 Sep 70	?	?	+	+	+
LVH-23	28 Sep 70	?	?	ND	ND	ND
LVH-24	27 Sep 70	?	?	ND	ND	ND

TABLE II

EFFECTS OF ANTISERUM TO TRYPANOSOME RHODESIENSE ON TRYPANOSOMES ISOLATED  
FROM MAN IN AREAS OUTSIDE KENYA

STRAIN	DATE ISOLATED	REGION	ANTISERUM (LVH-1)
ETH-1	30 Oct 73	So. Ethiopia	NEG
ETH-2	26 Nov 74	So. Ethiopia	NEG
WELLCOME	Over 20 yrs ago	Tanzania	NEG

TABLE III

EFFECTS OF ANTISERUM ON TRYPANOSOMA BRUCEI GROUP TRYPANOSOMES ISOLATED  
FROM CATTLE IN LAMBWE VALLEY

(+ INDICATES NEUTRALIZATION)

ISOLATE	ANTI-LVH-1	ANTI LVH-9	ANTI LVB-10B
LVB-15A	+		
LVB-16A	+		
LVB-18A	-		
LVB-2B	<u>+</u>		
LVB-3B	-		
LVB-4B	-		
LVB-10B	-		
LVB-24B	-		
LVB-36B	-		
LVB-5C	-		-
LVB-12C	+		
LVB-36C	-		
LVB-42C	<u>+</u>		
LVB-43C	-		
LVB-52C	-	-	
LVB-55C	-		
LVB-56C	-	-	
LVB-59C	-	-	
LVB-60C	-	-	
LVB-62C	+	+	-
LVB-65C	+		
LVB-66C	-		
LVB-72C	-		
LVB-77C	-		
LVB-78C	-		

## STUDIES ON TRYPANOSOMA RHODESIENSE IN CATTLE

**PROBLEM AND BACKGROUND.** During our initial immunological studies of the irradiated vaccine and antiserum production in cattle, we noted that some animals underwent a severe form of disease. In general, T. rhodesiense has been reported as being non-pathogenic for cattle. We have therefore carried out additional experiments to confirm our original findings and to compare the disease process in bovines with that which occurs in man.

**PROGRESS.** Five animals in our early study developed disease characterized by weight loss, fever, pleocytosis and CNS disorders. Uncoordinated movements, circling and opisthotonus were observed (Table 4 ). Fever and leucopenia were common during the onset of patent parasitemia and were followed by a leucocytosis. Terminal WBC levels were somewhat reduced (Table 5 ). Generally, there was an increase in packed cell volumes early in the disease although mild to moderate anemia developed as the infection progressed. High packed cell volumes terminally may have resulted from dehydration (Table 6 ). Total serum protein levels increased throughout the infection in 4 of the 5 animals. Total protein levels in animal 243 increased markedly for the first seven months but then subsided. The increased levels of protein were accounted for by increased Gamma globulin levels. Albumin levels dropped during the course of infection. No significant changes were noted in the Alpha or Beta globulin fractions (Table 7 ). A preliminary analysis of immunoglobulin levels indicated rises in both immunoglobulin G and M (Table 8 ). Cerebrospinal fluid from infected animals had increased levels of leucocytes made up primarily of lymphocytes. Total protein levels of cerebrospinal fluid were also increased (Table 9 ).

Gross alterations in the brain included thickened dull grey meninges while light microscopic examination revealed a moderate to severe meningoencephalitis with subcortical white matter, basal ganglia and mid-brain more severely altered than the cortical grey matter. A summary of the salient histological features is presented in Table 10 . Trypanosomes were isolated from the cerebrospinal fluids while these specimens were consistently negative for bacterial growth. These infections in cattle are of long-term duration as compared with those in man. However, the histologic lesions found in cattle are similar to those found in man in chronic trypanosomiasis. These lesions in cattle have not been previously described. In a more detailed study involving 10 infected and four control animals we have confirmed our earlier findings and will be able to offer a more descriptive report on the disease process in cattle at the completion of the present work.

Since the infection in cattle appears very similar to that found in man it may offer a model of the central nervous system pathology which has not been available heretofore. The implications of this disease

to the veterinarian in the field have not been clarified. Whether or not the T. rhodesiense trypanosomes are pathogenic in nature has not been shown.

Detection of parasites in T. rhodesiense infected cattle is difficult after the fourth month of infection if one relies on subinoculation of blood into rats. Small quantities of lymph node aspirate (usually less than 0.1cc expanded in 1.0cc of 10% fetal calf serum) injected into rats appears to be a more effective method of isolating the parasite late in the course of infection. Examination of Giemsa-stained lymph node smears is also less efficient than lymph node aspirate subinoculation. The results are summarized in Table // . The lymph node aspirate technique may be a useful adjunct in the detection of infections in field cases and the detection of infection in animals to be used in future immunization studies.



TABLE IV  
PATHOGENIC INFECTIONS OF TRYPANOSOMA RHODESIENSE IN CATTLE

ANIMAL NUMBER	INFECTING STRAIN	CLINICAL SIGNS	DURATION OF DISEASE (DAYS)	DETECTABLE PARASITES*	
				BLOOD LYMPH NODE	CSF
268	WELLCOME	1, 2, 5, 6 **	179	NEG	ND
6882	LVH-1	1, 6	227	POS	NEG***
243	LVH-1	1, 3, 4, 6	582	NEG	ND
7304	LVH-2	1, 4, 6	714	NEG	NEG
7307	LVH-9	1, 4, 6	279	POS	POS

\* AT NECROPSY

\*\* 1. UNCOORDINATED MOVEMENTS  
2. CIRCLING  
3. TREMOR

\*\*\* SPINAL CORD POSITIVE

4. HYPERSENSITIVITY  
5. OPISTHOTONOS  
6. WEIGHT LOSS

TABLE V  
LEUCOCYTES IN TRYPANOSOMA RHODESIENSE INFECTED CATTLE (X1000)

ANIMAL NUMBER	PRE INFECTION	SECOND WEEK	HIGHEST VALUE (AND DAY)	TERMINAL VALUE (AND DAY)
268	14.6	12.6	18.6 (36)	10.8 (179)
6882	11.2	9.1	18.8 (26)	9.4 (227)
243	9.6	7.7	34.3 (51)	18.7 (551)
7304	12.6	7.9	20.8 (558)	20.7 (714)
7307	15.4	23.4	25.8 (14)	15.6 (279)

6.

PACKED CELL VOLUMES IN TRYPANOSOMA RHODESIENSE INFECTED CATTLE (%)

ANIMAL NUMBER	PRE INFECTION	HIGHEST VALUE (AND DAY)	LOWEST VALUE (AND DAY)	TERMINAL VALUE (AND DAY)
268	31	36 (11)	27 (78)	40 (179)
6882	29	30 (37)	20 (227)	20 (227)
243	28	32 (513)	24 (204)	26 (551)
7304	28	33 (6)	25 (528)	33 (714)
7307	33	35 (10)	19 (279)	19 (279)

TABLE VII  
SERUM PROTEINS (G/100 ml) IN TRYPANOSOMA RHODESIENSE-INFECTED CATTLE

ANIMAL NUMBER	TOTAL PROTEIN		ALBUMIN		ALPHA		BETA		GAMMA	
	PRE-INFECT	TERMINAL	PRE-INFECT	TERMINAL	PRE-INFECT	TERMINAL	PRE-INFECT	TERMINAL	PRE-INFECT	TERMINAL
268	6.9	7.9	2.8	2.2	1.3	1.4	0.9	1.0	1.8	3.2
6882	5.9	8.3	2.2	1.3	1.0	1.1	0.8	0.9	1.8	5.0
243	7.0	7.0	2.1	2.1	0.9	1.1	0.9	0.8	2.4	3.1
7301	6.3	8.4	2.8	2.5	1.1	0.9	0.8	0.8	2.0	4.1
7307	7.2	8.1	2.8	2.1	1.3	1.0	0.8	0.9	2.2	4.1

TABLE VIII  
IMMUNOGLOBULINS (MG%) IN TRYPANOSOMA RHODESIENSE-INFECTED CATTLE

ANIMAL NUMBER	IGG		IGM		IGA	
	PRE-INFECTION	TERMINAL	PRE-INFECTION	TERMINAL	PRE-INFECTION	TERMINAL
268	1000	1500	225	3200	120	90
6882	4200	6000	450	850	90	50
243	3750	4200	850	450	90	68
7304	3200	6100	1300	1300	27	27
7307	1900	3750	600	1650	300	90

TABLE IX

LEUCOCYTES AND PROTEIN IN CEREBROSPINAL FLUID OF TRYFANOSOMA RHODESIENSE-INFECTED CATTLE

ANIMAL NUMBER	MID INFECTION		AT NECROPSY	
	WBC/mm <sup>3</sup>	TOTAL PROTEIN Mg%	WBC/mm <sup>3</sup>	TOTAL PROTEIN Mg%
6882	Not Done	Not Done	330	70
7304	542	150	512	242
7307	232	70	365	75
CONTROL	Not Done	Not Done	25	40

TABLE X

SUMMARY OF THE HISTOLOGICAL CHANGES IN THE CNS OF CATTLE INFECTED WITH TRYPANOSOMA RHODESIENSE

ANIMAL	CERVICAL SPINAL CD.	CERE- BELLUM	MID- BRAIN	CORTEX- OCCIPITAL LB.	BASAL GANGLIA	HYPO- CAMPUS	MID- CORTEX	CORTEX- OLFACTORY	PITUIT- ARY	SEE COMMENT NO.
243	1,3, 4,7	1,3-8	1,3-5 7,9	1-9,11	1-11	N/A	1-9 11,12	1-5 7,8	3,4 8,10	A
268	N/A	1,3-5 7,8	1,3 5,7	N/A	1-8,10 12	3,7	1-3,5 7,8	N/A	3,4 8,13	B
6882	3	1,3 5,7	3,7	1,3	3	3	N/A	N/A	3,8 13	C
7304	1,3 4	1,3-5 8,10	1,3-5 7	1-10	1-3,5-8 10-12	3,4 7	1-5, 7-9,11	1-9	3,4, 8,13	D
7307	1,3	1,3,5 7,8	1,2,6 7,11	1,3-5, 8	1,3	3,4 8	1-5,8, 9,11	1,3-5 8	3,13	E

NA: Not available or tissue sections not identified

1: Meningeal infiltrations

2: Subpial gliosis

3: Perivascular infiltrates

4: Mott's cells

5: Vasculitis

6: Periaxonal edema

7: Gliosis, focal

8: Gliosis, diffuse

9: Gemistocytic astrocytes

10: Malacia or Gitter cells

11: Vacuolation and/or cyst formation

12: Subependymal gliosis and/or malacia

13: Colloid cysts adenohipophysis

#### COMMENTS ON TABLE

A. Case 243. Lesions were most severe in the subcortical white matter but extensive lesions were present in the grey matter as well. Marked cystic cavitations primarily surrounding blood vessels near the external capsule of the basal ganglia, extensive astrocytosis of the white matter and exaggerated sulci indicates marked cerebral atrophy. The inflammatory reaction was mainly plasmacytic and Mott's cells were numerous. This animal had the most severe changes of the cases reviewed.

B. Case 268. Lesions in the brain sections examined were most severe in the white matter of midbrain and basal ganglia. The inflammatory reaction was primarily lymphocytic. Mott's cells were infrequent. The stroma of the choroid plexus of the 4th ventricle was edematous and the perivascular areas were infiltrated with lymphocytes.

C. Case 6882. The least severe histological changes were found in this case. The inflammatory reaction was primarily lymphocytic.

D. Case 7304. The lesions were similar in kind but slightly less severe than in animal 243. The inflammatory reaction was mixed plasma-lymphocytic with a few neutrophils, and most severe in myelinated areas of the brain. There was extensive subependymal gliosis, malacia and vacuolation near the lateral ventricles.

E. Case 7307. Lesions in this animal were similar in kind to animal 243 but less severe. The inflammatory reaction was primarily lymphocytic and much more extensive in the subcortical white matter, basal ganglia and midbrain, than in the grey matter.

TABLE XI

RELATIVE EFFECTIVENESS OF DIRECT LYMPH NODE SMEAR EXAMINATION COMPARED  
TO IP INOCULATIONS USING BLOOD AND LYMPH NODE ASPIRATES FOR DETECTION  
OF CATTLE HARBORING TRYPANOSOMA RHODESIENSE

DURATION OF INFECTION IN MONTHS		TYPE OF INFECTION		
		BLOOD (5ml) IP TO RATS	LYMPH NODE ASPIRATE IP TO RATS/MICE	LYMPH NODE SMEARS
4	I*	8/10***	NOT DONE	5/10
	C**	0/4	NOT DONE	0/4
5	I	2/10	10/10	5/10
	C	0/4	NOT DONE	0/4
6	I	1/10	10/10	7/10
	C	0/4	0/4	0/4
7	I	1/10	9/10	4/10
	C	0/4	0/4	0/4
8	I	0/10	7/10	2/10
	C	0/4	0/4	0/4
9	I	0/10	5/10	3/10
	C	0/4	0/4	0/4
10	I	1/9	4/9	2/9
	C	0/3	0/3	0/3
11	I	0/9	6/9	4/9
	C	0/3	0/3	0/3
12	I	0/9	5/9	3/9
	C	0/3	0/3	0/3
13	I	0/9	5/9	4/9
	C	0/3	0/3	0/3
14	I	0/9	4/9	3/9
	C	0/3	0/3	0/3

\* INFECTED ANIMAL

\*\*\* 8 of 10 Rats Showed Patent Infection by  
Day 30.

\*\* CONTROL



## ANEMIA IN TRYPANOSOMA CONGOLENSE-INFECTED CATTLE

**PROBLEM AND BACKGROUND:** Anemia is a characteristic of many trypanosome infections, varying in intensity according to the species of trypanosome as well as host. The anemia and resulting anoxia has been described by some investigators as the major cause of death. Our previous studies showed that T. congolense produced a rapid and severe anemia in Hereford cattle, making them a good study model. We have undertaken a thorough study of this anemia by routine hematological and radioisotopic methods in an attempt to define its etiology. Early in the course of infection an increase occurs in both mean corpuscular hemoglobin and mean corpuscular volume, coinciding with a decrease in hematocrit values (Table 12). Mean corpuscular hemoglobin concentrations did not change and a limited reticulocyte response was observed during this period. Later, however, the erythrocyte indices returned to normal even though the anemia persisted. Thrombocytopenia has been found to be a prominent feature of T. congolense infections in cattle and appears to be associated with high levels of trypanosomes in the peripheral blood. Chronically infected animals with low levels of parasites have less severe thrombocytopenia and thrombocytes are usually found in normal or elevated numbers when parasites can not be observed. Chemotherapeutic cure of animals with severe thrombocytopenia results in a rapid elevation of thrombocyte levels to higher than normal values. Leucocyte levels follow a similar but less marked pattern. The apparent half life of Chromium 51 labelled erythrocytes in infected animals is approximately half that found in control animals (Table 13). Chromium is found at higher levels in urine samples of infected animals indicating that the labelled erythrocytes have been destroyed and that cationic trivalent chromium is excreted by the kidney. The amount of chromium in fecal specimens was essentially the same in infected and control animals indicating that loss of erythrocytes due to hemorrhage did not occur. Total blood volumes did not differ between control and infected animals, (Table 14)

In order to measure erythropoiesis, ferrokinetic studies were carried out on normal and infected cattle. Animals were studied at 8, 17, 28, and 61 weeks after infection. The results are shown in Tables 15 to 18. Results of plasma iron turnover rate (PITR) are not yet ready for weeks 8 and 61. Results from week 17 indicate that the PITR of infected animals was 53% greater than that of the controls, indicating increased erythropoiesis. However, at week 18 the PITR of infected animals was only 38% of that of the controls indicating the presence of a severe dyshemopoiesis. Animals treated 8 weeks previously exhibited increased marrow activity. The reappearance of Fe-59 incorporated into erythrocytes followed a similar pattern. Whereas 50% of the injected dose of iron reappeared in controls in an average of 6.9 days, it took only an average of 4.9 days to appear in animals relatively early during infection (15-19 weeks). Later, however (32 weeks) 8 days were required

before 50% of the injected iron reappeared in erythrocytes. At 8 weeks after infection there was a marked hemodilution associated with lowered PCV values with the blood volume of the infected animals being approximately 50% greater than that of the controls. Near normal blood volumes were found in treated animals and those which self-cured (Table 18), 61 weeks.

These ferrokinetic studies support and confirm our earlier description of a hemolytic anemia with increased erythrocyte production early in the disease. In the later stages a dyshemopoiesis occurs with decreased erythrocyte production. The cause of this dyshemopoiesis may be related to iron metabolism since at this time the mean serum iron concentration in infected animals was 27 mg%.

Preliminary results indicate that a coagulopathy develops during the infection (Table 19). Although prothrombin times were similar in both infected and control groups, partial thromboplastin times became elevated in infected animals. The plasma protamine paracoagulation test was also positive in infected animals but not in controls. Accompanied by a thrombocytopenia, these changes indicate diffuse intravascular coagulation. Attempts are being made to study fibrinogen turnover rates to further elucidate the significance and extent of the coagulation defect.

TABLE XII  
SUMMARY OF HEMATOLOGY IN TRYPANOSOMA CONGOLENSE INFECTED CATTLE  
(Experimental Values Minus Control Values)

DAYS	0	8	17	24	37	47	61	82	128	166	201	310
PCV %	-2	-2	-9	-12	-17	-14	-16	-16	-17	-16	-15	-4
MCV $\mu^3$	-4	0	-2	+3	+3	+9	+6	+10	-5	-1	-3	-5
MCH $\mu\text{g}$	-1.0	+0.5	0	+1.5	+2.3	+3.1	+2.6	+3.3	-1.4	-0.8	-0.9	-2.1
MCHC %	0	0	+2	+1	+1	0	+1	-1	+1	0	0	0
LEUCOCYTES $\times 10^3$	0	-4	-3	-3	-4	-2	-3	-3	+4	+1	-1.5	+4
THROMBOCYTES $\times 10^3$	-70	-438	-347	-188	-314	-399	-329	-362	-212	-239	-106	-231

TABLE XIII  
ERYTHROCYTE SURVIVAL VALUES IN TRYPANOSOMA CONGOLENSE-INFECTED CALVES

(Average Half Life in Hours)

	DAY OF EXPERIMENT							
	0-15	16-30	31-45	46-60	75-90	91-105	130-145	146-160
INFECTED (2)	272	194	127	143	149	145	220	242
CONTROL (2)	322	341	307	316	341	350	335	346

TABLE XIV

BLOOD PARAMETERS IN TRYPANOSOMA CONGOLENSE-INFECTED CALVES AS MEASURED BY THE  $\text{Cr}^{51}$  LABELLED  
ERYTHROCYTE TECHNIQUE

		(Average ml. per Pound Body Weight)				
		WEEKS AFTER INFECTION				
		0	5	10	18	29
BLOOD VOLUME	Infected (2)	23.9	23.6	24.0	25.0	24.6
	Control (2)	23.1	23.6	22.9	24.3	25.1
ERYTHROCYTE VOLUME	Infected	8.6	5.4	5.3	5.7	6.6
	Control	7.9	7.4	7.7	8.6	9.0
PLASMA VOLUME	Infected	15.3	19.5	18.9	19.4	18.0
	Control	15.2	16.2	15.2	15.7	16.1

TABLE XV

FERROKINETIC STUDIES ON CATTLE INFECTED WITH TRYPANOSOMA CONGOLENSEEIGHT WEEKS POST INFECTION

ANIMAL NUMBER	PLASMA VOLUME (ml./Kg)	PCV (%)	BLOOD VOLUME (ml/Kg)	PLASMA IRON DISAPPEAR. RATE (T <sub>1</sub> - minutes)
7486	51.8	20	64.8	111
244	58.4	15	68.7	56
7390	52.0	20	65.0	84
6733	53.0	18	73.6	79
6711	81.4	14	95.0	92
MEAN	59.3	17	73.3	84
INFECTED				
233	50.4	27	69.0	155
6034	38.0	30	54.3	90
6927	40.4	30	57.7	71
MEAN	42.9	29	60.4	106
CONTROLS				

TABLE XVI

FERROKINETIC STUDIES ON CATTLE INFECTED WITH TRYPANOSOMA CONGOLENSESEVENTEEN WEEKS POST INFECTION

ANIMAL NUMBER	PLASMA VOL (ml/Kg)	PCV (%)	BLOOD VOL (ml/Kg)	SERUM IRON (mg/100ml)	PLASMA IRON DISAPPEAR.RATE (T <sub>1/2</sub> - min.)	PITR (mg/da/100ml blood)
INFECTED	6968	51.9	22	94	65	781.7
	6304	48.2	17	110	80	790.9
	161	47.4	27	100	115	439.9
	6565	53.6	22	36	32	608.1
	MEAN	50.3	22	85	73	655.0
CONTROLS	6733	38.4	33	96	117	381.0
	244	26.5	43	214	178	474.9
	MEAN	32.4	38	155	147	427.9

TABLE XVII

FERROKINETIC STUDIES ON CATTLE INFECTED WITH TRYPANOSOMA CONGOLENSETWENTY EIGHT WEEKS POST INFECTION

ANIMAL NUMBER	PLASMA VOL (ml/Kg)	PCV (%)	BLOOD VOL (ml/Kg)	SERUM IRON (mg/100ml)	PLASMA IRON DISAPPEAR.RATE (T <sub>1/2</sub> - min.)	PITR (mg/da/100ml blood)
6968	69.5	19	85.7	22	75	164.7
7319	74.7	20	93.6	29	75	213.8
8317	61.0	27	83.0	29	86	171.8
MEAN	68.4	22	87.4	26	79	183.4
INFECTED & TREATED AT 20 WEEKS	45.1	30	63.9	114	98	571.2
6565	55.3	19	67.9	88	75	581.4
66304	50.2	24	66.0	101	86	576.3
MEAN	35.6	31	51.6	134	147	434.4
CONTROLS	46.9	28	65.2	150	142	523.8
MEAN	41.2	30	58.4	142	145	479.1



TABLE XVIII  
FERROKINETIC STUDIES ON CATTLE INFECTED WITH TRYPANOSOMA CONGOLENSE  
SIXTY-ONE WEEKS POST INFECTION

ANIMAL NUMBER	PLASMA VOLUME (ml/Kg)	PCV (%)	BLOOD VOLUME (ml/Kg)	PLASMA IRON DISAPPEAR. RATE ( $T\frac{1}{2}$ - minutes)
7319	45.4	27	68.7	120
6968	50.9	24	67.1	56
8317	47.6	28	66.1	78
MEAN	48.0	26	67.3	85
6304	50.5	21	64.0	109
6565	38.9	31	56.4	82
MEAN	44.7	26	60.2	95
233	50.4	27	69.0	155
6634	38.0	30	54.3	90
6927	40.4	30	57.7	71
MEAN	42.9	29	60.4	105

TABLE XIX  
SUMMARY OF COAGULATION PROCEDURES ON TRYPANOSOMA CONGOLENSE-INFECTED CATTLE  
(Experimental Values Minus Control Values)

TEST	DAY OF EXPERIMENT										
	-41	-35	-20	+5	+9	+17	+24	+31	+37	+44	+51
PROTHROMBIN (Seconds)	+0.7	+0.3	+1.1	+0.1	+0.4	+0.4	-0.3	-0.2	+1.1	-0.3	ND
									+0.3	+0.3	+0.3
PARTIAL THROMBOPLASTIN (Seconds)	+5.4	-4.8	+0.2	+4.2	-7.3	-2.7	+1.9	+2.9	+14.3	+15.3	+12.0
									+10.7	+13.5	
THROMBOCYTES ( $\times 10^3$ )	ND	-65	-105	-333	-490	-306	-273	-201	-264	-304	-366
									-366	-499	

## IMMUNITY IN BOVINE TRYPANOSOMIASIS

**PROBLEM AND BACKGROUND:** Our initial results with T. rhodesiense showed that bovines could be immunized with irradiated trypanosomes. Similar experiments with T. congolense did not yield as strong an immunity but prepatent periods of immunized animals were extended and antibody developed after immunization. Recently we have found that immunization with irradiated trypanosomes is specific for the antigenic type of the immunogen. A second line of investigation involves infection followed by chemotherapeutic cure. In theory this method might offer a broad protection against many antigenic types. Similarly, passive transfer of immune serum should give a broad degree of protection. The experiments involving irradiated trypanosomes have been hampered because the only gamma emitting radiation source available to us is broken. Repairs are to be made by the International Atomic Commission sometime in the future.

**NATURAL IMMUNITY:** An age resistance appears to be present in cattle challenged with T. congolense (Table 20). Animals which survived the challenging infection were under 1.5 years of age. Animals older than 1.5 years did not survive. A sex difference also appeared with females of from 1 to 3 years of age having considerably longer survival times than those of males in the same age group. Prepatent periods were dependent on the number of parasites injected (Table 21), however, the duration of the disease was not. A ten-fold dilution of the challenge inoculum delayed the onset of parasitemia by one day.

**ACQUIRED IMMUNITY:** Cattle which had undergone varying periods of infection with T. congolense were rechallenged at varying periods after treatment with the stock strain of parasite. Immunity to subsequent challenge could be produced by this method (Table 22). The resistance was of relatively long duration and appeared to depend on both the duration of previous infections and the interval between treatment and rechallenge. Immune serum collected from chronically infected bovines conferred appreciable protection to mice against the stock strain of T. congolense. Fifty percent of antiserum treated mice did not become patent. When the globulin fraction of this serum was concentrated and administered to mice 90% of the recipients did not become patent. Column fractionation of the immune globulins indicated that the protective activity was primarily associated with the 7S gamma globulin fraction. In a preliminary experiment we have also been able to protect 2 calves by the transfer of immune serum.

TABLE XX  
THE EFFECT OF AGE AND SEX ON THE OUTCOME OF DISEASE

AGE	LESS THAN 1 YR		1 TO 2 YEARS		2 TO 3 YEARS		3 TO 4 YEARS		4 TO 5 YEARS	
	M	F	M	F	M	F	M	F	M	F
SEX										
NO. ANIMALS	3	2	6	4	4	5	2	1	0	2
SURVIVAL TIME DAYS	93	91	41	157	65	129	42	36	-	48
SELF CURES	2	1	2	0	0	0	0	0	-	0
AVERAGE MALE + FEMALE SURVIVAL TIMES	92		103		101		40		48	
TOTAL SELF- CURES	3		2		0		0		0	

TABLE XXI  
EFFECT OF NUMBERS OF TRYPANOSOMA CONGOLENSE INOCULATED ON THE PREPATENT  
PERIOD

AVERAGE DOSE PER 500 LBS	NUMBER OF ANIMALS	AVERAGE PREPATENT PERIOD IN DAYS
$3 \times 10^8$	2	2
$1.7 \times 10^7$	2	3
$7.0 \times 10^6$	3	3.3
$1.5 \times 10^5$	3	5.0
$1.2 \times 10^4$	17	5.6
$6.3 \times 10^3$	5	6.4

TABLE XXII  
ACQUIRED IMMUNITY TO TRYPANOSOMA CONGOLENSE

ANIMAL NUMBER	PARASITES PER 500 LBS	PREPATENT PERIOD (Da)	FIRST INFECTION		DURATION OF INFECTION (Da)	PCV AT TREATMENT	INTERVAL SINCE TREATMENT (Da)
			TYPE	PARASITEMIA			
151	1.3 x 10 <sup>5</sup>	5	Persistent		196	17	-
161	1.3 x 10 <sup>4</sup>	5	"		36	17	-
6304	1.9 x 10 <sup>4</sup>	5	"		77	19	-
6476	1.0 x 10 <sup>3</sup>	6	"		42	14	-
6565	8.4 x 10 <sup>4</sup>	6	"		42	22	-
265	1 x 10 <sup>4</sup>	5	"		82	15	-
267	1 x 10 <sup>4</sup>	5	"		48	13	-
314	6.8 x 10 <sup>6</sup>	3	"		49	17	-
SECOND INFECTION							
151	1 x 10 <sup>4</sup>	14	Relapsing		77	35	501
161	1 x 10 <sup>4</sup>	6	Persistent		Self cure	-	900
6304	1 x 10 <sup>4</sup>	6	"		189	12	859
6476	1 x 10 <sup>4</sup>	6	"		82	12	449
6565	1 x 10 <sup>4</sup>	8	"		145	20	449
265	1 x 10 <sup>4</sup>	18	Relapsing		Self Cure	-	296
267	1 x 10 <sup>4</sup>	13	"		Self Cure	-	299
314	1 x 10 <sup>4</sup>	14	"		Self Cure	-	202
THIRD INFECTION							
151	1 x 10 <sup>4</sup>	-	None		None	-	858
6304	1 x 10 <sup>4</sup>	-	"		"	-	169
6476	1 x 10 <sup>4</sup>	-	"		"	-	296
6565	1 x 10 <sup>4</sup>	-	"		"	-	228

PROJECT 3A161102B71Q Communicable Diseases and Immunology

TASK 00 Communicable Diseases and Immunology

Work Unit 173 Vaccine Development in Trypanosomiasis

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 DD DR&E(AR)656	
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75 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		
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				PREVIOUS		B. FUNDS (in thousands)	
NA				76		2	
B. NUMBER				FISCAL		177	
C. TYPE				CURRENT		179	
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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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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
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				NAME: Williams, James E, MAJ, MSC			
				NAME: Harrison, Daniel N.			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Yersinia pestis; (U) Plague; (U) Vaccines; (U) Immunization; (U) Serological tests; (U) Genetics							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text 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) 75 07 - 76 06 Strains of Y. pestis recently isolated from natural foci of plague were examined for virulence determinants and antibiotic sensitivities. Strains examined (43) from South Vietnam, Java, and the USA produced Fraction 1 antigen, VW antigens, pesticins, pigmentation and they were sensitive to streptomycin and tetracyclines. Experiments on latent disease are in progress. Rhesus monkeys vaccinated with the Army Plague Vaccine, USP, and thereafter challenged with typical encapsulated Y. pestis or with capsule-deficient Y. pestis remain under observation for latent disease. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							
Support in the amount of \$36,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

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

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 174 Ecology of Plague

Investigators.

Principal: COL Dan C. Cavanaugh, MSC

Associates: MAJ James E. Williams, MSC; CPT Lyman Roberts, MSC;  
Daniel N. Harrison, Ph.D.

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.

During the past reporting period, plague has exhibited a recrudescence in many endemic or enzootic areas of the Western United States. Reports of human disease and the results of rodent/flea surveys indicates that the plague bacillus (Yersinia pestis) has persisted in many locales either believed to be quiescent or entirely free of the danger of infection. An unusually high incidence of respiratory symptoms among the reported cases prompted some concern that Y. pestis with pneumotropic qualities might be involved. Also, it was considered of importance to test as many of the strains of Y. pestis as could be obtained for sensitivity to antibiotics.

Our observation that vaccinated laboratory animals frequently succumbed to a cryptic or chronic plague infection due to non-encapsulated (Fraction 1 deficient) mutant plague bacilli selected in vivo initiated studies in rhesus monkeys vaccinated with the presently approved Army (Cutter) plague vaccine to ascertain if this phenomenon occurred in primates. As these mutant plague bacilli appear to be a result of selection due to exposure to Fraction 1 antibody in vivo, all opportunities were sought to examine as many clinical specimens believed to contain Y. pestis as could be obtained to ascertain if such organisms might be widespread throughout the Western United States.

Additional studies directed towards the support of various field installations and the improvement of diagnostic reagents and procedures have continued. The above matters form the basis of this report.

# I. Virulence factors of Y. pestis.

Our interest in the non-encapsulated mutants occurring in the lesions of rodents succumbing to cryptic plague infection (see previous annual reports) stimulated interest in ascertaining how widespread such plague bacilli might be in nature. The present outbreak of plague in the United States provided the opportunity to study recently isolated Y. pestis in an attempt to resolve this question. Further, the unusual prevalence of plague pneumonia (at least 3 of 14 confirmed cases) in contrast to an anticipated 5% emphasized the requirement for the study to provide the early identification of potentially pneumotropic bacilli.

Table 1 presents data on virulence factors of recently isolated strains of Y. pestis. Strains were isolated from all reported cases of plague occurring in the United States. We received duplicate specimens on some cases which were submitted for these studies by both the State of New Mexico and the CDC Plague Laboratory at Fort Collins, Colorado. Strains of Y. pestis isolated by the World Health Organization (WHO) staff in Java from various rodents and fleas were also studied. The nine strains of Y. pestis obtained during 1974 from Vietnamese plague patients were isolated from clinical specimens, shipped directly from Saigon in Cary-Blair transport medium just prior to the fall of that city to the Communist forces.

Table 1. Virulence factors of some recently isolated plague bacilli.

Source of strains	Number strains tested	Virulence factors			
		F1	V/W	Pigment	PCF complex
United States, 1975					
Plague patients	20*	+	+	+	+
Rock squirrels	6	+	+	+	+
Java, 1972-1974					
Commensal rodents	2	+	+	+	+
Rodent fleas	6	+	+	***	+
Vietnam, 1974					
Plague patients	9	+	+	+	+

\* Includes some strains tested in duplicate.

\*\* One of six strains exhibited a preponderance of pigment-negative colonies.

\*\*\* All strains contained some pigment-negative colonies.

Inspection of the data contained in Table 1 shows that the strains involved in the current plague outbreak in the United States possess all of the known virulence factors attributed to classical plague bacilli. By contrast, the strains of Y. pestis isolated in Java are more variable. For example, the strains isolated from rodent hosts demonstrate a full complement of virulence factors while those isolated from vector fleas do not. Tests for the V/W complex of antigens on the bacilli isolated from fleas showed that many clones of the bacilli in these specimens lacked the V/W complex, so vital for virulence. The pigment-deficient strain of Y. pestis isolated from fleas in Java is also of interest, confirming, perhaps, the laboratory observations of the Soviet workers who reported that a selection towards non-pigmentation occurred in the mid-gut of infected fleas. The observation that Y. pestis isolated from the actual clinical specimens obtained from bubonic plague patients in Saigon also contained some pigment-negative clones of Y. pestis indicates that this may be a naturally occurring phenomenon in enzootic plague regions.

Classical LD<sub>50</sub> titrations were performed on the three strains of Y. pestis isolated from confirmed pneumonic plague patients. The titrations were conducted with cultures incubated at both 25C and 35C. It was of importance to consider the fact that pneumotropism might best be demonstrated with cultures incubated at 25C, thermal conditions which favor the elaboration of the antigens found on Y. pestis while within the mid-gut of the flea vector. The data (Table 2) show that the several strains of plague bacilli studied are of classical virulence. As previously observed (see previous annual reports) plague bacilli incubated at 25C are highly sensitive to phagocytosis and presumed destruction and LD<sub>50</sub> are often appreciably higher, reflecting this fact. Laboratory mice infected by the respiratory route often provide anatomical evidence of primary plague pneumonia at autopsy. While the laboratory mice available at WRAIR invariably demonstrate plague bacilli in their lungs at autopsy, no frank anatomical evidence of pneumotropism was observed in the mice utilized in these titrations.

In view of the considerable antibiotic pressure applied to the plague bacillus during the past few years in areas such as Vietnam, the Y. pestis described in Table 1 were tested for sensitivity to streptomycin, tetracycline, and several other drugs. The tests were by the disc method (Difco). Mueller Hinton agar was inoculated with 0.2 ml of a 24 hour culture of Y. pestis incubated at 25C, standardized photometrically as to density<sub>6</sub>. Viable cell counts for these cultures ranged from  $1 \times 10^5$  to  $9 \times 10^6$  per 0.2 ml. Similar cultures of Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923) were included as controls. The sensitivity tests were conducted at both 25C and the recommended temperature of 37C: The majority of plague

Table 2. Mouse LD<sub>50</sub> for three strains of Yersinia pestis isolated from pneumonic plague patients (Arizona or New Mexico, 1975).

Strain	Date	Culture incubated 18 hr at:	
		25C (flea simulation)	37C
751207	June, 1975	390*	9
752675	September, 1975	166	19
BAC001884	September, 1975	157	3
195/P (Control)		383	15

\* Number of plague bacilli per LD<sub>50</sub> dose.

outbreaks occur in areas where sophisticated laboratory facilities are not available. Sensitivity testing, however, might be mandatory and temperatures of 25C can usually be managed under almost any circumstances. The data (Table 3) show that the most recent isolates of the plague bacillus remain entirely sensitive to streptomycin and tetracycline, the drugs of choice for the treatment of plague. Further, both inoculation temperatures appear suitable for such tests. The in vitro tests also redemonstrate the hazards involved in the interpretation of such data in indicating that the plague bacillus is sensitive to penicillin (an absolutely worthless drug in the treatment of plague). Aureomycin (chlorotetracycline) is also of proven efficacy in the therapy of plague and this fact is reflected in the data. Neomycin, while effective in vitro if of unknown efficacy in vivo. An additional fact presented in Table 3 is reassuring. The capsule deficient strain CPS-1 isolated from cryptic plague infections is as susceptible to the effects of streptomycin and tetracycline as the parent strain 195/P.

## II. Efficacy of plague vaccine U.S.P. in preventing cryptic plague infections in primates.

Although capsule-deficient strains of Y. pestis cause latent or chronic disease in white laboratory rats vaccinated with the Army Plague Vaccine, U.S.P., it is not known if similar mutant plague strains will occur in vaccinated men who are thereafter exposed to the classical encapsulated form of Y. pestis. In view of the fact that vaccinated American military personnel received a potential 8-million man-years of exposure to plague infection in Vietnam, the phenomenon of long-term cryptic infection appearing in vaccinated subjects appeared to warrant further attention. Neither is it known if atypical capsule-deficient forms of Y. pestis can establish infections, including long-term cryptic infections, in exposed vaccinated subjects appeared to warrant further attention. Neither is it known if atypical capsule-deficient forms of Y. pestis can establish infections, including long-term cryptic infections, in exposed vaccinated subjects. Since capsule deficient forms of Y. pestis are transmitted in natural foci and, indeed, have caused fatal human disease, an inquiry into the efficacy of Plague Vaccine, U.S.P. against such variant Y. pestis also seemed indicated in a primate host representative of man. Thus, experiments were undertaken in rhesus monkeys vaccinated with the Army Plague Vaccine, U.S.P. to determine if capsule-deficient variants of Y. pestis arise spontaneously after virulent challenge with encapsulated plague bacilli, or if capsule-deficient variants of Y. pestis establish lethal or chronic infections after direct inoculation.

Table 3. Strains of *Yersinia pestis* tested for antibiotic sensitivities at 25C and 35C using the disk method

	Streptomycin						Tetracycline						Aureomycin						Neomycin						Penicillin G					
	2 mcg		10 mcg		30 mcg		5 mcg		30 mcg		30 mcg		25 C		35 C		30 mcg		25 C		35 C		30 mcg		25 C		35 C		5 units	
	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C	25 C	35 C
Six laboratory strains:**																														
range	9-15	15-19	17-22	22-28	16-24	16-25	28-34	29-35	26-31	23-29	26-30	30-35	21-28	29-47																
$\bar{x}$	11.33	16.17	20.00	25.83	21.17	19.83	30.00	31.00	28.67	25.17	27.67	32.33	25.50	36.50																
SD	2.16	1.60	2.10	2.23	1.83	2.93	2.45	2.28	1.97	2.32	1.51	1.97	3.02	5.86																
Eight strains isolated from rats & fleas in Java, 1972-1974:																														
range	10-15	14-21	18-23	25-30	16-26	16-22	24-36	29-35	23-33	25-30	25-35	29-36	20-32	28-40																
$\bar{x}$	12.63	16.88	20.63	27.50	19.50	19.75	29.25	31.75	28.63	26.25	28.75	33.13	26.63	33.50																
SD	1.60	2.47	1.92	1.51	3.38	1.75	3.81	2.25	3.96	1.67	4.20	2.80	3.46	4.28																
Six strains isolated from rock squirrels ( <i>S. variegatus</i> ) in New Mexico, USA, 1975:																														
range	11-12	14-17	19-21	26-28	16-20	17-20	25-28	28-31	25-29	23-27	25-27	31-32	23-24	34-42																
$\bar{x}$	11.33	15.50	20.17	26.50	17.50	18.00	26.50	30.17	27.00	24.33	26.33	31.17	23.50	38.50																
SD	0.52	1.05	0.98	0.84	1.52	1.10	1.22	1.33	1.67	1.37	1.03	0.41	0.55	2.88																
Twenty strains isolated from human cases in USA, 1975:																														
range	9-11	10-15	18-21	23-26	17-23	16-25	25-31	27-36	23-33	22-29	20-29	26-32	22-38	34-47																
$\bar{x}$	9.95	12.65	19.60	24.35	19.80	19.70	28.45	29.90	27.20	25.55	25.60	29.80	29.35	39.40																
SD	0.60	1.50	0.75	0.93	1.70	2.83	1.67	2.25	2.48	1.82	2.58	1.51	4.42	3.68																

Test criteria:\*\*\*

resistant

intermediate

≤ 11

12-14

≤ 14

15-18

≤ 12

13-16

≤ 11

12-21

\* Classical virulent strain 195/P, avirulent vaccine strain EV76(51f), capsule deficient CPS-1, urease positive MD 18, phage infected M1a-21, glycerol positive PKR 159.

\*\* All zones of inhibition measured in mm.

\*\*\* Matsen, J.M. & Barry, A.L. 1974. Chapter 46 in Manual of Clinical Microbiology ("Susceptibility testing: diffusion test procedures"). Criteria for 10 units Penicillin G are given above.

Twenty of 24 rhesus monkeys were vaccinated with the Army Plague Vaccine, U.S.P. (1 ml im with booster injections of 0.5 ml at 4, 8, and 20 weeks) until serological titers of antibody were achieved that are normally adequate to protect against lethal challenge. The 4 remaining animals served as unvaccinated controls. Three months after vaccination, challenges with virulent Y. pestis were performed. Two unvaccinated animals and 14 vaccinated animals were challenged with the encapsulated Y. pestis strain 195/P, while 2 unvaccinated and 6 vaccinated animals were challenged with the capsule-deficient strain CPS-2a (Table 4). Mouse titrations done concurrently gave mouse LD<sub>50</sub> values of 18 organisms for the 195/P inoculum and 9 organisms for the CPS-2a inoculum. In spite of the high virulence of challenge inocula for mice, only one monkey infected with strain 195/P died. The relative high resistance of rhesus monkeys to lethal plague found in these experiments also has been observed by other workers. Although only a single death occurred, one monkey challenged with strain 195/P exhibited a large (3 X 4 cm) bubo at the site of inoculation on the lower left abdomen a month after challenge. A second monkey, also challenged with 195/P, had a swelling of approximately 1 cm diameter in the left groin. Of those monkeys challenged with the non-encapsulated CPS-2a strain, one displayed a hard, fibrous area at the site of inoculation, but no swelling was evident. These manifestations of disease resolved completely, and the 3 monkeys appeared normal, 3 months after infection. Surviving monkeys are examined for symptoms of cryptic disease at regular intervals. Sera collected at appropriate intervals are being stored for testing at the completion of the study.

### III. Bacteriological studies.

Preliminary experiments (see previous annual reports) have demonstrated that bacteriophages specific to various strains of the plague bacillus or to various serotypes of Y. pseudotuberculosis could, perhaps, be utilized in both genetic studies and in an epidemiologic typing system. These studies also confirmed the fact that temperature of incubation greatly influenced the ability of these bacteriophages to lyse host organisms and indicated that media for such experiments should be free of blood which was found to be inhibitory.

Forty-five strains of Y. pestis and 27 strains of Y. pseudotuberculosis utilized in the above studies were available for testing. Since it was desirable to include several phages in the study, and, detect conditional situations where an effect is observed at one temperature but not at another, the WRAIR multipoint phage applicator was employed. Eighteen hour culture of the test organisms were photo-metrically standardized and inoculated on brain heart infusion agar (Difco). The Y. pestis "H" and the Y. pseudotuberculosis "IV" phages

Table 4. Rhesus monkeys challenged sc with Y. pestis after vaccination with plague vaccine USP

<u>Y. pestis</u> strain used for subcutaneous challenge	Number challenged with given IHA titer						Deaths
	< 1:16*	1:16	1:32	1:64	1:128	1:2048	
Encapsulated, virulent strain 195/P ( $4.5 \times 10^6$ <u>Y. pestis</u> per monkey)	2	3	3	3	4	1	One monkey that had a titer of 1:16 at challenge died 6 days later
Nonencapsulated, virulent strain CPS-2a ( $4.4 \times 10^6$ <u>Y. pestis</u> per monkey)	2	2	0	4	0	0	None

\* Nonspecific hemagglutination was observed in some monkey sera at serum dilutions of 1:4 and 1:8.



6  
were propagated on suitable host strains and adjusted to contain 10<sup>6</sup> PFU/ml. Divided among the various wells of the phage applicator reservoir in test patterns considered most suitable for reliable tests the phages were then inoculated onto the lawns of the host organisms prepared as described above. Inoculated plates were then incubated at temperatures shown by previous experiments to be suitable. Results are given in Table 5. The influence of temperature is evident. In the experiment, numerous instances where "H" phage applied to lawns of Y. pseudotuberculosis or "IV" phage applied to lawns of Y. pestis produced partial lysis were observed. It is unknown if this resulted either the appearance of mutant resistant organisms or, perhaps, organisms capable of a lysogenic relationship with the test phages. The test system will be extended to include other Yersinia phages and the "rough specific phages" of the Enterobacteriaceae. Such studies should produce results reflecting rough groups of cell-wall constituents, identify mutant organisms and increase our fund of knowledge on the genetics of the plague bacillus.

#### IV. Diagnostic reagents.

Increased interest in the indirect hemagglutination test for detection of antibody to the F1 antigen of Y. pestis has prompted a study on the feasibility of providing lyophilized reagents suitable for field and laboratory use. With the cooperation of the Dept of Biologics Research, lyophilized reagents were prepared, and tested over an 18 month period. In the 3 lots prepared and studied, antigenic titer of lyophilized reagent, as measured with standardized test sera, was identical ( $\pm 2$  dilutions) to that of aliquots of the various lots reserved for such tests prior to drying. The dried reagents have remained stable over the 18 month test period. While further testing is required to determine the absolute reliability of such preparations, the technique should permit the utilization of a useful test in areas removed from central laboratories.

Table 5. Sensitivity of various strains of Yersinia pestis and Y. pseudotuberculosis at different temperatures of incubation.

Temperature of incubation C°	<u>Y. pestis</u> lawns (45 strains)		<u>Y. pseudotuberculosis</u> lawns (27 strains)	
	Bacteriophages tested*		Bacteriophages tested	
	"H"	"IV"	"H"	"IV"
10	0	100**	0	74(10)
15	44(94)	100(10)	0	70(26)
17	98(98)	100(76)	0	74(30)
20	100	100(83)	4	78(24)
22	100	100(100)	7(50)	74(30)
25	100	69(42)	7(50)	70(68)
27.5	100	5(50)	15(33)	44(75)
30	100	5(50)	22(100)	22(30)
32.5	100	2(100)	26(57)	15(100)
34	100	2(100)	26(57)	15(100)
37.5	98(7)	0	33(66)	11(100)

\* Bacteriophage "H" is the classical bacteriophage utilized in identification of the plague bacillus. Bacteriophage "IV" is a bacteriophage regarded as specific for Y. pseudotuberculosis Type IV

\*\* Numbers indicate percentage of strains tested that were lysed by the indicated bacteriophage. Numbers in parenthesis denote the percentage of instances where lysis was incomplete.

## V. Miscellaneous studies.

A continuing effort towards producing suitable serologic reagents for the detection of cryptic plague infection has, so far, been unrewarding. During this reporting period, several lipopolysaccharide antigens have been prepared with non-encapsulated Y. pestis isolated from diseased rodents and from various serotypes of Y. pseudotuberculosis. The lipopolysaccharide antigens have been shown to be serologically active in gel diffusion tests but await further testing.

A number of specimens for diagnostic studies were received during the present reporting period, including 390 rodent sera collected in Brazil by USAMRU (Belem). Fraction 1 antibody was not detected in any of the sera. The collection, however, was of interest in that it contained the sera of species which had not been previously studied by the IHA technique. The Dept of Tropical Medicine (LAIR) submitted a series of specimens consisting of 136 rodent carcasses, 31,000 fleas, and 960 sera for plague studies. Y. pestis was not isolated from any of the specimens submitted. Antibody to the F1 antigen of the plague bacillus was not detected in any serum specimen collected prior to the fall of 1975. At that time, the serum survey was extended to include carnivore sera and F1 antibody was detected in the serum of a coyote at a titer of 1:256 and in the sera of a feral house cat (titer 1:8) and a domestic dog (titer 1:8). Additional sera, collected early in 1975, also contained specimens in which F1 antibody was detected; one coyote (titer 1:128); 9 domestic dogs (1 with a titer of 1:64; 4 with 1:16; 3 with 1:8 and 1 with 1:4). A serum obtained from a bobcat contained antibody at a titer of 1:8 as did a specimen obtained from a ground squirrel. Specimens displaying titers of 1:8 are considered equivocal. In this instance, however, such titers were, in a measure, reassuring: the domestic dog serum collected in the fall of 1975 with an antibody titer of 1:8 was re-bled in February, 1976, and a titer of 1:4 was demonstrated in this serum. In our experience, recent infection results in a rapidly rising titer. The titer of 1:8 in the ground squirrel serum was the only such result observed in the tests involving some 884 rodent sera. Unfortunately, fleas were not available from the area in which the squirrel was collected for isolation attempts. The specimens of carnivore sera indicate the presence of plague somewhere in the vicinity of the collecting site. However, the home ranges of the animals involved requires consideration. Animals, such as coyotes, travel extensively in their search for food. Observations on the duplicate sera collected from a single domestic dog indicate exposure (if an equivocal titer can be considered to reflect such a condition) was not recent.

## VI. Conclusions and recommendations.

The current plague outbreak involving the United States is cause for concern inasmuch as several military installations are present in the involved areas. It is advisable to maintain adequate surveillance to prevent human disease. The strains obtained in the present episode are fully virulent although entirely susceptible to the time-proven therapy employing either streptomycin or the tetracyclines. Studies on Y. pestis isolated from several widespread geographic locales were unsuccessful in yielding clones of non-encapsulated plague bacilli which are found in cryptic plague infections, although interesting variants were obtained from several clinical specimens and from organisms isolated from rats and fleas in Java, confirming our opinion that Y. pestis varies from area to area in terms of virulence factors.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION <sup>a</sup>	2 DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
				DA OB 6537	76 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>	8 DISSEM INSTR <sup>a</sup>	9A SPECIFIC DATA CONTRACTOR ACCESS	9B LEVEL OF SUM
75 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	3A161102B71G	00	175			
B. CONTRIBUTING							
C. <del>XXXXXXXX</del>	CARDS 114F						
11 TITLE (Precede with Security Classification Code) <sup>a</sup>							
(U) Pathologic Manifestations of Zoonotic Diseases of Military Importance							
12 SCIENTIFIC AND TECHNOLOGICAL AREA <sup>a</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. DATES/EFFECTIVE:				PRECEDING		B. FUNDS (7n Thousands)	
B. NUMBER <sup>a</sup> NA				FISCAL YEAR		6	
C. TYPE:				CURRENT		112	
D. KIND OF AWARD:				77		8	
E. CUM. AMT.						147	
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				Division of Pathology			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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				SOCIAL SECURITY ACCOUNT NUMBER			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: REARDON, MAJ, M.			
				NAME: JOHNSON, MAJ, A. J.			
22 KEYWORDS (Precede EACH with Security Classification Code) (U) Leishmaniasis; (U) Trypanosomiasis; (U) Radioisotope							
(U) Sarcosporidiosis; (U) Brain; (U) Kidney; (U) Schistosomiasis; (U) Wildlife;							
(U) Immunopathology; (U) Electron microscopy; (U) Reticuloendothelial System							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified number. Precede text of each with Security Classification Code)							
23(U) To study and define pathogenesis of natural and experimental trypanosomiasis, leishmaniasis, schistosomiasis, and sarcosporidiosis in a variety of animal hosts. To study spontaneous renal disease in the owl monkey. Provide anatomic pathology support for wildlife epidemiological surveys in the TransAmazon and other military installations. All projects under study are of military importance as zoonotic diseases or animal models for human disease.							
24(U) Studies will utilize conventional gross and histopathology, clinical pathology, electron microscopy, histochemistry, immunohistochemistry and radioisotopic techniques.							
25(U) 75 07-76 06 The pathogenesis of experimental Leishmania donovani infection in the cynomolgus monkey, hamster and dog is currently being defined. The pathogenesis of experimental Trypanosoma rhodensiense infection in the dog is being defined. Ultra-structural neuropathology studies of experimental Trypanosoma congolense infection in African cattle are in progress. Histochemical stains are being utilized to differentiate seven species of schistosomes in tissues. The pathogenesis of immune-complex glomerulonephritis is being defined in C4 deficient guinea pigs. The mechanism of bovine and ovine abortion induced by Sarcocystis fusiformis is currently under study. The hemodynamic physiological parameters utilizing 51Cr have been defined for the owl and cynomolgus monkeys. This project is completed, and baseline data will be applied to future studies in simian malaria. Anatomic lesions are being identified and statistical date compiled on wildcaught animals as part of a large epidemiological survey.							
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 75-30 June 1976. Support in the amount of \$30,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

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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.

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 175 Pathologic Manifestations of Zoonotic Diseases of Military Importance

Investigators.

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Background.

To define, study, diagnose, and control known and potential diseases of military importance common to both man and animals. The source of research material is derived from naturally occurring and experimentally induced diseases. High priority is given to the development of new animal models for human disease. The major effort is directed toward defining the pathogenesis of these diseases utilizing gross pathology, histopathology, clinical pathology, ultrastructural pathology, histochemistry, immunohistochemistry, and radiosotopic techniques.

During the reporting period, research activities have been concerned with: (1) Pathology of Trypanosoma rhodesiense infected C4-deficient guinea pigs; (2) Pathology of experimental Trypanosoma rhodesiense infection in the dog; (3) Electron microscopic studies of central nervous system lesions in cattle experimentally infected with Trypanosoma rhodesiense; (4) Comparative pathology of sarcosporidiosis; (5) <sup>51</sup>Cr hemodynamic studies in owl and cynomolgous monkeys; (6) Pathology of experimental Leishmania donovani infection in the cynomolgous monkey (Macaca fascicularis); (7) Electron microscopic study of Leishmania donovani infected hamsters; (8) Comparative histochemistry of Schistosoma sp.; (9) TransAmazon epidemiological survey; (10) Wildlife disease surveys; (11) Electron microscopic study of a spontaneous disease in the owl monkey (Aotus trivigatus).

(1) Pathology of Trypanosoma rhodesiense Infected C4-Deficient Guinea Pigs

In a feasibility study to acquire information on the role of complement activation by the "alternate" or "classic pathway" in trypanosomal induced glomerulonephritis, C4-deficient guinea pig mutants infected with Trypanosoma rhodesiense are employed. Using C4-deficient animals it is possible to see if the glomerulonephritis can develop in the absence of the "classic pathway" of complement activation which requires C4. The study involves light and ultrastructural analysis of the glomerular injury as well as immunohistochemical localization of IgG, IgM, C3 and C4 within the glomerular basement membrane and mesangium of both C4-deficient mutants and parenteral complement non-deficient guinea pigs.

Summary of Results: By light microscopy the initial onset and severity of the glomerular injury are remarkably similar in both C4-deficient and complement non-deficient guinea pigs.

Normal uninfected guinea pigs have glomeruli devoid of immunoglobulins and complement while infected animals have heavy deposits of IgG, IgM and C3 at 2, 4, 6 and 8 weeks post-infection. The glomeruli of C4-deficient guinea pigs are completely devoid of C4 in both controls and infected, however, heavy deposits of C4 are found in infected non-deficient guinea pigs.

Electron microscopic analysis of glomerular injury is two-thirds completed, the lesions are remarkably similar in both C4-deficient and complement non-deficient guinea pigs.

In progress are determinations of total complement and C3 serum levels in normal controls and infected C4-deficient and non-deficient guinea pigs.

(2) Pathology of Experimental Trypanosoma rhodesiense Infection in the Dog

This study is being conducted to evaluate the gross pathology, histopathology, clinical pathology, immunopathology and electron microscopic pathology of T. rhodesiense infection in the dog and its pathogenesis in this species.

Summary of Results: To date, dogs have been inoculated with 100,000 organisms of the Wellcome strain intravenously and have developed a clinical syndrome consisting of fever, anorexia, emaciation, lymphadenopathy and edema of the muzzle, face, head, neck, legs and abdominal wall. Conjunctivitis, chemosis, panophthalmitis, signs of generalized central nervous system disease, anemia and acute death were seen in individual cases. Preliminary clinical pathology findings demonstrate decreases in packed cell volume, total red blood cell counts, hemoglobin, total white blood cell counts (initially), platelets, and glucose. Elevations were seen in sedimentation of erythrocytes, white blood cells counts (later) and globulins. Counts of the parasites seem to follow a cyclic fever curve.

Gross necropsies and tissue collection for histopathology, electron microscopy and fluorescent antibody examination are now being done, and the phase of the experiment will be completed by the end of this fiscal year. The need for further studies beyond those planned in this experiment cannot be anticipated until these data are evaluated.

(3) Electron Microscopic Studies of Central Nervous System Lesions in Cattle Infected with Trypanosoma rhodesiense

The objective of this experiment is to investigate the electron microscopic pathology of T. rhodesiense in the bovine. Cerebrum, cerebellum, and brain stem will be examined. This work in cattle may



add to the pathophysiology of the human infection since the organisms appear to be similar when studied with neutralization tests.

Summary of Results: To date, 1 control and 2 infected animals have been sacrificed and central nervous system tissue prepared for electron microscopy. Although these animals were not perfused in vivo, several differences have been observed. Perivascular inflammatory cells and a multifocal gliosis is present in the infected, but not in the control animal. Directions have been forwarded to Kenya for an in vivo method of brain perfusion for electron microscopy that should make the identification of more subtle changes creditable.

#### (4) Comparative Pathology of Sarcosporidiosis

Protozoan parasites of the genus Sarcocystis are found within striated muscle, including the heart, of a high percentage of cattle, sheep and swine throughout the world. In the United States 75 to 98% of the cattle are reportedly infected. When beef infected with Sarcocystis is fed to carnivores, also man, coccidian sporocysts are shed in the feces. After sporocysts obtained from canine feces are fed to cattle, coccidian schizonts develop in vascular endothelial cells of many organs of the body and typical cysts of Sarcocystis are later found in striated muscle. Although Sarcocystis has been considered not very pathogenic, young calves fed sporocysts usually become severely ill and many die. Findings on the pathogenicity and life cycle of experimental Sarcocystis infection in calves and its similarities to Toxoplasma, a known pathogen in humans and domestic animals, has stimulated experimentation to determine the effects of Sarcocystis in both young and pregnant animals.

Summary of Results: Bovine abortion following experimental infections with Sarcocystis fusiformis from dogs was studied. Six 5-8 year old multiparous cows orally inoculated with sporocysts of Sarcocystis at 5 months or 6½ months after field breeding became ill with clinical signs of muscle tremors, anorexia, weight loss, excessive shedding of hair, temperature elevation, anemia and abortion.

Abortions were observed in two cows, one 41 days after inoculation and the other aborted on day 60. One cow became severely ill, recumbent and was euthanatized. A normal appearing fetus was observed in utero. One of two cows that did not produce calves had a macerated fetal skeleton in utero. One cow gave birth to a normal calf. Characteristic pathologic changes of sarcosporidiosis were observed within tissues from all cows.

No evidence of congenital transmission was found in histologic specimens obtained from fetuses or the calf. (Manuscript submitted and accepted for publication - J. Infectious Diseases).

Multiparous ovine ewes orally inoculated with sporocysts of Sarcocystis from dogs at 2 months after field breeding became severely ill with clinical signs of muscle tremors, hypersalivation, anemia, anorexia, weight loss, excessive shedding of wool, temperature elevation, abortion and death. Typical pathologic changes of sarcosporidiosis were observed



within tissues from all ewes. No evidence of congenital transmission was found in histologic specimens obtained from fetuses and lamb.

Immunologic glomerulonephritis in bovines following experimental infections with Sarcocystis fusiformis was studied. Glomerular injury associated with immunologic deposits within glomerular basement membrane and mesangium has been demonstrated with special histologic stains, electron microscopy and immunohistochemistry. The deposits are intramembranous and subepithelial. Heavy deposits of IgG and C3 have been demonstrated by immunofluorescence techniques. This experimental procedure is three-fourths completed.

These investigations consist of pathology support provided to the Animal Parasite Institute (API), USDA, Beltsville, MD. This work was initiated upon request to this department by API who has no diagnostic pathologist with expertise in protozoan diseases such as sarcosporidiosis and toxoplasmosis. These investigations have determined that two vertebrate hosts (including man) are required in the life cycle of bovine sarcosporidiosis and further elucidated the pathogenesis. Sarcosporidiosis, a multi-species disease like toxoplasmosis, is costly to the animal industry and a zoonotic disease.

(5) <sup>51</sup>Cr Hemodynamic Studies in Owl and Cynomolgous Monkeys

The owl monkey (Aotus trivigatus) has been used extensively in malaria research, particularly in studies of the metabolism, toxicology and physiology of various experimental compounds. It also has been used widely in research on the viral oncology and therapy of lymphomas and leukemias. The cynomolgous monkey (Macaca fascicularis) has been shown to be susceptible to infection with Leishmania donovani, developing hepatosplenomegaly and severe secondary anemia similar to that seen in man with visceral leishmaniasis. The cynomolgous monkey is also likely to substitute as a research animal for the rhesus monkey, which is rapidly becoming unavailable.

Because anemias develop in the above diseases in these species, and because many of the experimental chemotherapeutic compounds are hemolytic in nature, there is a need to study the red cell kinetics in these animals during infection with these diseases and during treatment with potentially hemolytic chemotherapeutic compounds. This study was conducted to establish the baseline value for blood volumes and erythrocyte half-life survival times in these species using standard methods. The values obtained, and an evaluation of the procedures used, will facilitate future studies of the pathogenesis and chemotherapy of these diseases in these monkeys.

Summary of Results: Red cell volumes and half-life survival times were determined by tagging erythrocytes with <sup>51</sup>chromium and reinjecting them into autologous animals. Plasma volumes were determined by the isotope dilution technique using radioiodinated serum albumen (RISA-<sup>125</sup>I). Three

male and 2 female adult owl monkeys had a mean red cell volume of 24.66 ml/kg, a mean plasma volume of 40.58 ml/kg, a whole blood cell volume of 65.24 ml/kg, and a mean <sup>51</sup>chromium erythrocyte half-life survival time of 11.6 days. Two male and 3 female adult cynomolgous monkeys had a mean red cell volume of 14.96 ml/kg, a mean plasma volume of 35.74 ml/kg, a mean whole blood volume of 50.52 ml/kg, and a mean <sup>51</sup>chromium erythrocyte half-life survival time of 13.6 days. This project is completed. Results will be utilized in future experiments in these animals when hemolytic anemia is expected.

(6) Pathology of Experimental *Leishmania donovani* Infection in the Cynomolgous Monkey (*Macaca fascicularis*)

The objective of this experiment was to determine the susceptibility of the cynomolgous monkey to infection with *Leishmania donovani* and compare host response in this species with previous results obtained in other animal models.

Summary of Results: When cynomolgous monkeys were exposed intravenously to large inocula of amastigotes of *Leishmania donovani* obtained from infected hamster spleen, they invariably exhibited clinical illness similar to that observed in the human disease. The initial clinical sign usually was splenomegaly in 3 to 7 weeks post-exposure followed shortly by the onset of pancytopenia and significant weight loss. Typical parameter changes included a 6 to 12-fold increase in spleen weight, a drop in platelet count from  $5-6 \times 10^5$  to less than  $1 \times 10^5/\text{mm}^3$ , a decrease in packed cell volume from 32-40% to 15% or less, a drop in total white blood count from  $5-10 \times 10^3$  to  $1-3 \times 10^3/\text{mm}^3$ , and weight loss approximately 10-35%. Animals usually died within 6 to 13 weeks post-exposure. Parasites were demonstrable microscopically from liver, spleen, bone marrow and numerous other organs at necropsy. Histopathologic lesions included severe diffuse granulomatous inflammation of spleen, liver, and the abdominal lymph nodes. Non-suppurative interstitial pneumonia and epididymitis also were evident. Sub-meningeal hemorrhages occasionally were observed. These results suggest that this monkey model may be suitable for testing of candidate anti-leishmanial compounds and also in studies relating to the biology and pathogenesis of the disease.

(7) Electron Microscopic Study of *Leishmania donovani* Infected Hamsters

The objective of this experiment was to investigate the host-parasite relationship; especially the morphology of the organism in tissues of the super-infected hamster. This information will be used as baseline data for future ultrastructural studies of *Leishmania donovani* in several animal models.

Summary of Results: To date, 2 infected hamsters have been sacrificed and the liver and spleen prepared for electron microscopy. The presence and morphology of the organism has been documented in splenic macrophages. Liver is now being sectioned to examine for hepatocellular pino-

cytosis.

(8) Comparative Histochemistry of *Schistosoma* sp.

This study was undertaken to evaluate the usefulness of acid-fast stains in distinguishing between different species of schistosomes in fixed tissues. To date, specimens of seven species of schistosomes in tissues from one or more of seven hosts have been collected. Both spontaneous and experimental infections are being studied. Routine H&E sections have been screened on all tissues to determine the number of ova, level of preservation and host reaction. Acid-fast stains utilizing two different methodologies commonly used in histopathology laboratories are being prepared. This project is one-half completed. Complete results will be reported at a later date.

(9) TransAmazon Epidemiological Survey

Histopathology support is being provided the TransAmazon WRAIR team in the survey of endemic and potentially zoonotic diseases in the area. To date, 600 specimens have been received, processed and are being evaluated.

Twenty-seven species are represented and are from two primary collection areas. In those specimens examined to date, there does not appear to be widespread disease as determined by light microscopy. Complete evaluation, however, will be necessary along with correlation of lesions and serological observations being made by other portions of the WRAIR team. Future plans for this project are to continue histopathology support and complete evaluation of data already collected.

(10) Wildlife Disease Surveys

Collaborative support is being provided for projects based at Fort Stewart and Fort Gordon, Georgia. The purpose of these projects is to monitor diseases of zoonotic significance using wildlife residing on military reservations as sentinel animals. Diseases being monitored are those transmitted to man and those potentially a threat to livestock grazing on or adjacent to these reservations. To date, tissue samples have been collected and examined microscopically on 45 white-tailed deer and 16 swine. Serum samples are presently being examined for serological evidence of exposure to significant pathogens. This project will continue with examination of animals killed during the fall 1976 hunting season. At that time, sufficient specimens will have been collected to evaluate the data and describe disease trends or potential problem areas.

(11) Electron Microscopic Study of a Spontaneous Renal Disease in the Owl Monkey (*Aotus trivagatus*)

The objective of this study is to investigate the ultrastructural pathology of a spontaneous occurring renal disease in a breeding colony of owl monkeys. Electron microscopy in this instance could be helpful

in establishing the etiology of the disease process. Owl monkeys, in addition, are valuable medical research animals and the recognition and understanding of their diseases is an important step to insure the success of the breeding colony.

**Summary of Results:** A renal disease characterized by non-suppurative interstitial nephritis and a mild-to-severe proliferative glomerulonephritis has been observed. Glomerular changes compatible with an immune mediated disorder can be visualized. The deposition of immune-complex deposits have been seen in all 3 monkeys examined to date. These observations have directed future studies towards the identification of the antigen involved.

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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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. Radioimmune assay inhibition test for antibody to core antigen of Hepatitis B (anti-HB<sub>C</sub>)

HB<sub>C</sub>Ag harvested chimpanzee liver was used as a source of antigen for a radioimmune inhibition test for anti-HB<sub>C</sub>. A 20% liver suspension was prepared in a hypotonic (.01 M) phosphate buffered saline. After homogenization, the liver was clarified by centrifugation and the supernatant was used as a source of antigen.

IgG anti-HB<sub>C</sub> derived from an asymptomatic HB<sub>S</sub>Ag carrier without anti-HB<sub>S</sub> activity was labeled by the method of Purcell *et al* (1974). The following reagents were added, in order, to a test tube: 20 uliters of 0.2 M phosphate buffer, pH 7.4; 200 uCu of high-specific activity <sup>125</sup>I (in 5 uliters); 10 ug of the protein to be labeled (in 1 to 10 uliters); 15 uliters of a solution of chloramine T (3.5 ug/uliter); 20 uliters of a solution of sodium metabisulfite (4.8 ug/uliter); and 20 uliters of a solution of sucrose (22.5%) and potassium iodine (2 mg/ml). After the addition of chloramine T, the reaction was allowed to proceed for 15 seconds before being terminated by the addition of sodium metabisulfite. The mixture was applied to the top of a column (0.9 by 15 cm) packed with Sephadex G-200, and equilibrated with phosphate-buffered saline,

pH 7.4 with 0.1% sodium azide. The protein was eluted with the same buffer. Fractions containing the first peak of radioactivity were pooled and diluted with an equal volume of 1% bovine serum albumin. This stock mixture was stored at 40C and diluted 1:2 with 1% bovine serum albumin just before use.

The micro-solid-phase radioimmunoassay was a modification of the method of Purcell *et al* (1974). The wells of polyvinyl microtiter plates were coated with diluted (1:100) anti-HB<sub>C</sub> derived from the same human source as mentioned above in phosphate buffered saline in 4 hours at 370C. After washing with saline, the sensitized wells received 200 ul of 1% bovine serum albumin in saline, followed by incubation overnight at 40C. After washing with phosphate buffered saline (PBS) the wells were inoculated with 50 ul of HB<sub>C</sub>Ag and the plates were incubated at 40C for 24-48 hours. Following another PBS washing, 50 ul of each patient's serum was added to duplicate wells. The plates were incubated at 40C for 24 hours, then washed with PBS and 50 ul of radiolabeled anti-HB<sub>C</sub> globulin was added to each well and incubated at 370C for 4-6 hours or 40C overnight. The plates were given a final PBS wash and the wells were cut with scissors and transferred to gamma counting tubes and counted in Nuclear Chicago (Searle) gamma counter for 1 min. Each plate contained four wells of anti-HB<sub>C</sub> negative serum as a control for plate variation. The iodinated anti-HB<sub>C</sub> preparation was shown not to react with normal or chimpanzee liver by direct RIA where these antigens were substituted for HB<sub>S</sub>Ag. Serum containing only anti-HB<sub>S</sub> (gave high counts of radioactivity by AusAb test (Abbott)) did not inhibit the reaction. Marked reduction in radioactivity with unlabeled anti-HB<sub>C</sub> antibody but not with normal serum was indicative of the specificity of the reaction. A sample was considered to have anti-HB<sub>C</sub> if a 50% (3S.D. below negative control mean) or more reduction in CPM of labelled anti-HB<sub>C</sub> occurred. Negative and positive controls were run on each plate. All sera were run in duplicate. An estimate of the sensitivity of the RIAI test for anti-HB<sub>C</sub> as compared to complement fixation test (serum panel from Bureau of Biologics, Bethesda, Maryland) is found in Table 1.

## II. Serodiagnosis of Hepatitis B infection by antibody to core antigen (anti-HB<sub>C</sub>)

Highly sensitive tests for presence of hepatitis B surface antigen (HB<sub>S</sub>Ag) and antibody (Anti-HB<sub>S</sub>) have significantly improved our understanding of the basic epidemiology of hepatitis B infection. Recently, antibody to the core antigen (HB<sub>C</sub>Ag) has been described (Almeida *et al* 1971, Hoofnagle *et al* 1973). This antigen-antibody system appears to be of great potential in the further elucidation of hepatitis B infection since anti-HB<sub>C</sub> is produced early in the course of viral infection as opposed to the presence of anti-HB<sub>S</sub> which is detected at variable times following infection.

Table 1 - Comparison of anti-HB<sub>c</sub> detection by CF and RIAI

<u>Sample No.</u>	<u>RIAI Titer Anti-HB<sub>c</sub></u>	<u>CF Titer Anti-HB<sub>c</sub></u>
101	1:64	1:8
102	Neg	Neg
103	1:512	Neg
104	1:2048	1:32
105	Neg	Neg
106	1:4096	1:1024
107	Neg	Neg
108	Neg	Neg
109	1:2048	Neg
110	Neg	Neg
111	1:2048	Neg
112	Neg	Neg
113	Neg	Neg
114	Neg	Neg
115	1:4096	1:10,384
116	1:1024	1:16
117	1:256	1:16
118	1:32	Neg
119	1:4096	Neg
120	1:4096	1:8

Because of scarce quantities of HB<sub>s</sub>Ag, the serological analysis of epidemiologically important populations for anti-HB<sub>c</sub> has been limited. Anti-HB<sub>c</sub> testing afforded the opportunity for increased efficiency of serodiagnosis of HBV infection and allowed for an estimate of the onset of HBV infection in longitudinal surveys.

Sera were obtained from military personnel at Ft. Hood, Texas from four different subpopulations:

a. Acute phase sera were collected from admission blood samples of soldiers hospitalized for treatment of acute hepatitis during 1973 and 1974. Previous surveys had demonstrated that this sustained outbreak of hepatitis was due to hepatitis B virus (Allen et al 1974). Eighty five patients whose blood was found to be negative for hepatitis B surface antigen (HB<sub>s</sub>Ag) by radioimmune assay were tested for anti-HB<sub>c</sub> as described below. Also 25 of 60 patients with HB<sub>s</sub>Ag in acute sera were selected for anti-HB<sub>c</sub> serology.

b. A second group of patients were identified with acute hepatitis at Ft. Hood. Sera were obtained from these patients when ill and a second sample collected 2-33 months later. All samples were tested for HB<sub>s</sub>Ag, anti-HB<sub>s</sub> and anti-HB<sub>c</sub>.

c. Soldiers (2333) entering Ft. Hood in Feb - Apr, 1974, were bled every 4 months for one year. Total soldiers available for rebleeding at 4, 8 and 12 months were 1915, 1210 and 900 respectively. A complete description of this study will be the subject of a separate publication. This population was tested for HB<sub>s</sub>Ag and anti-HB<sub>s</sub>. Individuals found to be positive for surface antibody on any specimen had all available sera tested for anti-HB<sub>c</sub>. In addition 215 soldiers from this group, negative for HB<sub>s</sub>Ag and anti-HB<sub>s</sub> were tested for anti-HB<sub>c</sub>.

d. In February, 1973, the prevalence of HB<sub>s</sub>Ag and anti-HB<sub>s</sub> was determined in military units with and without a hospitalized index case of hepatitis B. In this study, sera from 377 members from units with an index case and 520 sera from control units were tested for anti-HB<sub>c</sub>.

Anti-HB<sub>c</sub> was found in 34 of 85 sera (39%) in patients with acute HB<sub>s</sub>Ag negative hepatitis. All but one of the 25 acute HB<sub>s</sub>Ag positive cases examined were found to be anti-HB<sub>c</sub> positive (Table 2).

Table 2 - Detection of anti-HB<sub>c</sub> in acute hepatitis by RIAI

	<u>Total Tested</u>	<u>Anti-HB<sub>c</sub>+</u>
HB <sub>s</sub> Ag-	85	34 (39%)
HB <sub>s</sub> Ag+	25	24 (96%)



Persistence of anti-HB<sub>C</sub> following acute icteric hepatitis is demonstrated in Table 3. Most individuals (21/22) with acute HB<sub>S</sub>Ag positive hepatitis had anti-HB<sub>C</sub> initially. Anti-HB<sub>C</sub> was present in convalescent sera 3-33 months after the onset of illness in 20 of 22 patients. Of 19 acute HB<sub>S</sub>Ag negative hepatitis cases, 8(37%) had anti-HB<sub>C</sub> initially; only one of which persists in follow-up specimens. Of the remaining 12 individuals with neither HB<sub>S</sub>Ag or anti-HB<sub>C</sub> present during acute illness, 3 did seroconvert to anti-HB<sub>C</sub> by 3, 4, and 15 months respectively after illness. Anti-HB<sub>S</sub> seroconversion without anti-HB<sub>C</sub> was however noted in 2 others. Both positive and negative groups were comparable in mean time of followup and in the range of time to obtaining the convalescent sera.

Table 3 - Persistence of anti-HB<sub>C</sub> and seroconversion to anti-HB<sub>S</sub> in acute HB<sub>S</sub>Ag positive and negative hepatitis.

	(Acute Sera)	Mean and Range of Months to Conval- escent sera	(Convalescent Sera)	
	Number with Anti-HB <sub>C</sub>		Number with Anti-HB <sub>C</sub>	Number with Anti-HB <sub>S</sub>
22 HB <sub>S</sub> Ag+ cases	21	8.5 (3-33)	20	12*
19 HB <sub>S</sub> Ag- cases	7	9.4 (2-25)	7**	6

\* One patient, anti-HB<sub>C</sub>+, anti-HB<sub>S</sub>- in which HB<sub>S</sub>Ag persisted

\*\* 4 of 7 same patients as acute phase

The efficiency of anti-HB<sub>C</sub> in defining onset of subclinical hepatitis B is described in Table 4. The data is derived from 2333 soldiers followed for 1 year. Only individuals with follow-up sera available are included in this table. Of the total tested at 4, 8 and 12 months, were 48 soldiers who developed anti-HB<sub>S</sub> at 4, 8 or 12 months respectively following their arrival at Ft. Hood. Twenty-two of these 48 had anti-HB<sub>C</sub>. Eighteen of the 22 anti-HB<sub>C</sub> positive soldiers had anti-HB<sub>C</sub> present on arrival at Ft. Hood. Those soldiers (38) with anti-HB<sub>S</sub> upon entering Ft. Hood and who remained positive for anti-HB<sub>S</sub> over the follow-up period, 92% (35) had anti-HB<sub>C</sub> and all remained positive throughout. In 37 individuals who lost anti-HB<sub>C</sub> after being initially positive, anti-HB<sub>C</sub> was present only in 17. In the fourth group listed in Table 2, 215 randomly selected soldiers negative for anti-HB<sub>S</sub> and HB<sub>S</sub>Ag, were examined for anti-HB<sub>C</sub>. The only bias in the selection process was that individuals had to have

a follow-up specimen at eight and/or twelve months. Out of 215 individuals negative for all surface markers (anti-HB<sub>s</sub>/HB<sub>s</sub>Ag), 22 had anti-HB<sub>c</sub>. Fifteen of these 22 soldiers developed anti-HB<sub>c</sub> while at Ft. Hood. The remaining 7 individuals (3.2%) represent the prevalence of anti-HB<sub>c</sub> without HB<sub>s</sub>Ag, or anti-HB<sub>s</sub> entering the endemic area.

Table 4 - Detection of Anti-HB<sub>c</sub> in soldiers with subclinical hepatitis B over one year.

<u>Anti-HB<sub>s</sub> Status over one year course</u>	<u>Number</u>	<u>Total Anti-HB<sub>c</sub>+</u>	<u>Number Anti-HB<sub>c</sub>+</u> <u>Initially</u>	<u>Number Developing anti-HB<sub>c</sub> while in endemic area</u>
Negative to Positive	48	22	18	4
Positive for one year	38	35	35	0
Positive to Negative	37	17	17	0
Never Positive for Anti- HB <sub>s</sub> and/or HB <sub>s</sub> Ag	215	22	7	15

In Table 5, additional evidence for HBV infection beyond that which can be obtained by surface antigen or antibody was noted in the prevalence survey of index and control units. Twenty soldiers (13 index unit, 7 control units) were identified as having only anti-HB<sub>c</sub>. Since no other specimen was available it is not possible to state when the HBV exposure occurred.

Table 5 - Prevalence of anti-HB<sub>c</sub> in military platoons with current acute hospitalized case of hepatitis B and control platoons.

	<u>Number Studied</u>	<u>HB<sub>s</sub>Ag+</u>	<u>Anti-HB<sub>s</sub>+</u>	<u>Anti-HB<sub>c</sub></u>
Index case platoons	377	5	53	46 (13)*
Control platoons	520	5	70	57 (7)*

\*Individuals identified in respective units without anti-HB<sub>s</sub> or HB<sub>s</sub>Ag as evidence of HBV infection.

In summary, the present RIAI test for anti-HB<sub>C</sub> detection provides a useful tool for the study of the seroepidemiology of both clinical and subclinical hepatitis B infection. It is apparent from this data that the detectable anti-HB<sub>S</sub> response following acute and/or subclinical hepatitis B infection is variable. Unlike most other viral infections, hepatitis B has a relatively prolonged clinical course with slow development of anti-HB<sub>S</sub>. Such observations on the host immune response to wild strains of the virus are thus important in the context of planning vaccine efficacy trials. The presence of anti-HB<sub>C</sub> will be a major piece of serological evidence of hepatitis B infection in individuals immunized with the HB<sub>S</sub>Ag vaccine.

### III. Gamma globulin surveillance for anti-HB<sub>S</sub> titer.

In May 1975 (NEJM 293: 1093-1094) a series of articles were published concerning the use of gamma globulin with varying anti-HB<sub>S</sub> titers in persons with parenteral and non-parenteral exposure to HBV. Although the conclusions derived from these studies were difficult to interpret because of the non-comparable populations and the multiplicity of variables inherent in the experimental design, recommendations were made for the use of high titered immune serum (anti-HB<sub>S</sub>) globulin and/or standard commercial gamma globulin with a titer of anti-HB<sub>S</sub>. (Table 6) Since the efficacy of standard gamma globulin containing anti-HB<sub>S</sub> in prevention of drug associated Hepatitis B has not been established to be effective for use in the military situation of illicit drug use, the military has not extensively used gamma globulin to prevent hepatitis B. Because immune serum globulin in at least one study has been shown to only temporarily decrease in the incidence of acute hepatitis B by delaying the incubation period and decreasing the development of passive active immunity, the use of gamma globulin in the military should be carefully evaluated.

All newly acquired lots of gamma globulin are now monitored for anti-HB<sub>S</sub> level. (Table 7) This information will be available to preventive medicine personnel in the field. At the present time, gamma globulin is the only potential preventive measure available until a safe hepatitis B vaccine is available. When new techniques become available, military lots of gamma globulin will be tested for titers of antibody to hepatitis A and possibly C in the future. Such information will allow the military to select lots of gamma globulin that will be most efficacious in aborting hepatitis A outbreaks.

### IV. Prevalence of HB<sub>S</sub>Ag and Anti-HB<sub>S</sub> in Transamazon colonists.

In conjunction with USAMRU, Belem, the prevalence of HB<sub>S</sub>Ag and Anti-HB<sub>S</sub> was determined in a sample of 569 colonists along the transamazon highway west of the Town of Maraba. Prevalence of HB<sub>S</sub>Ag and anti-HB<sub>S</sub> by age and sex distribution is shown in Table 8.

Table 6 - Indications for use of Hyperimmune Hepatitis B Globulin or Standard Globulin

(Editorial: Alter, Barber and Holland NEJM 293: 1093-1094, 1975)

- Indications - 1. Acute intense exposure to HBV
- (a) Parenteral inoculation with HB<sub>s</sub>Ag in blood or secretions.
  - (b) Intimate contact with patients who have acute HB<sub>s</sub>Ag positive hepatitis.
2. Repeated HBV exposure (Dialysis Wards, Endemic Areas)
- (a) Standard gamma globulin may be more efficacious if passive active immunity is increased.

Contraindications

- 1. Individuals positive for anti-HB<sub>s</sub> (relative contraindications)
- 2. Post-transfusion hepatitis
  - (2) Few cases exist at present with RIA techniques that eliminate HB<sub>s</sub>Ag+ blood units.

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Table 7 - Lots of gamma globulin monitored for anti-HB<sub>s</sub> titer (AusAb test)

	<u>Anti-HB<sub>s</sub> Titer</u>
M 4483	1:256
M 4835	1:512
M 4862	1:512
M 4878	1:256
M 5146	1:512
M 5162	1:256
MO 4638 7A	1:2

Table 8 - Age-Sex Prevalence of HB<sub>s</sub>Ag and Anti-HB<sub>s</sub>, Maraba Colonists

Age	M A L E			F E M A L E		
	No. tested	No. with HB <sub>s</sub> Ag	No. with anti-HB <sub>s</sub>	No. tested	No. with HB <sub>s</sub> Ag	No. with anti-HB <sub>s</sub>
1-4	25	1	7	27	0	3
5-9	67	1	14	50	2	16
10-14	50	3	11	54	2	18
15-19	22	2	9	29	1	15
20-29	24	0	7	39	5	13
30-39	41	2	22	37	1	20
40-59	56	4	32	34	1	20
60+	8	0	3	6	2	2
Totals	293	13 (4.4%)	105 (35.8%)	276	14 (5.1%)	107 (38.7%)

The overall prevalence of HBV infection is listed in Table 9. The prevalence of markers of infection is prevalent in this Brazilian population; this data indicates that hepatitis B indeed past evidence of HBV infection was found in only a slightly lower proportion of Brazilians (10%) by age than in a lower socioeconomic group in Bangkok, Thailand. Since these colonists have only recently settled along the Transamazon highway, it is not certain whether the prevalence data reflect HBV transmission in their previous or current residence. Sera obtained 1 year after this initial bleed will be tested to determine the incidence of HBV infection along the highway. Such information will enable preventive medicine personnel to quantitate the degree of endemicity of hepatitis B infection. Eventually the sera will be used determining the prevalence and incidence of hepatitis A in this area.

#### V. Clinical Service

The hepatitis lab has supported the renal transplant and the

general medical service at WRAMC. Diagnostic serological services of detecting anti-HB<sub>s</sub>, anti-HB<sub>c</sub> and HB<sub>s</sub>Ag subtyping have been made available to clinicians in specific instances when the information is of benefit to the care of individual patients and is not readily available in the routine clinical laboratory. Recently all patients and staff of the renal ward were tested for HB<sub>s</sub>Ag and anti-HB<sub>s</sub>. The detection of anti-HB<sub>s</sub> in staff members will be important in assigning personnel to work with known antigenemic patients and thus prevent the spread of hepatitis B to other susceptibles.

Table 9 - Prevalence of HBV in Maraba Colonists

Age	No. With			
	No. Tested	HB <sub>s</sub> Ag <sup>+</sup> Anti-HB <sub>s</sub>	Anti-HB <sub>s</sub> alone	Total (%)
1-4	52	1	10	11 (21)
5-9	117	3	29	32 (27)
10-14	104	5	28	34 (33)
15-19	51	3	22	25 (49)
20-29	63	5	19	24 (38)
30-39	78	3	40	43 (55)
40+	104	7	53	60 (58)

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Disease and Immunology

Work Unit 176 Mechanisms of Transmission of Hepatitis Viruses

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6538	76 07 01	DD-DR&E/AR)636	
3 DATE PREV SUMMARY	4 R NO OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8A ORIGIN INSTR	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
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10 NO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	61102B	3A161102B71Q	00	177			
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C. CONTRACTOR	CARDS 114F						
11 TITLE (Precede with Security Classification Code)							
Characteristics of Attenuated Dengue Viruses							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS							
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17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER				FISCAL YEAR		C. FUNDS	
C. TYPE				CURRENT		D. FUNDS	
A. KIND OF AWARD				76		122	
F. CUM. AMT.				77		154	
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 CD&I			
				ADDRESS * Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME Joy, R.J.T., COL, MC				NAME * Harrison, Venton R.			
TELEPHONE 202-576-5551				TELEPHONE 202-427-5109			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
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				NAME: Hampton, Charles M.			
23 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Arbovirus; (U) Dengue; (U) Vaccine; (U) Immunity; (U) Attenuation; (U) Tissue Culture							
24 TECHNICAL OBJECTIVE * 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. (U) The objective is development, production, and assay of live-attenuated vaccines against classical strains of dengue viruses. The major types (1,2,3, and 4) of this virus are endemic throughout populated areas of the world, and although mortality rates are low, the incapacitation effected by these viruses and their associated sequelae could have serious impact on military time-tables and troop mobility.</p> <p>24. (U) Selected strains are subjected to multiple passages and frequent cloning in tissue culture systems, to produce pure progeny characterized by reduced virulence and adequate antigenicity, that will serve as candidate vaccine seed virus.</p> <p>25. (U) 75 07 - 76 06 A dengue (Type 2) vaccine seed was prepared in FRhL cells and tested thoroughly for the presence of adventitious agents. The vaccine virus was also used in preliminary experiments, prior to vaccine production, to establish parameters for growth and stability. Lot 1 of live-attenuated dengue 2 vaccine was prepared in FRhL cell roller flasks and freeze-dried after attenuation markers were examined. Safety tests of the vaccine were performed by in vivo and in vitro methods in accordance with FDA Regulations on Viral Vaccines. Virulence tests performed in lower primates with both seed virus and final vaccine indicated that attenuation characteristics did not change through the final vaccine production cycle. The vaccine may be considered ready for human trials. As a potential candidate vaccine strain, a human isolate of dengue 3 virus was subjected to four passages in primary monkey kidney cells. On passage it was observed that the dengue 3 virus increased in titer and the appearance of a mixed population of large and small plaque progeny correlated with a decrease in temperature sensitivity. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$26 000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>							

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 177 Characteristics of Attenuated Dengue Viruses

Investigators.

Principal: Venton R. Harrison, M.S.

Associate: Kenneth H. Eckels, Ph.D.; Charles M. Hampton

Description.

Our primary objective is the development, production, and assay of live-attenuated vaccines against classical strains of the dengue virus. The major types (1, 2, 3, and 4) of this virus enjoy widespread geographical endemicity throughout populated areas of the world, and although mortality rates are low, the incapacitation effected by these viruses and their associated sequelae could have serious impact on military time-tables and troop mobility. Selected strains of the dengue viruses are subjected to multiple passages and frequent cloning in susceptible tissue culture systems, to produce pure progeny characterized by reduced virulence and adequate antigenicity, that will serve as candidate vaccine seed virus.

Progress.

I. Dengue-2 vaccine strains.

A. Vaccine seed preparation.

Fetal rhesus lung (FRhL) pass-21 cells from Lederle Laboratories did not pass mycoplasma testing and passage of D-2, S-1, p-19a seed was repeated in FRhL pass-18 cells from Merrell-National Laboratories. Table 1 lists the data for three successive passages of S-1 in FRhL cells resulting in a vaccine seed virus. Difficulties with reversion were linked with efforts to harvest reasonably high titers of S-1 seed from the FRhL cells. Inoculation with a low MOI was necessary to prevent reversion to large plaque virus.

The S-1, FRhL-3 vaccine seed virus was tested for adventitious microbial agents. Tests for bacterial and mycoplasmal contaminants were negative after inoculation of the seed in trypticase soy, thioglycollate, and mycoplasma broths. Tests for adventitious viral agents were done in adult and suckling mice and in four types of cell cultures. Young adult mice remained healthy after receiving a combination intracerebral and intraperitoneal inoculation of S-1 seed virus. Suckling mice inoculated by the same routes using neutralized S-1 virus also

remained healthy over a period of 3 weeks. Tests of the S-1 seed after two passages in WI-38, primary rabbit kidney, primary rhesus monkey kidney, and primary green monkey kidney cell cultures were also negative for cytopathic or morphologic changes in the cell monolayer.

#### B. Growth and stability of the S-1 vaccine seed.

Preliminary experiments with the S-1 vaccine seed virus were done prior to vaccine production to better establish parameters necessary for high yields of small plaque virus with low reversion to large plaque virus. Roller flasks of FRhL cells were inoculated with S-1 vaccine seed virus at a low MOI (0.000013). Flasks were divided into 3 sets: a) day 4 media change; b) day 7 media change; c) no media change. The results of this experiment are recorded in Table 2.

Table 1. Passage of S-1, p-19a virus in FRhL cells.

Passage	Inoculation MOI	Harvest (day)	PFU/ml		Suckling mouse	
			35C	39.5C	IC	LD <sub>50</sub> /ml
1	0.01	7	$1.4 \times 10^4$	0		ND
2	0.003	9	$3.0 \times 10^5$	$2.5 \times 10^1$		ND
3 (vaccine seed)	0.00005	10	$4.2 \times 10^3$	0		<50% death with undilute seed

Table 2. Growth parameters of the S-1 vaccine seed in FRhL roller flasks.

Media change (day)	Harvest (day)	PFU/ml		Number of flasks containing large plaque virus
		35C	39.5C	
4	6	$6.9 \times 10^4$ *	0	0/3
	8	$3.7 \times 10^4$	0	2/10
7	8	$7.7 \times 10^4$	0	1/2
no change	8	$2.3 \times 10^2$	0	0/2

\* Average PFU titer for flasks containing only small plaque virus.

A growth curve of S-1 in FRhL roller flasks shows an optimal harvest time of 6 to 7 days after a media change on day 4 (Table 3). From the data in Tables 2 and 3, it is evident that a media change followed by a harvest 1 to 2 days later is necessary to boost the titer of S-1.

Table 3. Growth curve of the S-1 vaccine seed in FRhL roller flasks.

Day	PFU/ml (35C)
4 (media change)	$7.1 \times 10^{2*}$
5	$1.9 \times 10^4$
6	$6.9 \times 10^4$
7	$7.7 \times 10^4$
8	$4.4 \times 10^4$

\* Average titer of 3 flasks.

Another set of experiments was done to determine optimal conditions for storage of the bulk fluid harvest of S-1 virus after growth in FRhL cells. A minimal holding period after harvest of 7 days was necessary for plaque tests to be completed and large plaque revertant fluids to be discarded. Three methods of holding were tested: a) 4C, no freeze; b) shell freeze in acetone and dry ice and hold at -70C; c) plug freeze in liquid nitrogen and hold at -70C. All fluids at the time of harvest received a final concentration of 2.5% human serum albumin (HSA) in order to stabilize infectivity of the virus. The results for holding S-1 virus harvests under these conditions are listed in Table 4. After 7 days, the frozen harvests were thawed and plaqued along with the harvests held at 4C. A sample of S-1 fluids from each set of harvests was also freeze dried and, after 18 days, rehydrated and plaqued. All harvests were then held at 4C and another sample taken for plaquing at 14 and 21 days. Stability of S-1 infectivity was excellent by any of the storage methods with usually no more than a 50% reduction in titer (Table 4). Freeze drying was also feasible after the 7 day holding period at either 4C or -70C.

The preliminary experiments indicated an optimal harvest time for S-1 in FRhL roller flasks of 6 days after a media change on day 4. The harvests usually had a titer of  $> 5 \times 10^4$  PFU/ml and no more than 20% of the inoculated flasks contained large plaque virus. Harvests held at 4C or -70C for 7 days resulted in a minimal loss of infectivity as long as HSA was used as a stabilizer in the harvest fluids.

Table 4. Stability of S-1 virus harvests by storage at 4C or -70C.

Storage	Harvest(day 6)	Days post storage			
	PFU/ml	7	14	21	freeze dried
4C, a	$5.0 \times 10^5$	$2.1 \times 10^5$	$3.6 \times 10^5$	$3.4 \times 10^5$	$1.7 \times 10^5$
	b $4.4 \times 10^5$	$1.8 \times 10^5$	$3.3 \times 10^5$	$2.5 \times 10^5$	$1.8 \times 10^5$
	c $7.0 \times 10^5$	$1.8 \times 10^5$	$4.4 \times 10^5$	$3.7 \times 10^5$	$3.0 \times 10^5$
shell freeze, a	$3.7 \times 10^5$	$2.8 \times 10^5$	$5.5 \times 10^5$	$3.0 \times 10^5$	$3.4 \times 10^5$
	b $1.1 \times 10^{6*}$	$7.5 \times 10^5$	$1.4 \times 10^6$	$1.1 \times 10^6$	$5.0 \times 10^5$
plug freeze, a	$3.2 \times 10^5$	$1.5 \times 10^5$	$3.7 \times 10^5$	$2.6 \times 10^5$	$2.0 \times 10^5$
	b $7.0 \times 10^5$	$2.6 \times 10^5$	$3.9 \times 10^5$	$4.2 \times 10^5$	$2.0 \times 10^5$

\* Contained large and small plaque virus.

#### C. Vaccine preparation.

Lot 1 of D-2, S-1 vaccine was prepared using procedures established in preliminary experiments. FRhL (Merrell-National, p-4) roller flasks were inoculated with the S-1 vaccine seed virus diluted to 1:20 resulting in an MOI of  $5 \times 10^{-4}$ . Media was changed on day 4 and vaccine fluids were harvested on day 6. HSA (2.5%) was immediately added upon harvest and culture fluids centrifuged at 2,000 rpm for 20 min. Following filtration through a 0.45  $\mu$  Nalgene membrane, vaccine fluids from each roller flask were held separately at 4C and plaque assays were performed on the fluids from each flask. Due to centrifuge tube breakage, low virus titers, and large plaque virus reversion, a large proportion of the harvested fluids had to be discarded. The remaining fluids were pooled and, after removal of aliquots for reference storage, were freeze dried by Dr. S. Berman, Dept Biologics Research. No further HSA was required prior to freeze drying since a final concentration of 2.5% was present from the day of harvest. A total of 326 X 3 ml vials was prepared. Twenty-five were used for final container tests for sterility, mouse inoculation, and plaque titer.

Lot 1, D-2 vaccine was tested for safety following the procedure outlined previously for D-2 vaccine seed virus. All tests were successfully completed without detection of any adventitious agents. In addition, neutralization tests using anti-D-2 reference ascitic fluid (NIH) identified the S-1 vaccine fluids as a D-2 type. The final product, after rehydration and plaquing of 10 individual freeze dried vials of S-1 vaccine resulted in an average plaque titer of  $8.5 \times 10^4$  PFU/ml with no detectable large plaque virus present. The log suckling mouse (SM) intracerebral LD<sub>50</sub>/ml was 3.9 and the SM LD<sub>50</sub>/PFU ratio was 0.08.

D. In vivo studies with selected strains of D-2 virus in rhesus monkeys.

It was previously shown (WRAIR Annual Progress Report 74-07 - 75 06) that the rhesus monkey serves as the best model for the determination of in vivo virulence by subcutaneous inoculation. In order to monitor the attenuated status of the D-2 strain used for vaccine production, 2 rhesus monkeys were inoculated subcutaneously with dengue-2 vaccine seed virus (D-2/S-1/FRhL3a) and 5 monkeys were inoculated in a similar manner with the final vaccine product. All monkeys were determined to be D-2 antibody-free prior to inoculation. Blood samples were collected from all monkeys on 10 consecutive days following inoculation for the determination of circulating virus levels and at 15, 30, and 45 days post-inoculation for CF and HI antibody content. As controls, 2 monkeys were inoculated with the parent virus (D-2/PGMK6). It is clearly evident from the results listed in Table 5 that the attenuation characteristic was retained through the vaccine production cycle. It may be of interest to note that the 2 monkeys receiving vaccine seed virus were solidly protected against a challenge 90 days later with the parent virus (D-2/PGMK6).

E. Neurovirulence tests of live-attenuated D-2 vaccine.

In accordance with FDA Regulations on Viral Vaccines, Part II, SubChapter F, Part 630, pertaining to neurovirulence testing of live-attenuated viral vaccines, 11 male rhesus monkeys previously shown to be free of D-2 antibody, were inoculated in the following manner with final vaccine: 0.5 ml intrathalamically into each hemisphere and 0.5 ml intraspinally. Two control monkeys received similar inoculations with virus-free growth fluids. All monkeys were held 19 days and observed daily for evidence of CNS involvement or other physical abnormalities. Upon completion of the required holding period, all animals appeared to be in overt good health, and a serum sample was taken for evidence of D-2 replication and antibody stimulation (Table 6). At this time, under deep pentothal anesthesia, each monkey was perfused with 10% formalin and appropriate tissues from: a) lumbar cord, b) cervical cord, c) lower medulla, d) upper medulla, e) mesencephalon, and f) motor cortex, were removed and examined histologically for evidence of viral pathology. Results of these tests are fully described in the following report submitted by the Dept of Pathology, WRAIR (Dr. Tatsuo Hase and Dr. Paul K. Hildebrandt).

Gross and histologic findings of the brains and the spinal cords of the monkeys injected with attenuated Dengue-2 virus into the bilateral thalami and the lumbar spinal cords.

This study consists of 11 rhesus monkeys which were injected with 1 cc each of attenuated Dengue-2 virus containing culture medium into the bilateral thalami and the lumbar spinal cords, and 2 rhesus monkeys which were injected with the same amount of culture medium without virus as the control.

Intrathalamic injection was done stereotaxically under ketamin anesthesia and injection into the spinal cord was made according to the procedure of routine spinal puncture.

Formalin-fixed brains are examined by successive coronal sectioning at 3 to 4 mm thickness, and, as in the previous experiment, the following sections of the brain are selected for histological examination:

- OP (R&L) - at the level of the optic chiasm
- CP (R&L) - at the level of the middle of the cerebral peduncle
- BP (R&L) - at the rostral end of the brachium pontis
- BP2 (R&L) - at the caudal end of the brachium pontis

In addition, sections are taken from the medulla, C-4, T-6, T-12, L-1, and L-2.

In gross examination of the brains, the pia arachnoids and the brain surfaces are not remarkable. The points of needle entry are identified as small pits with minimal meningeal reaction most frequently in the anterior central gyri, 3 mm anterior from the central sulci and 3 mm lateral from the edges of the sagittal fissure. In coronal sectioning, needle tracks are observed passing through the cerebral cortex, the subcortical white matter, the corpus callosum, the lateral ventricle, and the medial thalamus. Injection of the material into the medial thalamus appears to have pushed some of the injected material either laterally into the internal capsule or inferiorly into the subthalamic region or inferoposteriorly into the path of the medial lemniscus of the midbrain or superiorly to the choroid plexus of the lateral ventricle and the subcortical white matter along the needle track, because lesions are found exclusively in one or more of the above regions in addition to the thalamus.

In histological examination of H&E sections, the lesions show principally a picture of repair process consisting of proliferation of glial cells and capillaries. Mild inflammatory reactions of edema and inflammatory cell infiltration are present in and around the lesions. Perivascular mononuclear cell reaction is minimal to mild, and a typical picture of perivascular cuffing is rarely seen. The inflammatory reaction is limited in the areas of lesions. It shows some variation among the cases; however, no clear demarcating is recognized between the experimental and control groups in blind study. The histological finding of the spinal cord is similar to that of the brain and mainly consists of proliferation of glial cells and capillaries with mild inflammatory cell infiltration at the site of injection, and are interpreted as being a response to the injection of foreign material. There is no evidence of a viral inflammatory reaction, and no difference in lesions could be detected between experimental and control animals.

Table 5. Summary of data on rhesus monkeys inoculated with D-2 vaccine seed virus and D-2 live-attenuated vaccine.

Monkey No.	Inoculum and dose	Viremia on day post-inoculation										1/CF			1/HI		
		1	2	3	4	5	6	7	8	9	10	15	30	45	15	30	45
937	Seed virus (D-2/S-1/FRhL3a) 3 X 10 <sup>3</sup>	-	-	-	-	-	-	-	-	-	-	8	8	8	20	10	10
938	"	-	-	-	-	-	-	-	-	-	-	16	32	8	40	20	20
867	Vaccine (D-2/S-1/FRhL4a) 3.8 X 10 <sup>4</sup>	-	-	-	-	-	-	-	-	-	-	16	16	32	20	20	20
810	"	-	-	-	-	-	-	-	-	-	-	8	8	16	<10	10	10
853	"	-	-	-	-	-	-	1*	-	-	-	32	32	32	20	20	40
792	"	-	-	-	-	-	-	-	-	-	-	16	32	32	20	20	40
179	"	-	-	-	-	-	-	-	-	-	-	16	16	16	<10	10	10

\* PFU/0.2 ml



Table 6. Summary of serological data on rhesus monkeys used for neurovirulence testing of D-2 vaccine.

Monkey No.	19 days post-inoculation *	
	1/CF	1/HI
335	128	40
340	64	40
744	128	20
568	64	40
741	32	20
345	64	40
341	64	80
976	256	320
333	64	20
036	64	80
185	128	160
Controls **		
176	< 4	< 10
849	< 4	< 10

\* Each monkey received 0.5 ml of live-attenuated vaccine in the right and left thalamus and lumbar spine enlargement.

\*\* Control monkeys received a placebo of virus-free growth fluids via same routes of inoculation described above.

F. In vitro markers for the S-1 clone.

It has been our hope to more fully understand the relationships between virulence and in vitro characteristics of D-2 virus so that applications can be made toward development of safer and more effective vaccines. D-2 clones designated S-1 (vaccine strain) and C-2a have been isolated and are temperature sensitive (ts) when compared to a parent virus. Although both S-1 and C-2a had similar plating efficiencies ( $10^{-4}$ ), they differed in mouse virulence with C-2a having an LD<sub>50</sub> similar to the parent virus (Table 7). The finding that

Table 7. Characteristics of D-2 clones.

Clone	Plaque size	Mouse neurovirulence SMIC LD <sub>50</sub> /PFU	Plating efficiency 39.5C/35C
S-1	1-2 mm	0.01	$10^{-4}$
C-2a	2-3 mm	0.2	$10^{-4}$
GM-6 (parent)	2-4 mm (mixture)	0.5	$10^{-1}$

C-2a had a higher mouse virulence while still being ts made it interesting for in vitro marker comparison with S-1.

Growth in primary green monkey kidney (PGMK) cells showed a lower non-permissive temperature for C-2a when compared to S-1 (Table 8). The temperature had to be raised to 40C in PGMK cells to significantly effect S-1 replication. At 39.5C, C-2a titers were markedly reduced, and at 40C replication was completely shut off. Replication of the parent at the higher temperatures occurred with thermal inactivation accounting for the lower titers at 39.5C and 40C.

Attempts to characterize the S-1 and C-2a mutants as RNA(+) or RNA(-) using conventional methods of labelled uridine incorporation into virus-specific RNA were not successful. A quantitative CF test using anti-poly I·C serum was successful in detecting low levels of dsRNA in virus-infected cells (Schwartz and Stollar, 1969). PGMK cells infected with S-1, C-2a, or parent viruses were collected and suspended in CF test buffer and sonicated for a short period to break cells. The uncentrifuged extracts were used as the source of antigen in quantitative CF tests with anti-poly I·C serum obtained from Miles Laboratories. The extent of complement fixation was calibrated using known amounts of synthetic poly I·C. These values were plotted and used as a standard curve for graphical determinations of unknown amounts of dsRNA.

Table 8. Replication of DEN-2 clones in PGMK cells.

Clone	Day post inoc	Day post inoc (PFU/ml)		
		35C	39.5C	40C
S-1	1	$4 \times 10^2$	< 5	< 5
	2	$3 \times 10^4$	$4 \times 10^2$	< 5
	3	$3 \times 10^4$	$5 \times 10^2$	$2 \times 10^1$
C-2a	1	$2 \times 10^2$	$4 \times 10^1$	< 5
	2	$9 \times 10^3$	$2 \times 10^1$	< 5
	3	$2 \times 10^4$	5	< 5
GM-6 (parent)	1	$2 \times 10^4$	$5 \times 10^3$	$1 \times 10^3$
	2	$1 \times 10^5$	$1 \times 10^4$	$5 \times 10^2$
	3	$1 \times 10^5$	$4 \times 10^3$	$1 \times 10^1$

Table 9 lists the ng amounts of dsRNA found in infected cells. These are net weights resulting from the difference between uninfected cell and infected cell values. The kinetics of S-1, dsRNA production were hard to follow, however, there appeared to be an accumulation of dsRNA at all three temperatures with no clearing after 3 days. With C-2a there was some accumulation but it was cleared by the third day. The parent appeared not to accumulate dsRNA as much as the two mutants, except possibly at 39.5C.

Involvement of double stranded polynucleotides with interferon induction is well documented. Also, ts mutants in many cases have been found to be very good interferon inducers. Table 10 lists the results of pre-treating monkey kidney cells with heat inactivated supernatant fluids from growth curves of S-1, C-2a, and parent viruses. Challenge was with Semliki Forest virus (SFV) and the presence of interferon inferred by the reduction in the number and size of SFV plaques. The only detectable interferon found using this system was at 35C for all three viruses. The two ts mutants were not better interferon inducers

Table 9. Immunochemical detection of dsRNA in PGMK cells infected with DEN-2 clones.

Clone	Day post inoc	Net ng dsRNA/10 <sup>5</sup> cells (PFU/ml)		
		35C	39.5C	40C
S-1	1	180 (4 X 10 <sup>2</sup> )	90 ( <5 )	40 ( <5 )
	2	60 (3 X 10 <sup>4</sup> )	100 (4 X 10 <sup>2</sup> )	90 ( <5 )
	3	190 (3 X 10 <sup>4</sup> )	110 (5 X 10 <sup>2</sup> )	40 (2 X 10 <sup>1</sup> )
C-2a	1	160 (2 X 10 <sup>2</sup> )	20 (4 X 10 <sup>1</sup> )	50 ( <5 )
	2	100 (9 X 10 <sup>3</sup> )	100 (2 X 10 <sup>1</sup> )	<10 ( <5 )
	3	<10 (2 X 10 <sup>4</sup> )	<10 ( 5 )	<10 ( <5 )
GM-6 (parent)	1	<10 (2 X 10 <sup>4</sup> )	70 (5 X 10 <sup>3</sup> )	10 (1 X 10 <sup>3</sup> )
	2	10 (1 X 10 <sup>5</sup> )	70 (1 X 10 <sup>4</sup> )	20 (5 X 10 <sup>2</sup> )
	3	30 (1 X 10 <sup>5</sup> )	10 (4 X 10 <sup>3</sup> )	<10 (1 X 10 <sup>1</sup> )

Table 10. Interferon assay of DEN-2 clones.

Clone	Day post inoc	% SFV plaque reduction		
		35C	39.5C	40C
S-1	1	0	0	0
	2	10	0	0
	3	50	0	0
C-2a	1	0	0	0
	2	10	0	0
	3	30	0	0
GM-6 (parent)	1	20	0	0
	2	10	0	0
	3	80	0	0

than the parent. Also, there was little correlation between the presence of dsRNA in cell extracts and the amount of interferon induced under the same experimental conditions.

Another marker that was examined was the ability of the ts mutants to make viral-specific antigen at the nonpermissive temperature. Indirect fluorescent antibody techniques were used; other methods such as CF were not sensitive enough to detect small amounts of antigen. Per cent fluorescing cells in a total of 400 to 800 cells was scored for D-2 positive antigen. Due to the low MOI (0.1 to 1.0), per cent fluorescence was low. However, a statistically significant figure for positive fluorescence results from the large number of cells scored. From Table 11, S-1 gave positive fluorescence without plaque formation at 39.5C, one day post inoculation. However, this was not a nonpermissive temperature for S-1 and an increase in fluorescence and infectivity was seen at this temperature. The C-2a mutant produced positive fluorescence at 39.5C and 40C without release of infectious virus.

Table 11. Antigen production in PGMK cells infected with DEN-2 clones.

Clone	Day post inoc	% fluorescing cells (PFU/ml)		
		35C	39.5C	40C
S-1	1	15 ( $1 \times 10^3$ )	5 (<5)	ND (<5)
	2	40 ( $4 \times 10^4$ )	8 ( $9 \times 10^3$ )	<1 (<5)
C-2a	1	4 ( $5 \times 10^1$ )	4 (<5)	2 (<5)
	2	18 ( $3 \times 10^3$ )	1 (<5)	<1 (<5)
GM-6 (parent)	1	18 ( $6 \times 10^3$ )	9 ( $2 \times 10^1$ )	6 ( $2 \times 10^2$ )
	2	11 ( $5 \times 10^3$ )	16 ( $6 \times 10^2$ )	<1 ( $2 \times 10^1$ )

We have tried to broaden our understanding of some of the molecular events that occur during replication of D-2, ts mutants. One of these mutants, S-1, is most important since it has a high potential for use as a human vaccine virus: 1) Lower nonpermissive temperature does not always correlate with lower animal virulence. With C-2a and S-1 in PGMK cells, 39.5C and 40C were the respective nonpermissive temperatures. The C-2a mutant, however, had the higher suckling mouse neurovirulence

of the two clones; 2) With both mutants there is found what appears to be an accumulation of dsRNA which is not seen for the parent. However, this does not appear to be correlated with higher temperatures and is found also at 35C. This may suggest a defect in replication that may or may not be temperature sensitive; 3) Interferon levels in mutant-infected cells were no greater than in parent-infected cells. Higher levels of dsRNA did not correlate with increased interferon production; 4) Intracellular antigen production was seen with one of the mutants (C-2a) at the nonpermissive temperature and this may represent an RNA(+) mutant.

## II. Dengue-3 vaccine strains.

### A. Detection of an adventitious agent in D-3, parent seed stock.

Mr. D.R. Dubois, Dept of Biologics Research, originally detected a cytopathic agent in WI-38 cells inoculated with D-3 parent (PGMK pass 4) seed virus. Patches of rounded cells were observed in the WI-38 monolayer 30 days after inoculation. The agent was passaged in WI-38 cells and attained a titer of  $1 \times 10^5$  TCID<sub>50</sub>/ml after four passages. WI-38 cells inoculated with a second passage of the agent and stained with hematoxylin and eosin revealed nuclear and cytoplasmic inclusion bodies. Failure to neutralize the agent with D-3 hyperimmune ascitic fluid and the inability to produce plaques in LLC-MK2 cells ruled out the presence of D-3 virus.

Further isolation of a similar agent was made from Lederle Lot No. 0948 PGMK cells, which were used for passage of the D-3 virus. No cytopathic agent was found in the D-3 human acute serum which was used as the source of D-3 virus. A preliminary identification of a herpes or cytomegalovirus was made on the basis of cytopathology in WI-38 and stained microscopic analysis of infected cells.

In a letter to Lederle Labs, it was strongly recommended that whole cell suspensions of PGMK should be inoculated in WI-38 cells. Since the herpes or cytomegalovirus is cell-associated, any safety test of the PGMK cells should include this type of test.

### B. Repeat passage of D-3, CH53489.

An aliquot of the human acute serum, CH5348, containing D-3 virus was thawed and inoculated in PGMK and FRhL cells. The inoculum titered  $3.8 \times 10^2$  PFU/flask. The results after four passages in PGMK cells are listed in Table 12. Results of direct inoculation and passage in FRhL cells are shown in Table 13 for one passage. Work is now in progress to subpassage in FRhL cells from both the PGMK pass-4 seed and the FRhL pass-1 seed.

Table 12. D-3, CH53489 passage in PGMK cells.

Virus harv/pass	PFU/0.2 ml		EOP
	35C (morph)	39.5C	
D-3, day 7/PGMK-1	$4.9 \times 10^3$ (small, faint)	$10^1$	0.002
day 14	$5.0 \times 10^4$ (9L @ $10^{-2}$ )	$1.0 \times 10^2$	0.002
D-3, day 7/PGMK-2	$4.7 \times 10^4$ (18L @ $10^{-2}$ )	$6.3 \times 10^2$	0.013
day 12	$3.8 \times 10^4$ (14L @ $10^{-2}$ )	$2.7 \times 10^2$	0.007
D-3, day 5/PGMK-3	$3.2 \times 10^4$ (7L @ $10^{-3}$ )	$1.3 \times 10^3$	0.041
day 10	$7.5 \times 10^4$ (24L @ $10^{-3}$ )	$2.5 \times 10^3$	0.033
D-3, day 5/PGMK-4	$7.9 \times 10^4$ (28L @ $10^{-3}$ )	$3.4 \times 10^3$	0.043
day 9	$1.3 \times 10^5$ (38L @ $10^{-3}$ )	$3.9 \times 10^3$	0.030

Table 13. D-3, CH53489 passage-1 growth curves in FRhL cells.

Growth curve - a		Growth curve - b	
Harvest (day)	PFU/ml	Harvest (day)	PFU/ml
4 *	$2.0 \times 10^1$	7	$1.0 \times 10^1$
10	$3.5 \times 10^1$	14	$1.3 \times 10^2$
17	$5.5 \times 10^1$	21	$4.5 \times 10^2$
24	$2.5 \times 10^1$	28	$1.5 \times 10^2$
31	$7.0 \times 10^2$	35	$1.1 \times 10^4$

\* Media (EMEM) replaced upon each harvest.

The data from Table 12 indicate a rise in titer of a large plaque virus population upon passage. Correlated with higher large plaque titers is a decrease in temperature sensitivity. Large plaque virus was also selected for the one passage in FRhL cells. By the 31st day in FRhL cells, large plaques were seen in harvests from these flasks.

Table 14. Summary of data on rhesus monkeys receiving a primary challenge with selected strains of Dengue-3 virus followed by a secondary challenge with the parent Dengue-2 virus 180 days later.

Challenge virus	No. viremic		1/CF			1/HI		
	No. inoculated		15d	30d	45d	**	180d	**
Primary (selected strains of D-3)	9/12		57*	72	36	< 4	240	120
							80	< 10
Secondary (D-2/PGMK-6)	8/12		430	272	180		403	340
								254

\* Geometric mean for 12 monkeys.

\*\* Titers obtained on day prior to secondary challenge.



C. Cross protection in rhesus monkeys given various D-3 virus strains.

It was previously observed (WRAIR Annual Progress Report 74-07 - 75 06) that a considerable degree of cross protection was elicited between monkeys challenged with selected strains of both D-2 and D-3 viruses, providing the challenge interval did not exceed 60 to 90 days. However, in order to determine the longevity of this cross protection status, 12 monkeys inoculated with selected strains of D-3 virus were held 6 months and given a secondary challenge with the parent D-2 virus. From the results listed in Table 14, it is apparent that negligible, if any, residual cross protection remains over this period of time.

Summary.

A D-2 vaccine seed virus was prepared in FRhL cells and tested thoroughly for adventitious agents. The D-2 vaccine virus was also used in preliminary experiments, prior to vaccine production, to establish parameters for growth and stability of the virus. Lot 1 of D-2 vaccine was prepared in FRhL cell roller flasks and freeze dried after attenuation markers were examined. Safety of the D-2 vaccine was confirmed by in vitro and in vivo techniques. Tests of the vaccine seed and vaccine virus in rhesus monkeys indicated that attenuation characteristics did not change through the final vaccine production cycle and that the vaccine is effective in stimulating D-2 antibodies.

In order to more fully understand the relationship between virulence and in vitro characteristics of D-2 virus, two ts mutants were examined. Mouse virulence, restrictive temperatures in primary cell culture, dsRNA production, interferon induction, and antigen production were compared to the parent at permissive and nonpermissive temperatures. Results are discussed.

Dengue-3 virus from a human isolate was subjected to four passages in primary monkey kidney cells. Upon passage, the D-3 virus increased in titer and the rise of a mixed population of large and small plaque virus correlated with a decrease in temperature sensitivity.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 177 Characterization of Attenuated Dengue Viruses

Literature Cited.

Reference:

1. Schwartz, E.F., and Stollar, B.D. Antibodies to polyadenylate-polyuridylate copolymers as reagents for double strand RNA and DNA-RNA hybrid complexes. Biochem. Biophys. Res. Commun. 35: 115, 1969.

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 DD DR&E(AR)636	
3. DATE PREV. SUMM <sup>a</sup>	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>a</sup>	6. WORK SECURITY <sup>a</sup>	7. REGRADING <sup>a</sup>	8. DISSEM INSTN <sup>a</sup>	9. SPECIFIC DATA- CONTRACTOR ACCESS <sup>a</sup>	10. LEVEL OF SUM A. WORK UNIT
75 07 01	R. Imp.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
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>XXXXXXXX</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 Bioengineering 002300 Biochemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
63 08		76 06		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 *				FISCAL YEAR		7	
C. TYPE				CURRENCY		310	
D. KIND OF AWARD				76		8	
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				Division of Medicine			
				ADDRESS * Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME * Olsson, COL R.A.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-427-5121			
				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 (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
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.							
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.							
25. This department was abolished per letter, SGRD-MD, 16 Jan 76 subject: Elimination of Elements of the Walter Reed Army Institute of Research. This action was a result of a congressional reduction contained in the Department of Defense Appropriations Bill for FY 76. Departmental efforts were spent in completing and preparing reports of work begun in FY 75 and reported last year. No new projects were begun. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

<sup>a</sup> 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.

PII Redacted

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 announcement in January 1976 that the Department would be closed on or about 1 July adversely affected progress. Within 2 months 3 of the 6 investigators had left the laboratory for other jobs or retirement. The efforts of the remaining staff were directed to preparing reports of completed work for submission to professional journals. No new projects were begun and as investigative activity virtually halted, the scientific work described in the FY 75 Annual Report is unchanged. Thus, the record of progress is that of publications.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 085 Circulatory Responses to Disease and Injury

Literature Cited.

Publications:

1. Olsson, R. A.: Brief Reviews: Myocardial Reactive Hyperemia. Circ. Res. 37:263, 1975.
2. Olsson, R. A. and Patterson, R. E.: Adenosine as a Physiological Regulator of Coronary Blood Flow. Prog. Molec. Subcell. Biol. 4:227, 1976.
3. Olsson, R. A., Davis, C. J., Khouri, E. M., and Patterson, R. E.: Evidence for an Adenosine Receptor on the Surface of Dog Coronary Myocytes. Circ. Res. In Press.
4. Lowensohn, H. S., Patterson, R. E., and Olsson, R. A.: Exercise Performance During Dietary Potassium Depletion in Dogs. Fed. Proc. 35:529A, 1976.
5. Elliot, E. C., Bellamy, R. F., and Olsson, R. A.: Coronary Venous  $pO_2$  and  $pCO_2$  Following Myocardial Infarction in Unanesthetized Dogs. Fed. Proc. 35, 1976.
6. Patterson, R. E., Haut, M. J., Montgomery, C. A., Lowensohn, H. S., McQuilken, C. T., Djuh, Y. Y., and Olsson, R. A.: Muscle Glycogen Metabolism in Potassium-Deficient Dogs with Paralysis: Effects of Deoxycorticosterone Acetate. Clin. Res. 24:38A, 1976.
7. Olsson, R. A., and Patterson, R. E.: Evidence for an "Adenosine Receptor: on the Coronary Myocyte Surface Physiologist 18:399, 1975.

Manuscripts Submitted for Editorial Consideration.

1. Lowensohn, H. W., Patterson, R. E., and Olsson, R. A.: Exercise Performance During Dietary Potassium Depletion in Dogs. To J. Clin. Invest.
2. Elliot, E. C., Bellamy, R. F., and Olsson, R. A.: Coronary Venous  $pO_2$  and  $pCO_2$  Following Myocardial Infarction in Unanesthetized Dogs. To Ann. J. Physiol.
3. Gregg, D. E., and Bedynek, J. L., Jr.: Compensatory Changes in the Heart During Progressive Coronary Artery Stenosis. Proc. Workshop on Pathogenetic Mechanisms of Angina Pectoris, Pisa, 1976.

4. Wray, H. L.: Cyclic Adenosine 3', 5'-Monophosphate Stimulation of Membrane Phosphorylation and  $\text{Ca}^{2+}$ -activated,  $\text{Mg}^{2+}$ -dependent ATPase in Cardiac Sarcoplasmic Reticulum. To J. Biol. Chem.
5. Lowensohn, H. S., Khouri, E. M., Gregg, D. E., Pyle, R. L., and Patterson, R. E.: Phasic Right Coronary Artery Blood Flow in Conscious Dogs with Normal and Elevated Right Ventricular Pressures. To Circ. Res.
6. Khouri, E. M., Olsson, R. A., Bedynek, J. L., Jr., and Bass, B. G.: An Implantable Semiconductor Beta Radiation Detector. To Am. J. Physiol.
7. Olsson, R. A., Gentry, M. K., and Davis, C. J.: Inhibition of Cardiac Microsomal 5'-Nucleotidase by Lectins. To Biochim. Biophys. Acta.



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>		2. DATE OF SUMMARY <sup>a</sup>		REPORT CONTROL SYMBOL: DD FORM 1498 (AR) 636	
3. DATE PREV SUMMARY		4. KIND OF SUMMARY		5. SUMMARY SECURITY <sup>a</sup>		6. WORK SECURITY <sup>a</sup>		7. REGRADING <sup>a</sup>	
75 07 01		D. Change		U		U		NA	
8. NO. CODES <sup>a</sup>		9. PROGRAM ELEMENT		10. PROJECT NUMBER		11. TASK AREA NUMBER		12. WORK UNIT NUMBER	
A. PRIMARY		61102A		3A161102B71R		02		086	
B. CONTRIBUTING									
C. <del>XXXXXXXX</del>		CARDS 114F							
13. TITLE (Precede with Security Classification Code) <sup>a</sup>									
(U) Military Hematology									
14. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>a</sup>									
008800 Life Support    002600 Biology    003500 Clinical Medicine									
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D. NUMBER <sup>a</sup>				E. TYPE				F. AMOUNT:	
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G. KIND OF AWARD:				H. CUM. AMT.				9	
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								404	
20. RESPONSIBLE OOD ORGANIZATION					21. PERFORMING ORGANIZATION				
NAME <sup>a</sup> Walter Reed Army Institute of Research					NAME <sup>a</sup> Walter Reed Army Institute of Research				
ADDRESS <sup>a</sup> Washington DC 20012					ADDRESS <sup>a</sup> Division of Medicine Washington DC 20012				
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22. GENERAL USE					SOCIAL SECURITY ACCOUNT NUMBER				
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					NAME: Ahr, MAJ D. J.				
					NAME:				
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(U) Coagulation; (U) Malaria; (U) Blood; (U) Anemia									
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)									
23. (U) Define the hematologic pathophysiology of infectious diseases of military importance, of trauma, burns and shock, and to identify modalities to restore hemostasis.									
24. (U) Procedures include biochemical methods, <u>in vitro</u> cell-free and membrane-dependent systems, large and small laboratory animal models, and studies of human subjects.									
25. (U) 75 07 - 76 06 Alterations in intracellular metabolism and membrane biophysics were studied in erythrocytes from rhesus monkeys during the course of a synchronous infection with <u>P. knowlesi</u> . The effect of purified <u>S. dysenteriae</u> 1 toxin on protein synthesis by a cell-free system isolated from rat liver was studied, and the specific effect of the toxin was localized. Alterations in glycogen metabolism in the muscles of potassium-depleted dogs were studied. Effects of benzene and certain of its derivatives on synthetic enzymes were examined. The dependence of bone marrow erythroid colony formation on pyridoxine was established in pyridoxine-deficient animals. The relationship between reduced pyridoxal kinase levels in certain populations and increased resistance to malaria was studied using two <u>in vitro</u> systems. The hypercoagulable state was examined in a group of patients with severe vascular disease. The effects of several medications on platelet function and metabolism were examined. Interaction of plant lectins with glycolipids in liposomes was studied. Cell surface alterations in lymphocytes transformed with E-phytohemagglutinin and concanavalin-A, in lymphocytes from patients with chronic lymphocytic leukemia, and in the abnormal cell of leukemic reticuloendotheliosis were studied. DNA excretion by purified lymphocyte populations (T and B cells) was studied. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$110,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.									

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AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PII Redacted



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, David H. Boldt, John A. Kark, Robert G. Knodell, August J. Salvaço, 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) study of normal blood cell metabolism and its alteration by disease or by toxic agents, (2) examination of blood cell surface membrane composition and function in normal and disease states, and (3) coagulation.

Progress:

1. Metabolism of normal blood cells and its alteration by disease or by toxic agents

The major emphasis of our metabolic studies during the past year has been on examining alterations in the metabolism of blood cells and certain other host tissues produced by infecting agents (bacterial or parasitic) or by toxic agents such as medications or environmental pollutants. The particular metabolic pathways we focused on were (a) purine nucleotide metabolism, (b) glucose metabolism, (c) cell-free protein synthesis, (d) pyridoxine (vitamin B6) metabolism, (e) heme metabolism, and (f) glycogen metabolism.

a. Purine nucleotide metabolism and b. glucose metabolism

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 a collaborative effort between CPT H. K. Webster (Div Biochemistry), LTC M. J. Haut (Dept Hematology, Div Medicine), MAJ L. Martin (Div CD&I), LTC P. Hildebrandt (Div Experimental Pathology), and Dr. J. Durocher (Pennsylvania Hospital). Young adult male rhesus monkeys are placed in a restraining chair and allowed to chair-adapt for 2 weeks. An indwelling catheter is inserted in the inferior vena cava 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 stabilize. 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 two or three cycles (48-72 hours). To date, 16 animals have been studied. They include the following groups: infected and followed through two or three cycles (parasitemia about 35%) before treatment; infected and treated at a low parasitemia; treated without having been infected; phlebotomized but non-infected (anemia controls); and non-chaired, non-infected (squeeze-cage controls).

Studies performed on these animals included measurement of the mono-, di-, and trinucleotides by high performance liquid chromatography (HPLC); assays of red cell glycolytic enzymes, particularly the kinases; measurement of red cell filterability and surface charge; and assays of selected glycolytic intermediates. The most exciting data so far are the results CPT Webster has obtained on nucleotide profiles in infected and in non-infected chloroquine-treated erythrocytes using HPLC. The most significant of these are: (1) a progressive depletion of the nucleotide pools in the parasitized erythrocyte, with the exception of ATP. ATP is spared until schizogony, when it drops rapidly; (2) the presence of these nucleotide pool changes during the pre-patent period of infection; (3) alterations in the levels of hypoxanthine and inosine intermediates during the intraerythrocytic invasion cycle, presumably because of their roles as precursors in the purine salvage pathway; (4) a lowering of adenine nucleotide levels in erythrocytes of chloroquine-treated animals. Measurement of glycolytic enzymes in erythrocytes from these monkeys revealed an increase in activity of pyruvate kinase and hexokinase in all animals following inoculation with stabilate, usually beginning before parasitemia was patent. In some animals, glucose-6-phosphate dehydrogenase and phosphofructokinase were elevated as well. Data from our laboratory and those of others suggests that the increased enzyme activity is due to contribution of parasite enzymes.

#### c. Cell-free protein synthesis

MAJ Steinberg from our department and Dr. B. P. Doctor of Div Biochemistry have successfully collaborated on a number of studies involving protein synthesis by cell-free extracts from mammalian tissues. The most provocative of these is the demonstration of protein synthesis inhibition by Shigella dysenteriae 1 toxin. The effect of this toxin, purified to near homogeneity, was examined for its possible effect on in vitro mammalian protein synthesis, using a cell-free extract from rat liver. It was shown to have no effect on aminoacylation of tRNA. However, the transfer of amino acids from AA-tRNA to ribosomes or polysomes to effect the polypeptide chain synthesis was inhibited in the presence of microgram quantities of toxin. This was evidenced by inhibition of

poly-U-directed polyphenylalanine synthesis using washed ribosomes as well as the synthesis of nascent polypeptide chains using polysomes and mixtures of amino acids. Unlike diphtheria toxin,  $\text{NAD}^+$  was not required by S. dysenteriae 1 toxin for its activity in this reaction. This study was a cooperative effort of CPT M. Thompson and Dr. B. P. Doctor of Div Biochemistry, MAJ Steinberg from our department, and Drs. P. Gemski and S. Formal from Div CD&I.

In collaboration with COL Diggs from Div CD&I, Drs. Steinberg and Doctor also developed cell-free systems for synthesis of parasite protein by extracts from (1) trypanosomes pathogenic to humans (T. rhodesiense) grown in rats and isolated from their blood stream by a column method, and (2) P. berghei-infected mouse erythrocytes.

A third collaborative project between MAJ Steinberg and Dr. Doctor was their examination of the effect of dilantin on protein synthesis in the rat brain. They found that DPH had no effect on poly-U-directed polyphenylalanine formation or natural mRNA-directed amino acid incorporation into polypeptide in rat brain. DPH also had no effect on the rate of aminoacylation of purified rat brain tRNA's and did not inhibit DNA-dependent RNA synthesis as catalyzed by RNA polymerase. Pretreatment of rats with DPH in vivo did not affect poly-U-directed polyphenylalanine synthesis in rat brain preparations.

#### d. Pyridoxine metabolism

Our major goals in studying pyridoxine metabolism are (1) to understand the metabolism of vitamin B6 in the normal bone marrow and peripheral blood cells, (2) to determine how this metabolism is altered by disease states, toxic agents (such as medications or environmental pollutants), or nutritional deprivation, and (3) to examine the effect of alterations in B6 metabolism on the course of parasitic infections. During FY 75 and the first half of FY 76, our work in this area concentrated on the first two goals. During that time, we developed a rapid, sensitive fluorometric assay for pyridoxal kinase, determined the enzymatic basis for low plasma pyridoxal-5'-phosphate in patients taking isoniazid, examined vitamin B6 metabolism in a large group of patients with idiopathic refractory sideroblastic anemia, and preleukemia-associated sideroblastic anemia, and performed extensive biochemical, ultrastructural, and bone marrow culture studies on animals rendered B6-deficient by diet alone.

Since January 1976 our vitamin B6 studies have shifted focus. We are now directing the majority of our efforts in B6 metabolism toward understanding the role of this vitamin in malaria

infection. This research involves four specific projects: (1) study of in vitro proliferation of P. falciparum and P. knowlesi in human erythrocytes with high versus low pyridoxal kinase activity; (2) examination of vitamin B6 metabolism in P. knowlesi freed from erythrocytes by saponin lysis; (3) a study of the effect of nutritional or drug-induced coenzyme B6 deprivation on the virulence of P. knowlesi infection in rhesus monkeys; and (4) a field study in Nigeria in which certain genetic polymorphisms common to people indigenous to the area will be examined for their protective value against malaria. The next several paragraphs describe first our completed patient and animal studies, and then our more recent efforts on the role of vitamin B6 in malaria.

Pyridoxal kinase activity was shown to be decreased in 16/17 patients taking isoniazid, but not in 16 untreated controls. Changes in erythrocyte pyridoxine phosphate oxidase or B6 phosphatase did not occur. A decrease of 25% in activity measured at optimum pyridoxal concentration (unphysiologically high), accompanied by a 50% drop in plasma PLP, was consistent with the hypothesis that a derivative of INH causes significant inhibition of pyridoxal kinase during administration of isoniazid for prophylaxis of active tuberculosis.

Plasma and red cell concentrations of PLP, erythrocyte pyridoxal kinase activity, erythrocyte synthesis of PLP from pyridoxine, and response of these parameters to pyridoxine treatment were determined for 25 patients with pyridoxine refractory sideroblastic anemias, 18 idiopathic cases and 7 secondary to hematologic malignancies. Low plasma PLP levels were found in nearly all subjects prior to treatment; during pyridoxine therapy they rose to a variable degree. Pretreatment red cell PLP levels were normal; after treatment they rose to 5-10 times the normal fasting concentrations. In all but two patients the ability to synthesize PLP (either from pyridoxal or pyridoxine) was normal or increased. The remaining two subjects were within the low normal range. All patients taking pyridoxine (including these two subjects) had an elevation of red cell pyridoxal kinase activity to 2-3 times the normal activity. We concluded that the circulating erythrocytes of patients with refractory sideroblastic anemia responded to pharmacologic doses of pyridoxine by synthesizing adequate amounts of PLP. Since no reticulocyte responses occurred, heme synthesis in these disorders is seldom--if ever--limited by inability to synthesize enough erythrocyte PLP. However, these results do not exclude the possibility that a population of nucleated erythroid cells dying in the bone marrow do have such a disorder.

To investigate the possibility that the above patients had defective enzyme function with physiologically low substrate levels, kinetic studies were performed on dialyzed hemolysates from 7 control subjects and 5 patients with refractory sideroblastic anemia. Linear initial PL kinase reaction velocities were obtained with varied PL and MgATP concentrations (at constant free  $Mg^{+2}$ ). Apparent  $V_{MAX}$  and apparent Michaelis constants ( $K_{PL}$  and  $K_{ATP}$ ) were derived by statistical analysis (Cleland, Adv Enz 29: 1, 1967). Hemolysates from 7 normals gave  $V_{MAX} = 45.7 \pm 2.8$  nM PLP/g hgb/hr,  $K_{PL} = 4.1 \pm .17$   $\mu M$ ,  $K_{ATP} = 5.1 \pm .98$   $\mu M$ . Apparent  $K_M$  values were normal in 5 cases of RSA but  $V_{MAX}$  was elevated (76 to 171). PL kinase activity determined in intact red cells was proportional to  $V_{MAX}$  found in hemolysates (1:2.7) for 4 patients and 3 normals.

Six dogs and six monkeys were studied during the production of severe vitamin B6 deficiency by the feeding of a chemically defined diet, and during recovery by administration of pyridoxine. New experimental diets were devised incorporating modern knowledge of trace nutrient requirements, dietary stability, amino acid balance, lipid requirements, and fiber requirements. These diets (Teklad #TD-74200 and TD-74201, made to our specifications) provided all nutrients other than vitamin B6; no other nutritional deficiencies developed in these animals. Plasma and red cell PLP concentrations fell profoundly during nutritional deprivation. Classic findings of sideroblastic anemia developed in all B6-deprived animals: microcytic, hypochromic red cells with low reticulocyte counts, increased bone marrow iron, abnormal sideroblasts, and responsiveness to vitamin B6 alone. Only the dogs developed severe anemia; monkeys showed severe protein catabolism and infections while anemia was only mild. A technique was developed for closed needle aspiration of bone marrow of rhesus monkeys through dense cortical bone of the femur into the rich medullary cavity. Monkeys were anesthetized with Ketamine and a tract into the marrow was made by drilling with a bone-setting pin. Sophisticated and precise techniques were developed for the measurement of three enzymes of heme synthesis (delta-amino-levulinic acid synthetase, d-ALA-dehydrase, and heme synthetase) in collaboration with Dr. H. Bonkowski of Dartmouth University School of Medicine. These techniques were used successfully on the liver biopsies and bone marrow aspirates obtained from our animals. Plasma clot erythroblast culture with rhesus monkey and dog bone marrows proved to be very successful; good 3-day proliferation and maturation through at least four cell divisions to mature red cells was obtained reproducibly. Rhesus monkey bone marrows in plasma clot tissue culture resembled the functional structure of erythroid maturation: an erythroblast clump attached around a central reticuloendothelial "nurse cell". This unique property will permit basic studies of the nurse-cell:erythroblast relationship which were not previously feasible. An erythroid proliferative defect was found in B6 deprivation; this was not

corrected by erythropoietin but was corrected quantitatively by vitamin B6 to a rate of proliferation far above that of control animals. As was hypothesized, older animals subjected to vitamin B6 deficiency did not develop neurologic or hepatic disease (clinical and histologic observations) as was seen in the classic experiments with very young animals.

In vitro proliferation of P. falciparum and P. knowlesi in human erythrocytes with high versus low pyridoxal kinase activity is being examined in collaboration with two groups: COL C. Diggs and MAJ D. Haines of Div CD&I, WRAIR, and Dr. L. Miller's group at NIAID, NIH. Preliminary experiments showed such a rapid uptake of B6 by erythrocytes from conventional culture media that further work has been curtailed pending restandardization of the culture systems with media containing varying amounts of B6 ranging from none to several times that found in normal media. A protocol has been written for study of the effect of nutritional or drug-induced coenzyme B6 deprivation on the virulence of P. knowlesi infection in rhesus monkeys.

To determine if low pyridoxal kinase activity confers resistance to malarial infection, our department has arranged to participate in a collaborative study with the University of Ibadan, Ibadan, Nigeria and the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland. In this study, 100 children with severe infection with P. falciparum and 150 age-matched controls will be intensively studied, particularly with regard to levels and kinetics of erythrocyte pyridoxal kinase, and complete blood group phenotyping. Our basic hypothesis is that the low pyridoxal kinase activity found in Blacks is high enough to provide adequate coenzyme B6 for normal erythrocyte functions, but is unable to supply enough coenzyme B6 to meet the parasite's needs. Therefore the parasite develops more slowly in the erythrocyte, and the host has more time to marshal its immunologic defenses.

#### e. Heme metabolism

Studies of heme metabolism have centered on two projects. One of these involves sequential measurement of several enzymes involved in heme biosynthesis, and of several porphyrin intermediates in the liver and bone marrow of animals rendered B6-deficient by administration of a chemically defined diet. In the other project, the effect of certain hydrocarbons on the activities of enzymes involved in heme synthesis is being examined. This is part of a general attempt to develop in vitro systems which will predict in vivo toxicity of environmental pollutants produced during munitions manufacture. To date, the effect of benzene, nitrobenzene, toluene, and xylene on delta aminolevulinic acid (ALA) synthetase, ALA

dehydrase, and ferrochelatase (heme synthetase) have been determined. These four compounds were found to inhibit the in vitro synthesis of heme by ferrochelatase, but to enhance the formation of delta aminolevulinate and porphobilinogen by ALA synthetase and ALA dehydrase respectively. The next step planned in this study is examination of the effect of several water-soluble breakdown products found in effluents from factories making trinitrotoluene. Several of these substances are structurally similar to toluene. We have made arrangements to procure samples of five of these through the environmental group at USAMBRTL.

#### f. Glycogen metabolism

Collaborative studies with MAJ Patterson, Dr. Lowensohn, and COL Olsson (Dept Cardiorespiratory Diseases, Div Medicine), MAJ Montgomery (Div Experimental Pathology), and Y. Y. Djuh (Dept Endocrinology, WRAMC) on the biochemical basis for muscle weakness in potassium deficiency have been successfully completed. Defective muscle glycogen metabolism is implicated in pathogenesis of weakness in potassium-deficient states because of (1) changes in muscle glycogen content in potassium-deficient rats, (2) decreased lactate production and muscle glycogen in exercising potassium-deficient dogs receiving deoxycorticosterone acetate (DOC), and (3) inhibition of glycolytic enzymes in vitro by incubation in potassium-deficient media. Muscle glycogen was evaluated in dogs receiving a potassium-deficient diet. Four received a diet alone (group I) and 5 received the diet plus DOC, 20 mg/day (group II). In group I, serum and muscle  $K^+$  decreased 40% from controls ( $p < 0.005$ ), and 3 of 4 dogs became paralyzed and died (mean survival 7 mos.). Muscle pathology suggested mild denervation atrophy, but no significant change occurred in body weight, Mg, phosphorylase (PY), muscle or serum phosphorus (P) or  $Mg^{++}$ , serum CPK or  $CO_2$  or electromyograms (EMG). In contrast, all dogs in group II became paralyzed and died within 2 months. The group II dogs developed CPK  $> 1,000$  mU/ml and pathologic findings of necrotizing myositis with regeneration consistent with steroid myopathy. Serum and muscle K did not differ from group I, but PY decreased 27% ( $p < 0.03$ ) and serum  $CO_2$  increased 36% ( $p = 0.001$ ). Body weight, Mg, serum and muscle P and  $Mg^{++}$  and EMG did not change significantly in group II. The data indicated that  $K^+$  depletion per se does not alter muscle glycogen metabolism, serum CPK or  $CO_2$  or EMG, but causes paralysis by another mechanism. Adding DOC to a potassium-deficient diet creates a different model in which PY is inhibited, perhaps by DOC and/or metabolic alkalosis.

#### 2. Examination of blood cell surface membrane composition and function in normal and disease states

Study of the role that alterations in lymphocyte surface membranes play in expression of cell-mediated immunity continues to be a major



effort in our department. We have invested a great deal of our effort in this area of research since we feel that examinations of the changes induced in normal lymphocytes during transformation, and the alterations of lymphocyte membranes in certain diseases associated with altered immune responsiveness will enable us to perceive more clearly the role of the lymphocyte in mediating host response to infection, and to eventually devise means for increasing or decreasing the intensity of this response.

A major probe for such studies is binding of plant lectins. Lectins are proteins from plants 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 the number of lectin receptors per cell as well as affinity constants for lectin binding. In addition, (1) measurement of glycosyl transferases (ecto-enzymes involved in adding sugars to membrane glycoproteins and glycolipids) and (2) examination of other types of cell-cell interaction (such as excretion of DNA by stimulated lymphocytes) have been used with a great deal of success this past year as probes of the lymphocyte surface membrane. The major areas of effort in this past year's lymphocyte studies have been (1) alterations in surface characteristics of lymphocytes transformed by plant mitogens; (b) DNA excretion by purified lymphocyte populations; (c) characterization of the surface membranes of the chronic lymphocytic leukemia (CLL) cell and the abnormal cell found in leukemic reticuloendotheliosis; (d) interactions of plant lectins with glycolipids in liposomes; and (e) lectin-induced cytotoxicity.

a. Alterations in surface characteristics of lymphocytes transformed by plant mitogens

In earlier studies (FY 75) in this area, we showed that normal lymphocytes transformed with either E-PHA or con-A had increased receptor sites for six different lectins. The increase was much more dramatic in the con-A-treated lymphocytes. Studies using various specific inhibitors indicated that these surface changes did not depend on DNA, RNA, or new protein synthesis, and did not require an intact microtubular system. During the past year, studies have been completed showing that the new receptors emerging in E-PHA transformed cells are due to exposure of formerly cryptic receptor sites, and not due to synthesis of new receptors in transformed cells. Con-A transformed cells, however, appear to synthesize new receptors.



In addition, cell surface glycosyl transferases were examined in both E-PHA and con-A transformed cells. Glycosyl transferase activity was normal or decreased for both endogenous and exogenous acceptors in E-PHA transformed cells, but was increased for both types of acceptors in con-A transformed cells. Puromycin did not inhibit the increased glycosyl transferase activity normally seen in con-A transformed cells. Extraction of cell surface glycosyl transferase with Triton X-100 increased the activity for exogenous acceptors 10-to 30-fold in both untransformed and transformed cells.

b. DNA excretion by purified lymphocyte populations

A large proportion of DNA synthesized in vitro by lymphocytes stimulated with plant mitogens or specific antigens is selectively excreted from the cells. This excreted DNA has been shown to differ substantially from intracellular DNA based on physico-chemical parameters. It has been speculated that excreted DNA may serve as a means of communication among lymphoid cells participating in immune reactions. Therefore, separated human lymphocyte populations were examined for DNA synthesis and excretion in response to stimulation by the plant mitogen phytohemagglutinin (PHA).

Human peripheral blood mononuclear cells were isolated from healthy volunteers by isopycnic centrifugation on Ficoll-Hypaque. Purified populations of T and B lymphocytes were separated from the mononuclear cells by a combination of affinity chromatography and rosette sedimentation techniques. Resultant T and B cell populations were greater than 95% pure as judged by surface markers. Macrophage depletion was accomplished by passage of mononuclear cells over columns packed with nylon fibers followed by incubation of the non-adherent cells in glass petri dishes. The resultant nonadherent macrophage-depleted population contained fewer than 0.3% peroxidase positive cells. Cells stimulated with PHA for 3 days were pulse-labeled with  $^3\text{H}$ -thymidine, then returned to culture for an additional 3 days, at which time the distribution of radiolabeled DNA between cells and media was determined. When human peripheral blood mononuclear cells were cultured with PHA, 50%-70% of  $^3\text{H}$ -thymidine incorporated by the cells was excreted into the media. Approximately 90% of the excreted radiolabel was acid-precipitable and alkali-stable, indicating that this material was DNA.

Macrophage-depleted mononuclear cells stimulated with PHA demonstrated decreased incorporation and excretion of  $^3\text{H}$ -thymidine; yet, as in unseparated mononuclear cell cultures, 50%-70% of incorporated radiolabel was subsequently excreted into the media. Magnitude of DNA synthesis and excretion in macrophage-depleted populations could be partially restored by addition of a small number of glass-adherent cells to these cultures.

Separated T cells responded well to PHA stimulation and subsequently excreted 50%-70% of newly synthesized radiolabeled DNA. By contrast, purified B cells stimulated with PHA synthesized and excreted little radiolabeled DNA. As in the pure T cell cultures, DNA excreted by the B cells, although quantitatively little, still represented 50%-70% of the  $^3\text{H}$ -thymidine originally incorporated into these cells. Therefore the pattern of DNA synthesis and excretion by PHA-stimulated human T and B lymphocytes is identical despite a great difference in the magnitude of the response. When 5%-20% T cells were added to the pure B cell cultures, the magnitude of both DNA synthesis and excretion approached the level observed in pure T cell cultures.

Thus, both human T and B lymphocytes synthesized and excreted DNA in response to plant mitogen stimulation. Magnitude of DNA excretion paralleled synthesis. Both macrophages and T lymphocytes were required for maximal DNA synthesis and excretion in response to PHA stimulation. The data indicate that DNA excretion is a general property of lymphocytes stimulated to undergo DNA synthesis by plant mitogens. These studies were done in collaboration with MAJ R. MacDermott and G. Nash from the Dept Gastroenterology, Div Medicine.

c. Characterization of surface membranes of the CLL cell and the abnormal cell found in leukemic reticuloendotheliosis

In earlier studies with CLL cells (FY 75), we found that the number of receptors present per cell for a battery of five plant lectins was significantly abnormal when compared to normal purified human B cells. Specifically, receptors for E-PHA, con-A, and WGA were decreased in CLL membranes, receptors for L-PHA were increased, and receptors for RCA-I were equivalent to normal B cells. During the past year experiments have been performed to investigate the mechanism responsible for these altered complex carbohydrates which serve as lectin receptors. Various glycosyl transferases serve as cell surface ectoenzymes and are felt to function in part by adding on specific carbohydrate residues to developing oligosaccharide chains on both glycolipids and glycoproteins. Sialotransferase, galactosyl transferase, and N-acetyl-hexosaminyltransferase activities for both exogenous and endogenous (intact cell surface) acceptors were examined both in CLL cells and in normal lymphocytes. CLL cells had reduced endogenous and exogenous glycosyl transferase activity for the three enzymes tested. This suggests that the cause of reduced lectin receptors in this disease is in part due to reduced carbohydrate content of glycoproteins due to deficient synthesis of terminal sugar residues in developing oligosaccharide chains. Preliminary studies of  $^3\text{H}$ -labeled cell surface sialic and galactose residues, and specific measurement of surface carbohydrate members confirms this data.

Functional and structural features of the abnormal cell in leukemic reticuloendotheliosis were performed in collaboration with MAJ R. MacDermott (Dept Gastroenterology, Div Medicine) and Dr. J. E. Valeski (AFIP). A patient with a WBC of 30,000 containing 95% malignant cells was studied intensively. Studies included cell markers, PHA responsiveness, cell adhesion phagocytosis with EM morphology, and a variety of lymphocyte and monocyte functional studies (not done before) which included antibody-dependent cellular cytotoxicity, lectin-induced cytotoxicity, MLC, and CML. The results indicated that the malignant cell was a hybrid with both lymphocytic and monocytic features and function.

d. Interaction of plant lectins with glycolipids in liposomes

A panel of five plant lectins with different binding specificities was used to examine interactions of lectins with glycolipids in liposomes. The studies indicate that lectins can specifically recognize and bind to certain glycolipids. R. communis agglutinin bound specifically to liposomes containing either mixed beef brain gangliosides or globoside I. By contrast, neither the erythroagglutinating phytohemagglutinin nor the leukoagglutinin from Ph. vulgaris showed significant binding to liposomes containing either glycolipid. Concanavalin-A bound to liposomes with or without glycolipid substituents. Although wheat germ agglutinin bound well to liposomes containing mixed beef brain gangliosides, purified  $GM_1$  ganglioside proved to be the most effective receptor for this lectin. In addition wheat germ agglutinin bound well to liposomes containing globoside I or ceramide trihexoside but only in small amounts to ceramide dihexoside, di- or monogalactosyl diglyceride in liposomes. The amount of wheat germ agglutinin bound to liposomes containing ceramide trihexoside was directly proportional to their glycolipid content. These results suggest that steric factors as well as specific saccharide structures may be important determinants of lectin-glycolipid interactions. These studies were done in collaboration with LTC C. Alving and Dr. R. Richards (both from Div CD&I).

e. Lectin-induced cytotoxicity

MAJ Boldt collaborated in studies of lectin-induced lymphocytotoxicity with a group led by MAJ R. MacDermott (Dept Gastroenterology, Div Medicine). The ability of lectins to induce lymphocytotoxicity of autologous human red cells was investigated using an overnight chromium release assay. Crude phytohemagglutinin-P (PHA) induced a moderate degree of cytotoxicity (18%). After fractionation of PHA into erythroagglutinating (E-PHA) and leukoagglutinating (L-PHA) components, E-PHA induced cytotoxicity (35%) but L-PHA did not. Since both E-PHA and L-PHA are mitogens, this indicates that the lymphocytotoxicity is not due to nonspecific activation of

lymphocytes. In addition, wheat germ agglutinin (WGA), a nonmitogenic lectin, produced marked autologous lymphocytotoxicity (79%), a further indication that cytotoxic capacity is independent of mitogenic capability. Cold and inhibitors of glycolysis or the electron transport chain inhibited the observed cytotoxicity from 60% to 100%, showing that a metabolically active effector cell is necessary. Using anti-Fab immunoabsorbent cell chromatography and E-rosette separation techniques followed by nylon wool depletion, isolated populations of T, B, and Null lymphocytes were obtained. All three populations participated in WGA- or E-PHA-induced lymphocytotoxicity. These studies demonstrated that autologous lymphocytotoxicity of human red cells can be induced in vitro by both mitogenic and nonmitogenic lectins with metabolically active T, B, or Null lymphocytes.

Further studies were performed to determine the target cell specificity in lectin-induced lymphocytotoxicity. The target cells used included human autologous RBC's, human allogeneic RBC's and xenogeneic RBC's from sheep or chickens. While E-PHA induced cytotoxicity for all cell types (33% to 46%), L-PHA caused human lymphocytes to kill only xenogenic cells (54% to 62%); conversely, WGA induced killing of only human red cells (80% to 81%). These differences allowed further dissection of possible control mechanisms. In each instance, killing of specific target cells could not be inhibited by unlabeled RBC's which would not themselves be killed. Both E-PHA and WGA bound well to all RBC types but L-PHA bound poorly to chicken RBC's and not at all to human or sheep RBC's. Lectin-induced agglutination of RBC's correlated with binding ability. Thus, lectin-induced lymphocytotoxicity retains specificity in the presence of different RBC types but is not mediated by lectin binding to or agglutination of the RBC's. Furthermore, preincubation of RBC's with the lectins followed by washing did not result in cytotoxicity. However, when lymphocytes were preincubated with WGA, 55% cytotoxicity was induced toward human cells with no killing of xenogeneic cells. These experiments demonstrated that the target cell specificity associated with lectin-induced lymphocytotoxicity is related to a prearmed lymphocyte that seeks out and kills the appropriate target cell.

### 3. Coagulation

Investigations in the coagulation laboratory were concentrated in four areas: (a) the hypercoagulable state, (b) studies of the effect of medications on platelet function, (c) studies on factor VIII and its role in von Willebrand's disease, and (d) clotting abnormalities in animals with parasitic infection.

#### a. Hypercoagulability

Hypercoagulability is a serious complication of a number of disorders that 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 severe vascular disease (in collaboration with the WRAMC vascular surgery department). This study, begun in FY 75, has been continued and refined. Extensive coagulation profiles in patients with significant symptomatic, recurrent peripheral vascular disease were obtained. PT, PTT, TT, AT3, fibrinogen, factors VIII and XI and platelet responses to low doses of platelet-aggregating agents were performed. Observations in 45 consecutive patients have been analyzed completely to date. Significant elevations in levels of fibrinogen (56%) and coagulation factors VIII (36%) and XI (38%) were observed along with a significantly short PTT in 47% of patients. Marked enhancement in platelet response to epinephrine and ADP in vitro was observed in 64% and 55% respectively. When significant abnormalities in two or more tests are used as a criterion for laboratory evidence for hypercoagulability, abnormalities in this profile correlated positively with clinical criteria in 91% of these patients. False positive results were estimated to be present in 2% and false negative results in 7%. Preliminary analysis on this group of patients which is now increased to 60 patients shows no significant changes from the last complete analysis. These data indicate that significant laboratory abnormalities can be demonstrated in approximately 90% of selective patients with clinical histories suggestive of hypercoagulability. These data suggest that direct selective therapy for treatment of specific abnormalities observed in coagulation and platelet function tests may afford better long-term benefit to patients with recurrent vascular disease.

Early data suggest that the use of ASA, an antiplatelet agent, is of long-term benefit to those patients presenting with marked hyperresponsiveness of platelets in vitro. A long-term, random study is anticipated comparing the use of ASA versus coumadin as maintenance therapy in this group.

#### b. Studies of effect of medications on platelet function

Bleeding time, platelet aggregation, platelet adenine nucleotide levels and coagulation factor assays were studied in three consecutive patients receiving mithramycin for embryonal testicular carcinoma. These studies demonstrated a severe drug-dependent, reversible hemorrhagic diathesis associated with (1) prolongation of the bleeding time, (2) decreased platelet aggregation responses to

ADP, collagen and epinephrine, and (3) depleted platelet stores of ADP in the absence of thrombocytopenia. These abnormalities were temporally correlated with the onset of mucocutaneous bleeding in all patients and are similar to those described in congenital platelet "storage pool disease". Careful monitoring of the bleeding time will allow interruption of therapy prior to the development of severe bleeding diathesis.

Similar studies in random patients treated with other chemotherapeutic agents have given similar results, particularly in patients given combination chemotherapy for breast cancer.

The methodology used for studying toxicity of cancer chemotherapeutic agents is obviously equally applicable to studying platelet toxicity of antiparasitic agents, antibiotics, or toxic agents (either microbial toxins or environmental pollutants), and we hope to apply these techniques to one or more of the latter during the coming year.

c. Studies on factor VIII and its role in von Willebrand's disease

Experiments were conducted to elucidate the molecular events occurring during the interaction between human platelets, coagulation factor VIII, and the antibiotic ristocetin. Attempts to label ristocetin with  $I^{125}$  were unsuccessful.  $^3H$ -labeled ristocetin was used in a series of experiments employing agarose gel chromatography with normal human platelets in plasma, washed platelets, and plasma alone.  $^3H$ -ristocetin failed to bind to platelets or any major plasma protein component.

$I^{125}$ -human factor VIII was prepared after purification of factor VIII from commercially available VIII concentrates by agarose column chromatography. Nonspecific binding to human platelets was observed in a series of platelet aggregation experiments only in the presence of ristocetin. Platelet aggregation and VIII binding to platelets were related linearly over a 3 log range of platelet and VIII concentration and were dependent on the amount of ristocetin present.

Preliminary studies suggest that ristocetin produces a macromolecular VIII complex in the absence of platelets. When platelets are present, VIII binds nonspecifically to the platelet membrane. When ristocetin is added, the platelets are aggregated as innocent bystanders within the macromolecular VIII complex. Further studies including attempts to radiolabel ristocetin and examine the reactivity of chemically altered VIII should provide pertinent clues to the structure and function of factor VIII.

d. Clotting abnormalities in animals with parasitic infection

A collaborative study is currently underway with the Div of Experimental Pathology on the induction of disseminated intravascular coagulation (DIC) in beagle dogs infected with trypanosoma rhodesiense. Biweekly blood samples are being tested for PT, PTT, TT, FSP, fibrinogen, fibrin monomer and coagulation factors V and VIII. Results are as yet incomplete.

A collaborative study is also underway with the Dept Med Zoology and the Div Experimental Pathology studying occurrence of DIC in cynomolgous monkeys infected with visceral leishmaniasis. Incomplete analysis of data so far reveals consistent evidence of the presence of DIC in infected, untreated anemias as manifested by a fall in fibrinogen, V, VIII, and prolongation of the PTT. FSP were increased. It is anticipated that this animal model will be useful for drug testing.



Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 086 Military hematology

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3 DATE PREV SUMMARY 75 07 01	4 KIND OF SUMMARY D. Change	5 SUMMARY SCTY <sup>a</sup> U	6 WORK SECURITY <sup>a</sup> U	7 REGRADING <sup>a</sup> NA	8A DISB'N INSTR'N 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		
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18 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
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22 KEYWORDS (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 <sup>a</sup> 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) 75 07-76 06 Factors influencing the responsivity and sensitivity of the tubuloglomerular feedback mechanism, important in the pathogenesis of acute renal failure, have been examined. Sodium restriction and hypotensive hemorrhage increased the magnitude of feedback; reductions in renal perfusion pressure or plasma volume did not. The role of prostaglandins in control of renal hemodynamics after pyrogen and bradykinin were examined. A balance between prostaglandin and renin release rates results in net control of total renal blood flow and intrarenal distribution. The effect of dithiothreitol on heavy metal induced acute renal failure in vivo was examined. This agent prevented heavy metal induced alterations in electrolyte transport, renin activity and prevented the development of acute renal failure. Histopathology and pathology, early after heavy metal induced acute renal failure, further support the concept that tubuloglomerular feedback mechanism is important in the pathogenesis of acute renal failure. Energetics of ion transport across renal epithelial tissues in vitro have been described. Na transport required glycolytic and H transport pentose energetics. For technical reports, see Walter Reed Army Institute of Research Annual Report, 1 July 75-30 June 76. Support in the amount of \$110,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76. <sup>a</sup> Available to contractors upon originator's approval.							

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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 maintenance 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

a. Based on previous work from this laboratory, a postulated sequence of events for the pathophysiology of the initiation of acute renal failure has been developed (15). In brief, the initiating event - whether it be a nephrotoxin (such as heavy metal salts), circulatory failure or cryptogenic - results in tubular epithelial (membrane) dysfunction characterized by decreased fluid and electrolyte absorption. As a result of this change in tubular fluid reabsorption there is an alteration in the composition of tubular fluid delivered to the macula densa segment of the distal nephron, manifested by increased tubular fluid sodium concentration. As a direct consequence of this alteration in tubular fluid composition, as sensed by the macula densa, there is an increase in renin-angiotensin system activity on the local level, with an increase in juxtaglomerular renin activity. The increased renin angiotensin activity mediates a change in renal hemodynamics, characterized by diminution in total renal blood flow with preferential outer cortical ischemia, resulting in a progressive decline in glomerular filtration rates. Despite a decrease in filtered sodium load, because of the present membrane dysfunction there is continued excess delivery of sodium to the macula densa with persistent increased renin activity.

Thus, the mechanism of tubuloglomerular feedback relates alterations in tubular fluid constituents to changes in glomerular filtration. The usual turn-off of this mechanism in acute renal failure is precluded by the persistence of tubular epithelial dysfunction. For this sequence of events to account for the pathophysiology of early acute renal failure epithelial transport dysfunction must be demonstrated in contrast to epithelial necrosis. In an *in vitro* model, the urinary bladder of the fresh water turtle, 2 heavy metal salts, uranyl nitrate and  $\text{HgCl}_2$ , inhibit active sodium transport by altering the entry of sodium across the apical membrane of the epithelial cells (6,7). This inhibition of sodium transport occurs without affecting the enzyme system that provide energy for transport or altering the passive permeability of the tissue to small ions. Furthermore, the inhibitory effect of these 2 heavy metal salts can be completely reversed by dithiothreitol, a dithiol sugar capable of maintaining sulfhydryl groups in reduced states and/or complexing heavy metals (6,7). Thus it is possible in this preparation to demonstrate a direct effect of heavy metal salts on epithelial transport function and to completely reverse this process with a simple organic compound. To further characterize the early membrane changes ultrastructural and histochemical studies were performed on proximal tubules for 15 minutes to 24 hours after the initiation of acute renal failure in rats with  $\text{HgCl}_2$  (8). Between 15 minutes and 3 hours mitochondria matrices were condensed all along the proximal tubules. By 6 hours the microvilli of the brush border were focally absent, mitochondria swollen and the endoplasmic reticulum dilated. By histochemical evaluation, as early as 15 minutes after administration of  $\text{HgCl}_2$ , alkaline phosphatase and 5' nucleotidase activity of the brush borders was decreased. By 6 hours these enzymes and succinic dehydrogenase activity were also decreased. These results support the hypothesis that  $\text{Hg}^{2+}$ , when given in sublethal doses, is associated with early histochemical and ultrastructural changes in the brush border of the proximal tubules, which may be related to early changes in sodium reabsorption and to the subsequent development of acute renal failure.

b. As proposed above the initiating event for heavy metal induced acute renal failure, membrane transport dysfunction results in a cascade of events that culminate in the diminution of glomerular filtration and azotemia. Presumably dithiothreitol, an agent which can reverse the membrane effect of  $\text{HgCl}_2$  and uranyl nitrate *in vitro*, could also reverse this membrane dysfunction induced by heavy metals *in vivo* and thereby ameliorate or prevent the subsequent development of acute renal failure. To evaluate this possibility rats treated with standard doses of uranyl nitrate or  $\text{HgCl}_2$  which induced a predictable pattern of acute renal failure (2) were compared with groups of rats treated 30 minutes after the administration of heavy metal with dithiothreitol. In these studies, dithiothreitol prevented or markedly ameliorated the heavy metal induced reduction in glomerular filtration rate, the rise in sodium excretion and the activation of the renin-angiotensin system activity. These observations demonstrate, for the first time, that heavy metal induced acute renal failure, *in vivo*, presumably mediated by activities of tubuloglomerular feedback mechanism can be markedly altered by the administration of a simple, relatively non-toxic compound, dithiothreitol.

c. Although activation of the tubuloglomerular feedback mechanism may be the major pathophysiologic process in the development of acute renal failure, other mechanisms need to be evaluated. In order to determine the potential role of transtubular "leak" of glomerular filtrate in the pathophysiology of uranyl nitrate induced acute renal failure the recovery of radioinulin injected into proximal tubular segments was determined. The recovery of microinjected  $^3\text{H}$ -inulin was determined in control and experimental groups of rats. The experimental group of rats was studied 2-6 hours after subcutaneous injection of uranyl nitrate, 10 mg/kg BW, as a 10 mg/ml solution in 150 mM NaCl. The microinjections were performed in random superficial proximal tubules, care being taken to assure complete transtubular injections of the 4.8 nl over a 1-2 minute interval. The mean recovery of  $^3\text{H}$ -inulin in 16 microinjections in 5 control rats was  $97.8 \pm 0.9\%$ . The recoveries from 6 rats studied 2-6 hours after uranyl nitrate was  $97.4 \pm 1.0\%$  in 14 consecutive microinjections, a value which is not significantly different from the control rats. The recovery of  $^3\text{H}$ -inulin did not seem to vary over the 2-6 hour period. The results of the present study confirm that over the time period studied and with a 10 mg/kg BW dose of uranyl nitrate that transtubular "leak" plays little if any role in the pathophysiology of this nephrotoxic model of acute renal failure. In order to determine if an elevation of intratubular hydrostatic pressure would increase the potential for transtubular leak, microinjections of radioinulin were performed in a second experimental group of rats 2 to 24 hours after uranyl nitrate during a mannitol diuresis. The following groups of animals were studied: control rats; animals injected with uranyl nitrate 24-27 hours previously. In each group the rats were studied under conditions of hydropenia or under a mannitol diuresis (5% mannitol in 75 mM NaCl solution at 1.0 ml/100g body weight for 30 minutes and then 0.5 ml/100g body weight). The ipsilateral recoveries of  $^3\text{H}$ -inulin in control hydropenic and mannitol diuretic rats were  $97.8 \pm 0.9\%$  and  $95.6 \pm 9.9\%$ , respectively, not significantly different from each other. In hydropenic rats 2-6, and 24 hours after uranyl nitrate recovery of microinjected  $^3\text{H}$ -inulin was  $97.4 \pm 1.0\%$  and  $94.2 \pm 1.2\%$  values not significantly different from each other or control values. In contrast, animals studied under a mannitol diuresis 2-6 hours or 24 hours after uranyl nitrate had ipsilateral  $^3\text{H}$ -inulin recoveries of  $89.7 \pm 2.0\%$  and  $84.3 \pm 4.0\%$ , respectively, values significantly lower than hydropenic or mannitol diuretic control rats. Therefore under conditions of a mannitol diuresis, a maneuver known to increase intratubular pressure, ipsilateral recovery decreased and contralateral recovery increased as compared to the control groups. These results as with the earlier study (above) are not consistent with significant transtubular "leak" of tubule fluid in the hydropenic rat with uranyl nitrate induced acute renal failure. The cause of the minor but significant tubule fluid "leak" in animals with uranyl nitrate acute renal failure undergoing a mannitol diuresis could be ascribed to: (1) increased intratubular pressure resulting in disruption of pre-existing cellular junctions which may have weakened due to either a direct effect of uranyl nitrate, or the instability of newly formed junctions in the case of regenerating or spreading tubular epithelial cells; (2) glomerular reperfusion, due to mannitol, of previously non-functioning nephron units with more advanced cellular necrosis. The



observation that areas underlying necrotic cells are covered by processes from adjacent cells is not consistent with the latter possibility. In any case the degree of "leak" demonstrated is not sufficient to explain the degree of renal insufficiency observed in this model of acute renal failure at either time interval of study, as has been suggested by others.

d. If tubuloglomerular feedback mediates the initiation phase of acute renal failure, normal and pathophysiological states that activate tubuloglomerular feedback may predispose to the development of acute renal failure. Preliminary studies were designed to define what physiological and pathophysiological state activate tubuloglomerular feedback. Tubuloglomerular feedback was assessed by micropuncture in rats by comparing the ratio of proximal single nephron glomerular filtration rate (SNGFR) to distal SNGFR (9-11). Variations in salt intake were first examined. Presumably high salt intake, which in rats minimizes juxtaglomerular renin activity and ameliorates heavy metal induced acute renal failure (3), should minimize tubuloglomerular feedback. The proximal distal SNGFR ratio under these conditions was not significantly different from unity suggesting that under the conditions of high salt intake tubuloglomerular feedback is suppressed. Low salt intake, on the other hand, which maximizes juxtaglomerular renin activity and potentiates the development of both clinical and heavy metal induced acute renal failure (3), should activate tubuloglomerular feedback. The proximal-distal SNGFR ratio from rats on a low salt intake as predicted was significantly greater than 1 ( $P < 0.01$ ). This study demonstrates that under the condition of low salt intake tubuloglomerular feedback is activated. Another pathophysiological state, hypotensive hemorrhage, a condition that may itself lead to the development of acute renal failure was also investigated. Only when blood pressure, after a 1% of body weight hemorrhage, in rats fell below 80 mmHg was there evidence for activation of tubuloglomerular feedback. Thus these studies lend further evidence that tubuloglomerular feedback is implicated in the pathogenesis of acute renal failure.

e. In addition to the effect of the renin-angiotensin system on renal hemodynamics, other modulators of renal blood flow may contribute to the observed hemodynamic alterations associated with acute renal failure. To examine the role of prostaglandins and kallikreins, humoral agents that may also play a significant role in the pathogenesis of acute renal failure (2), two separate experimental models in dogs were studied: triple typhoid toxin induced renal hyperemia and intrarenal arterial infusion of bradykinin. The intravenous administration of pyrogenic agents, such as triple typhoid vaccine, results in decreased renal vascular resistance and renal hyperemia in man and other mammals. The mechanism responsible for the increase in renal blood flow following administration of triple typhoid vaccine remains obscure even though a typical renal hyperemic response has been well documented. The similarity of the renal hemodynamic changes following the administration of prostaglandins of the A- and E-type to those observed during pyrogen induced renal hyperemia suggested the possibility of pyrogenic activation of renal prostaglandin synthesis and subsequent renal vasodilatation (12). The reported elevations of renal venous prostaglandins or prostaglandin-like material during endotoxin shock lends support to this suggested role for renal

prostaglandins in renal hyperemia induced by pyrogens. To determine whether pyrogen induced renal hyperemia is mediated by increased renal synthesis and release of prostaglandins, prostaglandin E and F levels were determined during triple typhoid vaccine induced renal hyperemia. The results of this study show that the i.v. administration of triple typhoid vaccine to anesthetized and conscious dogs result in a significant but unsustained increase in renal blood flow accompanied by a modest decline in systemic blood pressure. The administration of meclofenamate, an inhibitor of prostaglandin synthetase prevents both the development of renal hyperemia and the intrarenal blood flow redistribution when triple typhoid vaccine is administered. The hyperemic response and the increase in prostaglandin release suggest the formation of an endogenous, intermediate agent which mediated the increase in prostaglandin synthesis. The lack of a sustained renal hyperemic and decline in outer cortical blood flow suggests that the increased renin release "buffers" the effects of prostaglandins on renal hemodynamics. It has previously been demonstrated that marked cortical ischemia and decrease in total renal blood flow characterize the renal hemodynamic abnormalities associated with acute renal failure. While increased renin-angiotensin system activity may account for those renal hemodynamic alterations, diminished vasodepressor activity has not been completely evaluated. Thus abnormalities in prostaglandin synthesis or deficiencies of the kallikrein-kinin system must be considered as potential pathophysiologic mechanisms in the pathogenesis of acute renal failure. The following study was therefore undertaken to determine the interrelationship of the renal vasodepressor kinins and prostaglandins and renal vasopressor angiotensin II. Bradykinin was infused into the renal artery before or after inhibiting prostaglandin synthetase. Simultaneous measurements of renal blood flow, cortical blood flow distribution and the renal secretion of prostaglandins E and F and renin were obtained in both experimental groups. The results suggest that prostaglandins mediate bradykinin-induced increases in renal blood flow while the concurrent increase in renin-angiotensin system activity "buffers" the effect of kinin-induced alterations in renal blood flow.

f. As a consequence of acute renal failure alterations in acid base balance occur. Of importance is a basic understanding of the mechanism involved by which renal tissue participates in the control of the extracellular buffer reserve as a major defense mechanism for maintenance of extracellular pH. Investigations have been directed in order to define the energetics required for renal  $H^+$  and  $Na^+$  transport and the adaptive response to hypoxia-anoxia in an in vitro preparation, the urinary bladder of the fresh water turtle. Initial studies have demonstrated that  $Na^+$  transport is ultimately dependent on ATP generation but can be inhibited by a variety of means; uncoupling of oxidative phosphorylation, by a variety of agents, dinitrophenol, CCP, and salicylates (13). The energy supply for  $H^+$  transport appears to be dependent on an alternative pathway, the hexose monophosphate shunt. The rate of metabolism through the shunt, assessed by differential  $^{14}C^1$  vs  $^{14}C^6$  glucose decarboxylation, is directly related to the rate of  $H^+$  transport. Inhibiting  $H^+$  transport by a variety of means, inhibits metabolism through this pathway. Direct inhibitors of the shunt such, as

acetyphenylhydrazine inhibit H<sup>+</sup> transport. The presence of HMP shunt enzymes were also evaluated. The levels of G6PD and GPD in the epithelial cells of the bladder were 10 times greater than in erythrocytes. Further studies are in progress to determine the effect, if any, of heavy metals on these enzyme pathways.

## 2. Chronic Renal Disease and Transplantation:

a. As a result of chronic renal disease an impairment of cellular mediated immunity occurs. This results in an altered capacity to defend against infectious disease and alters the expected response to transplantation. In the past in an attempt to assess the severity of impaired cellular responsiveness the incorporation of radiolabelled thymidine was measured in isolated mononuclear cells cultured with plant mitogens (14). Recently newer techniques were developed to assess cellular responsiveness in lymphocytes from both normal and patients with chronic renal disease. The current approach is to measure the altered rate of metabolism of lymphocytes after exposure to plant mitogens or mitogens plus modifiers of blastogenesis. The metabolic rate was measured by the rate of  $^{14}\text{CO}_2$  evolution from  $^{14}\text{C}$ -glucose added to the incubation media. Preliminary studies have demonstrated that pokeweed mitogen consistently doubles the metabolic rate of cultured lymphocytes from normal individuals within 3 hours. Dibutyryl cyclic AMP ( $10^{-4}\text{M}$ ) abolishes this response. Lymphocytes from patients with chronic renal disease have only a 50% increment in their metabolic rate and this response is abolished by less than  $10^{-5}\text{M}$  dibutyryl cyclic AMP. This newer assay system therefore is capable of differentiating the responsiveness of lymphocytes from different populations and because of the simplicity of the method it will allow a clearer definition of the effects of dialysis on lymphocyte dysfunction.

b. Patients with both chronic and acute renal failure especially those undergoing hemodialysis therapy have altered rates of drug metabolism and clearance (15). Studies of the alterations in drug metabolism have been restricted to patients with stable chronic renal failure because of the high risk unstable state of individuals with acute renal failure. The prototype drug study was procainamide and its active metabolite N-acetyl-procainamide. Both drugs were measured by a rapid fluorimetric assay devised in this laboratory (16). Normal individuals with the capacity to rapidly acetylate INH had higher peak serum levels of N-acetylprocainamide than did slow acetylators. Peak serum procainamide levels were identical in both groups of normals. In individuals without renal function serum procainamide levels were higher than the above normal and the T<sub>1/2</sub> for disappearance prolonged. The serum levels of N-acetylprocainamide rose more slowly and reached higher levels than in normals. This metabolite was measurable for up to 124 hours after a single oral dose of procainamide. During clinical dialysis N-acetylprocainamide levels decreased and the calculated clearance was  $48 \pm 10$  cc/min. This study demonstrates that drug metabolism is altered in chronic renal disease and that dialysis can appreciably increase drug clearance.



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 <sup>a</sup>	2. DATE OF SUMMARY <sup>a</sup>	REPORT CONTROL SYMBOL	
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<p>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.</p> <p>24. (U) To approach this problem we developed a unique cell culture model.</p> <p>25. (U) 75 07 - 76 06 Results on the behavior of adenine nucleotides and energy charge in this model together with our previous findings led to the formulation of a new concept defining cellular growth control as the superposition of cell surface associated regulatory mechanisms, such as active transport, on internal metabolic interlocks representing phylogenetically earlier adaptations increasing survival under unfavorable conditions. This conceptual breakthrough explains a host of hitherto little understood phenomena of growth regulation in microorganisms, neoplastic cells and normal cells and opens the way for eventual clinical control and enhancement of adaptive cell growth underlying tissue repair, wound healing, recovery and survival following injury and stress associated with the mission of the military as stated in para 23 (U) above. At this point the USA Medical R&amp;D Command determined that this work had no direct or supporting military application and ordered the abolition of the Department of Cellular Physiology where the work was carried out and the withdrawal of the personnel authorizations supporting this program (cf. letter from CO, USAMRDC, to Dir. WRAIR, dated 16 Jan 76). For technical reports see Walter Reed Army Institute of Research Annual progress Report 1 Jul 75 - 30 June 76.</p>							

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Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 090 Cellular mechanisms of diseases

Investigators.

Principal: Andre D. Glinos, M.D.

Associate: Kenneth R. Warren, Ph.D., Edwin M. Bartos, Ph.D., Richard C. Robinson, B.A.

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 reported that high density suspension cultures of this cell line, developed in the Dept. of Cellular Physiology<sup>1</sup>, when maintained by daily media renewal without cell dilution remain stationary for prolonged periods of time with no structural alterations or loss of viability<sup>2</sup>. The kinetics of DNA synthesis and mitosis in these cultures is indicative of arrest in the  $G_0$  or early  $G_1$  phase of the cell cycle<sup>3</sup> and is associated with a markedly increased synthesis of specialized proteins characteristic of the WRL-10A phenotype<sup>4,5,6</sup>. The fact that with long established, transformed or neoplastic cell lines this response occurs in suspension but not in attached cultures where only "normal" cells become quiescent with increasing cell density led us to suggest<sup>3</sup> that growth regulation of cultured cells may in all cases involve changes in the availability of essential nutrients to the synthetic machinery of the cell: in the case of transformed cells in suspension, this could occur through depletion in the bulk media<sup>4,7</sup> while in attached cultures of normal cells it would involve microenvironmental gradients<sup>8</sup> and decreased transport through the cell membrane as originally discussed by Foster and Pardee<sup>9</sup>.

Subsequent work by Holley et al.<sup>10</sup> has confirmed that increase of cell density in suspension cultures of transformed or neoplastic cells is followed by quiescence in the  $G_0$  or  $G_1$  phase of the cycle. Also, in a number of cases, cell populations grown either in suspension<sup>11</sup> or in attached cultures<sup>12</sup> have been shown to accumulate in the  $G_0/G_1$  phase of the cycle and to be reactivated to grow in response to external withdrawal and supply of low molecular weight nutrients.

In contrast, while the growth regulatory role of internal concentrations of nutrients as affected by membrane transport has been conceptually expanded<sup>13</sup>, experimental evidence that macromolecular synthesis in the cell is indeed regulated in this fashion is still lacking.

In this paper it is shown that the adenine nucleotides, known to play a multiple role in macromolecular synthesis, undergo a characteristic change in high density, growth inhibited, suspension cultures of WRL-10A cells and that a similar change may be obtained in low density cultures by manipulation of certain nutrients in the media. The relevance of these and previous findings to growth regulation in normal cells is discussed.

#### Materials and Methods

The derivation of WRL-10A cells from the L-929 strain of mouse fibroblast and methods for producing high density growth inhibited populations and starvation plateau cultures have been reported elsewhere<sup>2,5</sup>.

Since all media renewal involved centrifugation of the cultures and resuspension in fresh media it was necessary to wait for a minimum of 2 hr before sampling the cultures in order to obtain proper dispersion of the cells.

Half ml samples were removed from the cultures and the intracellular adenine nucleotide levels determined according to the methods described by Swedes et al.<sup>14</sup>. Standards for adenylates were prepared in fresh unused media. Cellular protein and media glucose (Glc) and lactic acid were determined as described previously<sup>1</sup>. Nucleotide concentrations were expressed as fmoles/cell rather than protein because as reported earlier<sup>1</sup> the transition from exponential growth to high density populations did not alter the amount of total protein per cell which in these experiments averaged 230 pg.

### Results

When all low density suspension cultures of WRL-10A fibroblasts assayed for adenine nucleotides over a period of 2 yr are considered, a range of ATP concentrations between 6.5 and 10.5 fmoles/cell is noted. The ATP level in these cultures appeared to be directly related to the daily growth index, with the lower ATP levels exhibited by cultures growing by a factor less than 2. Generally, ATP comprised approximately 91%, ADP 6% and AMP 3% of the total adenylate ( $\Sigma$ Ad) of WRL-10A cells and when the adenylate energy charge  $(\text{ATP}) + 0.5 (\text{ADP}) / (\text{ATP}) + (\text{ADP}) + (\text{AMP})$ <sup>15</sup> was calculated, it was found to be 0.90 or greater in all log growing populations of such cells.

A representative experiment showing changes in the adenine nucleotides during the progression of suspension cultures of WRL-10A cells from low density exponential growth phase to high density growth inhibited populations, is illustrated in Table 1. While in the exponential phase no significant changes in adenine nucleotide levels or energy charge were detected, in the early phase of the transition the ATP and  $\Sigma$ Ad levels decreased in the interval between 2 and 23 hr, and then, subsequent to media renewal, increased to levels characteristic of exponential growth. Nonetheless, the adenylate energy charge remained stable and well poised at or above 0.89 throughout this period. In the late transition phase, the adenylates did not fluctuate significantly and growth continued but with a greatly reduced rate for 4 to 5 days, after which growth indices ranged between 0.95 and 1.05 and the population could be defined as truly stationary. During this phase, ATP and  $\Sigma$ Ad had stabilized at approximately 50% of the original values with only a minimal increase upon daily refeeding. The energy charge in late transition and stationary phase WRL-10A cells remained well poised with values at least as high as those observed in exponential growth.

As it has been shown previously, high density growth inhibited cell populations may be reactivated to grow by dilution to a density of between  $3$  and  $5 \times 10^5$  cells/ml<sup>3</sup>. In Fig. 1 it may be seen that under



these conditions  $\Sigma$ Ad began promptly to rise toward levels characteristic of logarithmically growing cells. By 2 hr  $\Sigma$ Ad had increased to 8.4 fmoles/cell and to 9.1 fmoles/cell by 4 hr. The adenylate energy charge remained constant throughout this period. In Fig. 1, it may also be seen that the first peak of mitotic activity after dilution occurred at 24 hr, consistent with our previous findings<sup>3</sup>.

Nutrient depletion must be considered a plausible putative mechanism for the growth inhibition observed in high density suspension cultures<sup>3</sup> and, accordingly, the course of adenine nucleotides, energy charge and growth were followed in low density cultures in which daily media renewal and dilution were omitted. A representative experiment illustrated in Fig. 2 shows that the culture continued to grow exponentially past the media renewal point at 24 hr, until 54 hr. At that time growth ceased and a cell density plateau was reached and maintained for the next 36 hr, after which the cell density began to decline. ATP and  $\Sigma$ Ad levels, which are virtually constant throughout the normal 24-hr renewal cycle of the exponential growth period (Table 1 and Fig. 2) began to decline immediately after renewal was omitted at 24 hr, and stabilized at approximately 50% of the original log values. After 90 hr as the cell density fell, ATP and  $\Sigma$ Ad rose, apparently as a consequence of cell lysis with uptake of labilized metabolites by the remaining viable cells. Even under these conditions of total nutrient deprivation the adenylate energy charge remained well poised with a mean for all time points from 0 hr until 54 hr of  $0.94 \pm .004$ . Not until 78 hr, i.e. 24 hr into the density plateau, did the energy charge decline below 0.9 reaching a value of 0.85. From this point on, the energy charge fluctuated between this low value and a high of 0.95, with a mean for all values from 78 hr to 120 hr, when the culture was terminated, of  $0.90 \pm 0.03$ . Thus, even with cell death affecting a part of the cell population, the energy charge of the remaining viable cells was maintained at high levels.

An alternative approach to total nutrient deprivation involves the selective omission of key media constituents utilized by the cells for energy production and macromolecular synthesis. In high density cultures, with populations at least 10X higher than in low density ones, Glc is exhausted at the end of the 24-hr media renewal cycle<sup>2</sup> and this could be related to the decrease of ATP and  $\Sigma$ Ad levels and the inhibition of growth. Accordingly, a low density culture was treated with media containing Glc at a concentration of 0.1 mg/ml instead of the normal 1.0 mg/ml thus simulating the decline of Glc availability which occurs in the high density populations. It was found that Glc was virtually exhausted at the end of the renewal cycle and that lactate production was eliminated. While these effects were also observed in high density stationary cultures<sup>2</sup>, there was no significant effect on either the rate of cell proliferation or the level of ATP.

Another key constituent of the media, glutamine, was found to be depleted in high density cultures considerably faster than Glc. Thus,

the glutamine concentration of a suspension culture with a density of  $6.5 \times 10^6$  cells/ml was found to decrease to less than 3% of its initial media concentration within 5 hr after media renewal (Glinos and Taylor, unpublished data). To determine the possible role of limited glutamine availability in the decrease of the adenine nucleotides and the growth inhibition characteristic of high density populations, a suspension culture of WRL-10A cells was carried for a period of 46 days with daily media renewal without dilution, using media containing approximately 3% of the normal 2 mM glutamine in Eagle's MEM. It was found that glutamine restriction resulted in considerable growth retardation so that the cell density level achieved at the end of the 45 days of growth was  $25 \times 10^5$  cells/ml compared to  $100 \times 10^5$  cell/ml, reached within 10 days by cell populations supplied with the normal amount of glutamine. Nevertheless, the decrease of the adenine nucleotides shown to accompany inhibition of growth in high density stationary populations (Table 1) and in the early phase of the starvation plateau (Fig. 2) was absent here, as the values of  $\Sigma$ Ad determined during the last 3 days of the culture averaged  $9.18 \pm 1.84$  fmoles/cell well within the range characteristic of low density exponentially growing populations.

When, however, exponentially growing low density suspension cultures of WRL-10A cells were renewed with Eagles MEM media lacking both Glc and glutamine, the ATP and  $\Sigma$ Ad levels fell by 23% within 4 hr and at 24 hr ATP was found to be  $3.49 \pm 0.02$  and  $\Sigma$ Ad  $4.06 \pm 0.15$  fmoles/cell respectively. While the adenylate energy charge remained quite high with values above 0.89, the cell population increased between 4 and 24 hr by a factor of 1.17, a marked reduction if compared to the normal rate of low density cultures growing in complete media (Table I, days 1-3 and Fig. 2, -48 to -24 hr).

### Discussion

The experiment summarized in Table I shows that as the cell density increased the values for ATP and  $\Sigma$ Ad at 23 hr became progressively lower but were restored after media renewal. The change in the size of the adenylate pool appears, thus, to be due to depletion of essential nutrients in the media. When the density of the culture reached  $10^7$  cells/ml the 2-hr values of ATP and  $\Sigma$ Ad were only slightly higher than at 23 hr because the large number of cells present and consequent rapid nutrient utilization make a transient restoration difficult to detect, especially since the culture cannot be sampled earlier than 2 hr after media renewal (cf. Methods). The direct dependence of the intracellular nucleotide concentration on external nutrients is further demonstrated through the rapid restoration of the adenylate pool size with dilution of the high density populations (Fig. 1) and the prompt lowering of the pool size in the starvation plateau (Fig. 2).

Concerning the identity of the nutrients, the results obtained implicate Glc and glutamine and since the characteristic change of the adenine

nucleotides was reproduced only when these 2 nutrients were withdrawn simultaneously, the metabolic processes involved must allow at least limited substitution of one nutrient by the other. While oxidative metabolism is such a process, the maintenance of the energy charge at very high levels indicates that unlike the Glc and amino acid starvation experiments of Live and Kaminskas<sup>16</sup> with Ehrlich ascites tumor cells it could not be the main process affected.

Another process to be considered is the *de novo* synthesis of purines where glutamine is essential. Since withdrawal of glutamine from the media of L cells results in induction of glutamine synthetase<sup>17</sup> and glutamate substrate resulting from transamination will always be present in cultures supplied with Glc and essential amino acids, it follows that only the simultaneous coordinated withdrawal or exhaustion of both glutamine and Glc would limit purine biosynthesis. Such a process would be expected to affect equally all three adenine nucleotides and permit the decline of the  $\Sigma$ Ad pool with maintenance of high energy charge observed.

With the exception of the glutamine alone withdrawal experiment, where the slow and prolonged reduction of the growth rate was probably due to interference with protein synthesis, in all other cases described in this paper changes of the growth rate were preceded by changes in the adenylate pool (Table 1, Figs. 1 and 2). Data relevant to a possible association of the concentrations of the adenine nucleotides with growth regulation in other types of mammalian cell cultures are inconsistent<sup>16,18-21</sup>. In phylogenetically earlier cells, however, nutritionally induced alterations of growth were found to be associated with changes in the concentrations of adenine nucleotides in many cases and Atkinson et al.<sup>22</sup> point out that it is primarily the total adenylate pool which is involved while the relative concentrations of the nucleotides and consequently the energy charge remain constant; also, that the variation of the size of the pool does not appear to be greater than 3- to 4-fold even in cases of starvation. This response is illustrated by the observations of Swedes et al.<sup>14</sup> on an adenine requiring mutant of *Escherichia coli* grown in a chemostat and which bear a striking similarity to the observations on mammalian cells reported here. In both cases manipulation of the nutrient media resulted in limiting the availability of purines to the economy of the cells followed by a moderate decrease of the total adenylate pool, inhibition of growth and maintenance of the energy charge at high levels. The latter indicates that the transition from active growth to the stationary phase does not represent a simple arrest of biosynthesis because of lack of raw materials but is the result of complex regulation involving well coordinated shifts in the relative emphasis of energy producing and energy utilizing processes<sup>15,22</sup>.

That the transition from exponential growth to the stationary phase in response to nutritional limitation is not due to the indiscriminate

cessation of all biosynthetic processes but is highly regulated, is further demonstrated by the reversible enzymatic differentiation exhibited under these conditions by many microorganisms<sup>23</sup> as well as by WRL-10A cells. The latter, of fibroblastic origin, have undergone phenotypic mutation and are capable of synthesizing components of the cholinergic system, but, only when maintained in suspension as high density growth inhibited populations<sup>5,6</sup>, Glinos and Bartos in preparation). This similarity of behavior of microorganisms and a long established transformed mammalian cell line grown in suspension, indicates that the internal metabolic interlock mechanisms involved in the regulation of growth and differentiation are capable of operating when cell surface associated mechanisms found in normal mammalian cells have not yet developed or have been lost. Phylogenetically, metabolic interlock mechanisms appear, therefore, to represent earlier adaptations increasing the probability of survival in unfavorable conditions.

Recently, Pardee<sup>24</sup> has demonstrated that in normal cells nutritional limitation causes arrest in the same location of the G<sub>1</sub> phase of the cell cycle as do conditions acting through the cell surface. He termed this location the restriction point (R-point) to indicate that it represents a key switching mechanism for cellular metabolism of paramount significance for cell survival under adverse conditions, growth, differentiation and neoplastic transformation. Viewed in this perspective, the findings described in this and previous papers<sup>2-8</sup> suggest that an important characteristic of the R-point in normal mammalian cells is the superposition of sophisticated cell surface associated growth regulatory mechanisms on phylogenetically ancient metabolic interlocks. Accordingly, it is proposed that one of the relatively early steps in carcinogenesis consists of loss of growth control mechanisms associated with the cell surface while the phylogenetically earlier metabolic interlocks are maintained. It is not, therefore, surprising that under the proper nutritional conditions arrest and prolonged quiescence in the G<sub>1</sub> phase can be demonstrated in some transformed or neoplastic cell lines as in the case of WRL-10A cells<sup>3</sup> and myeloma X563-5 cells<sup>10</sup>. Such a concept is also in good agreement with the common clinical experience of carcinoma *in situ* and the recent demonstration that whether certain tumor cells *in vivo* will remain in a quiescent state or will proliferate depends on the extent of vascularization of the areas in which they are located<sup>25</sup>.

#### Summary and Conclusions

The intracellular concentrations of adenine nucleotides were determined in suspension cultures of WRL-10A cells, a long established transformed line, during their progression from exponential growth to high density stationary populations. It was found that this progression was associated with a 50% reduction of the total adenylate pool while the energy charge remained at values above 0.90. This alteration was also

observed in the early phase of starvation of low density cultures and could be reproduced by simultaneous withdrawal of glucose and glutamine, indicating interference with *de novo* purine biosynthesis. In addition to the maintenance of the energy charge, high density suspension cultures of WRL-10A cells were previously shown to be arrested in the G<sub>1</sub> phase of the cell cycle and to exhibit markedly increased synthesis of specialized proteins characteristic of their phenotype. The transition from active growth to quiescence in response to nutritional limitation in these cells is, therefore, not due to the indiscriminate cessation of all biosynthetic processes but is highly regulated. The fact that comparable responses are known to occur on the one hand in microorganisms and on the other in normal mammalian cells is indicative of the operation in all cases of similar metabolic interlock mechanisms representing phylogenetically early adaptations increasing survival under adverse conditions. It is, therefore, proposed that one of the characteristics of the restriction point in normal mammalian cells is the superposition of sophisticated cell surface associated growth control mechanisms on phylogenetically primitive internal metabolic interlocks; according to this view, one of the early steps in carcinogenesis would consist of the loss of the former while the latter would be maintained. Such a process would account for the demonstrated G<sub>1</sub> arrest caused by nutritional limitation in some transformed and neoplastic cell lines as well as for carcinoma *in situ* and its dependence on the extent of its vascularization for continued quiescence or for tumor growth.

Table 1. Behavior of adenine nucleotides and energy charge during the development of high density growth inhibited populations

Growth phase	Cells/ml $\times 10^{-5}$	Growth	ATP/Cell (fmoles)		Ad/cell (fmoles)		Energy charge	
			2 hrs	23 hrs	2 hrs	23 hrs	2 hrs	23 hrs
Log (Day 1-2)	10.3	1.97	10.11 $\pm .23$	9.72 $\pm .60$	10.92 $\pm .26$	11.05 $\pm .97$	0.96 $\pm .02$	0.93 $\pm .02$
Early Transition (Day 3-5)	21.8	2.09	8.20 $\pm .16$	7.44 $\pm .27$	9.64 $\pm .16$	8.06 $\pm .10$	0.89 $\pm .01$	0.95 $\pm .02$
	37.4	1.52	8.13 $\pm .26$	5.48 $\pm .12$	8.94 $\pm .05$	6.18 $\pm .52$	0.94 $\pm .02$	0.92 $\pm .02$
	61.7	1.56	8.03 $\pm .16$	4.24 $\pm .20$	8.67 $\pm .07$	4.60 $\pm .15$	0.96 $\pm .01$	0.95 $\pm .02$
Late Transition (Day 6-10)	101.6	1.14	5.09 $\pm .42$	4.60 $\pm .31$	5.37 $\pm .40$	4.90 $\pm .34$	0.96 $\pm .02$	0.96 $\pm .01$
Stationary (Day 11-24)	102.4	1.05	5.62 $\pm .49$	4.94 $\pm .44$	5.98 $\pm .60$	5.34 $\pm .51$	0.96 $\pm .01$	0.96 $\pm .02$

The culture was centrifuged daily and resuspended in fresh media without dilution of the cell population. Cell viability estimated on the basis of the fraction of cells excluding nigrosin was <99.5% throughout. Samples for cell counts and nucleotide assays were obtained at 2 and 23 hr after media renewal as described under Methods. Cell count values shown represent the density of the culture at 23 hr. The growth index was computed from the ratio of the cell count at 23 hr to the count at 2 hr after media renewal. The first four entries represent individual daily values obtained during the logarithmic growth and early transition phase of the culture when changes in the cell density and adenine nucleotide levels were most pronounced. The last two entries represent mean values obtained during the 6-10 and 11-24 day intervals, corresponding to the late transition and stationary phases when daily changes in cell density and adenine nucleotide levels were minimal.

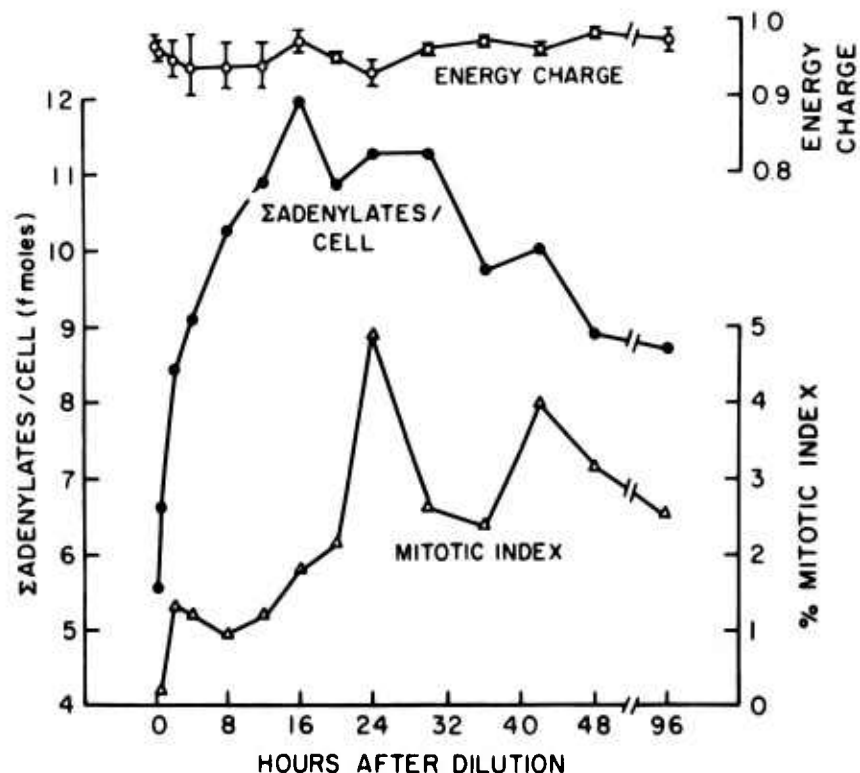


FIG. 1. Adenine nucleotides, energy charge and mitotic activity following dilution of high density stationary culture. A subculture was established from a high density ( $12.0 \times 10^6$  cells/ml) growth inhibited population of WRL-10A cells by dilution to  $4.7 \times 10^5$  cells/ml with fresh prewarmed media. The subculture was subsequently subjected to media renewal and dilution to approximately  $5 \times 10^5$  cells/ml at 24, 48 and 72 hr. The behavior of ATP was sufficiently similar to total adenylates ( $\Sigma Ad$ ) so that it could be omitted from the graph. The mitotic index was determined by counting at least 2000 cells fixed and stained in a mixture of crystal violet, citric acid and methylcellulose, as described previously<sup>3</sup>. The course of  $\Sigma Ad$  (closed circles), energy charge (open circles) and mitotic index (triangles) is shown.

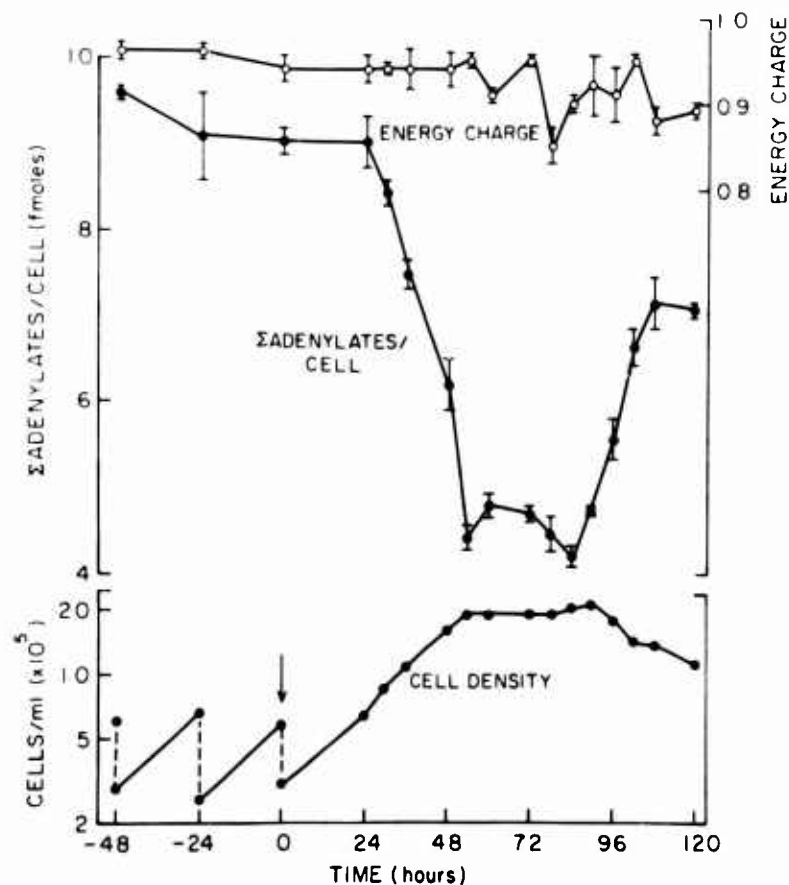


FIG. 2. Cell density, adenine nucleotides and energy charge during the starvation plateau of WRL-10A cells. The small closed circles represent cell population kinetics. Two days of logarithmic growth with daily media renewal and cell population dilution (broken lines at -48 and -24 hr) are shown. Media renewal and dilution was also carried out at 0 hr but was subsequently omitted. The resulting cell population kinetics reproduce a typical starvation plateau as described earlier<sup>2</sup>. Large closed circles and open circles represent the course of  $\Sigma$ Adenylates/cell and energy charge, respectively. The behavior of ATP was sufficiently similar to  $\Sigma$ Ad and was, therefore, omitted from the figure.



Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 090 Cellular mechanisms of diseases

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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-DR&E(AR)636	
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(U) Vaccine; (U) Enteric Bacteria; (U) Antigens (U) Virulence; (U) Salmonella; (U) Plasmids							
23. TECHNICAL OBJECTIVE <sup>a</sup> 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text 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) 75 07-76-06 The ability of a Salmonella tennessee strain to ferment lactose and sucrose was shown to be determined by a conjugally transmissible plasmid bearing the genes for both fermentation characters. Purified Vi antigen, acetic anhydride-treated Salmonella typhi endotoxin, and potassium methylate-treated S. typhi endotoxin employed as vaccines in Swiss white mice were shown not to protect these animals against challenge with a virulent S. typhimurium hybrid expressing S. typhi antigens. Examination of a representative of the major class of partially diploid hybrids formed in S. typhimurium matings with Escherichia coli showed that the added Salmonella genes were conserved by chromosomal insertion. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$63,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>							

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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. The ability of a Salmonella tennessee strain to ferment both lactose and sucrose was shown to be determined by a conjugally transmissible plasmid, deoxyribonucleic acid molecular weight  $164 \times 10^6$ , bearing the genes for both fermentation characters.
2. Purified Vi antigen, acetic anhydride-treated Salmonella typhi endotoxin, and potassium methylate-treated S. typhi endotoxin employed as vaccines in Swiss white mice were shown not to protect these animals against challenge with a virulent S. typhimurium hybrid expressing S. typhi antigens.
3. Examination of a representative of the major class of partially diploid hybrids formed in Salmonella typhimurium X Escherichia coli matings showed that the added Salmonella genes were conserved by chromosomal insertion near the site of the allelic E. coli genes.

Progress.

1. Plasmid-determined ability of a Salmonella tennessee strain to ferment lactose and sucrose.
  - a. Previously (Annual Report, WRAIR, 1974), we characterized a group of conjugally transmissible plasmids conferring the property of lactose fermentation on Salmonella strains which were isolated from clinical specimens. More recently (Annual Report, WRAIR, 1975), we showed that Salmonella strains with the equally unusual ability to ferment sucrose also contained transmissible plasmids which determined that ability. In the present study we investigated a strain of S. tennessee, also isolated from a clinical specimen, which possessed the ability to ferment both lactose and sucrose. We found that this strain, S. tennessee 2718-64, was able to transfer the sucrose fermentation property ( $Scr^+$ ) to an Escherichia coli recipient, WR302, at

low frequency ( $10^{-8}$  per donor cell). Although  $\text{Scr}^+$  WR3026 exconjugants were obtained infrequently, all of those isolated in several different matings were found to have received the lactose fermentation property ( $\text{Lac}^+$ ) as well. E. coli WR3026 exconjugants expressing the  $\text{Lac}^+$  and  $\text{Scr}^+$  characters were able in turn to transmit these characters in matings with S. typhi strain WR4200 and with a second E. coli recipient, WR3027, but, again, the transfer frequencies of either character were low ( $10^{-8}$  to  $10^{-7}$  per donor cell). Serial transfers of mating mixtures to fresh nutrient broth were carried out over periods of several days in the hope that newly formed exconjugants might prove to be higher-frequency donors. However, we were able to improve neither the frequency of transfer nor the consistency of those transfer frequencies from one mating to another. Nevertheless, we did observe one consistent feature in that all of the exconjugants obtained in these matings, whether selected for receipt of the  $\text{Lac}^+$  character or the  $\text{Scr}^+$  character, acquired both characters.

b. Spontaneous segregation of the inherited fermentation characters was observed occasionally among S. typhi WR4200 exconjugants, as well as among E. coli WR3026 and WR3027 exconjugants. In the case of the S. typhi exconjugants, all of 16  $\text{Lac}^-$  segregants observed as nonfermenting colonies on MacConkey lactose indicator medium were found also to have lost the  $\text{Scr}^+$  character; likewise all of 13  $\text{Scr}^-$  segregants obtained on MacConkey sucrose indicator medium had lost the  $\text{Lac}^+$  character as well. Joint loss of both characters appeared to be the general rule also among similar numbers of  $\text{Lac}^-$  and  $\text{Scr}^-$  segregants from the E. coli exconjugants, although we did obtain one segregant of an E. coli WR3026 exconjugant from which only the  $\text{Scr}^+$  character was lost. This segregant was able to transfer its  $\text{Lac}^+$  character at low frequency ( $10^{-8}$ /donor cell) to Salmonella typhi WR4200 and to E. coli WR3027.

c. By means of dye-buoyant density centrifugation, we detected covalently closed circular (CCC) deoxyribonucleic acid (DNA) in  $\text{Scr}^+$   $\text{Lac}^+$  exconjugants of E. coli WR3026 and WR3027. Examination of the isolated CCC DNA by electron microscopy revealed a single molecular species with a mean contour length of  $83.7 \pm 3.4 \mu\text{m}$ , corresponding to a molecular weight of  $164 \times 10^6$ . Several E. coli exconjugant segregants from which both the  $\text{Lac}^+$  and  $\text{Scr}^+$  characters had been lost reflected that loss, upon examination, in the disappearance of their CCC DNA. We examined also the E. coli WR3026 exconjugant segregant from which only the  $\text{Scr}^+$  character had been lost and found it to contain CCC DNA with a mean contour length of  $75.9 \pm 4.3 \mu\text{m}$  corresponding to a molecular weight of  $148 \times 10^6$ ,  $16 \times 10^6$  less than that determined for the exconjugants expressing both characters.

d. In view of the low frequency with which either the  $\text{Lac}^+$  character or the  $\text{Scr}^+$  character was transmissible as a selected marker in this study, we believe that the appearance of the unselected charac-

ter in every exconjugant examined argues against the possibility that these characters might be borne on separate plasmids. We think the most plausible interpretation is that the genetic determinants of both the Lac<sup>+</sup> character and the Scr<sup>+</sup> character are located on a single, conjugally transmissible plasmid replicating extrachromosomally as a CCC DNA molecule with a molecular weight of  $164 \times 10^6$ . This view is supported by the general pattern of segregation which results in joint loss of both characters and in the loss of the only CCC DNA form observed in those exconjugants. The Scr<sup>-</sup> Lac<sup>+</sup> exconjugant segregant represents, we think, an instance in which a portion of the DNA bearing the determinants of the Lac<sup>+</sup> character (but not, apparently, the genes determining transfer) has become dissociated and lost from this plasmid.

2. Ineffectiveness of Vi and chemically treated endotoxins as typhoid vaccines in mice challenged with a *Salmonella typhi*-*Salmonella typhimurium* hybrid.

a. The development of mouse-virulent *Salmonella typhimurium* hybrids expressing *S. typhi* antigens (Annual Report, WRAIR, 1973, 1974) has provided challenge strains that produce a true systemic typhoid infection in mice. Using these hybrids, we have developed an assay system with Swiss Webster white mice as test animals, which allows differentiation of the protective capacity of various typhoid vaccines. In the present study, we employed this assay system to examine the protective activity of purified Vi antigen and of chemically modified *S. typhi* somatic antigens in comparison with that of an acetone-treated vaccine prepared from *S. typhi* Ty2.

b. The acetone-treated vaccine (AK), prepared from *S. typhi* Ty2, was shown previously (Annual Report, WRAIR, 1973) to have significant protective effect against the death of mice challenged with virulent *S. typhimurium* hybrids that expressed *S. typhi* somatic antigens. Purified Vi antigen prepared from *Citrobacter freundii* was supplied by E. E. Baker, Boston University, Boston, Mass. Acetylated (Acet-RE) and methylated (Meth-RE) endotoxins prepared from *S. typhosa* strain 59 were supplied by S. Marcus, University of Utah, Utah Medical Center, Salt Lake City, Utah. The Acet-RE and Meth-RE vaccines are crude endotoxin preparations treated either with acetic anhydride or potassium methylate to reduce their toxicity while maintaining their immunizing activity(1).

c. Swiss Webster white mice (16-18g) were injected intraperitoneally with one dose (equivalent to  $5 \times 10^8$  organisms) of the AK vaccine and challenged one week later with a 2,500-organism dose of *S. typhimurium* hybrid H42. This hybrid expresses the *S. typhi* somatic antigens 9,12, the Vi antigen, and the flagellar d antigen, and its mean lethal dose is less than 50 organisms. The Vi, Meth-RE,



and Acet-RE vaccines were administered intraperitoneally in two doses given a week apart, and the mice were challenged with hybrid H42 1 week after administration of the second dose. The dosages of Vi, Meth-RE, and Acet-RE vaccines were similar, generally to those administered by Pistole and Marcus (1,2) in their mouse protection studies employing these vaccines against challenge with mucin-treated *S. typhi*. Although rather large doses of Vi, Acet-RE, and Meth-RE were employed in their studies and in ours, these doses were well within the limits of nontoxicity established for these antigens (1,2). Survivors were counted 21 days after challenge.

d. Although both of the chemically detoxified somatic antigen vaccines, as well as the purified Vi vaccine, were found by Pistole and Marcus (1,2) to confer significant protection for mice against a mucin-treated *S. typhi* challenge, it is apparent from the data presented in Table I that such was not the case against hybrid H42 in our assay system. Only the whole-cell AK vaccine significantly protected the mice in these experiments as compared with the non-vaccinated controls ( $p < 0.01$ ). The absence of protection by purified Vi antigen observed here is consistent with our previous findings (Annual Report, WRAIR, 1974) that the Vi antigen played no significant role in conferring protection against death in this system. Rather, it was the *Salmonella* somatic antigens which were shown to be important in conferring that protection.

### 3. Conservation of *Salmonella typhimurium* deoxyribonucleic acid by chromosomal insertion in a partially diploid *Escherichia coli* hybrid.

a. We showed previously (Annual Report, WRAIR, 1973) that the minority class of partially diploid *Escherichia coli* hybrids formed in *Salmonella typhimurium* Hfr X *E. coli* F<sup>-</sup> matings conserve the added *Salmonella* DNA extrachromosomally as covalently closed circular (CCC) molecules by association with some part of the sex factor, F. The majority class, however, conserved the *Salmonella* DNA in some manner which did not involve association with F or assumption of the CCC configuration. To ascertain what this manner of conservation was, we constructed, in the present study, a partially diploid *E. coli* hybrid, determined to be of the majority class by the absence of F and/or CCC DNA, which contained the *Salmonella rha*<sup>+</sup>, *metB*<sup>+</sup>, and *argH*<sup>+</sup> alleles as supernumerary genes. We then converted this hybrid to an Hfr strain of the P4X-6 type transfer orientation and designated it WR2080.

b. In matings between the diploid Hfr WR2080 and *E. coli* recipient strains, selection for any of the three *Salmonella* markers of the donor, *rha*<sup>+</sup>, *metB*<sup>+</sup>, or *argH*<sup>+</sup> always resulted in hybrids which received the other two markers as well. The inseparability of the



Salmonella alleles in these experiments contrasts with the behavior of their E. coli counterparts when transferred by the haploid E. coli Hfr, P4X-6. In the latter case, the E. coli, rha<sup>+</sup>, metB<sup>+</sup> and argH<sup>+</sup> alleles are readily separable by recombination in the hybrids. It is probable, therefore, that recovery of hybrids expressing any of the Salmonella alleles is dependent upon their conservation of the complete Salmonella genetic segment on which these three genes are borne. The minimum length of the segment encompassing these genes is 1.5 min.

c. In interrupted mating experiments, the entry times for the proximally transferred E. coli thr<sup>+</sup> and leu<sup>+</sup> markers of the diploid Hfr, WR2080, were the same as those of the analogous haploid Hfr, P4X-6. However, whereas P4X-6 transfers its argH<sup>+</sup> marker at 23.5 min. and its rha<sup>+</sup> marker at 25.0 min, the entry time of the Salmonella argH<sup>+</sup> marker of WR2080 was 25.3 min, and entry of its Salmonella rha<sup>+</sup> marker was time at 24.7 min. Entry of the E. coli ilv<sup>+</sup> gene of WR2080 occurred at 29.5 min, whereas entry of the ilv<sup>+</sup> gene of P4X-6 occurred at 28 min. In keeping with the idea that recovery of hybrids expressing the Salmonella alleles requires entry and conservation of the entire genetic segment on which they are located, we believe the 24.7 min rha<sup>+</sup> and 25.3 min argH<sup>+</sup> entry times represent variations of a common time of entry for both markers, which is about 25 min. The entry of the Salmonella alleles of WR2080 near the 25 min mark, coupled with the 1.5 min delay in the entry of its E. coli ilv<sup>+</sup> allele (as compared with P4X-6) is consistent with the view that a segment bearing the Salmonella genes is inserted in the WR2080 chromosome near the region occupied by the allelic E. coli genes.

d. By employing E. coli recipients which are poor acceptors of Salmonella DNA in crosses with the diploid Hfr WR2080, we were able to demonstrate a significant reduction of unselected inheritance of the recessive E. coli alleles near the site of the inserted Salmonella genetic segment. Thus, analysis of 100 E. coli hybrids selected for receipt of the ilv<sup>+</sup> marker of WR2080 showed a normal 51% unselected inheritance of the E. coli rha<sup>-</sup> allele, but inheritance of the neighboring metB<sup>-</sup> allele was somewhat reduced at 30%, and inheritance of the E. coli argH<sup>-</sup> allele of WR2080 occurred in only 5% of the hybrids. By comparison E. coli hybrids selected for receipt of the ilv<sup>+</sup> marker of P4X-6 showed the following unselected inheritance of these markers: rha, 56%; metB, 45%; and argH, 41%. In a cross in which inheritance of a Salmonella allele of WR2080 (rha<sup>+</sup>) could be scored along with inheritance of its recessive E. coli alleles (metB<sup>-</sup> and argH<sup>-</sup>) 29% of ilv<sup>+</sup> selected E. coli hybrids inherited the metB<sup>-</sup> allele and only 7% inherited the argH<sup>-</sup> allele. Inheritance of the Salmonella rha<sup>+</sup> marker in this cross was 8%. We believe that the low inheritance of the E.

coli argH<sup>-</sup> gene in these crosses is due to its proximity to the inserted Salmonella genetic segment, whose inheritance in these recipients occurs at the same low frequency. Our conclusion is that that the Salmonella chromosomal segment bearing the rha<sup>+</sup>, metB<sup>+</sup>, and argH<sup>+</sup> alleles is inserted in the chromosome of WR2080 near the original site of its argH<sup>-</sup> gene, thus creating a duplication of this region within the hybrid chromosome.

#### Summary.

1. A strain of Salmonella tennessee possessing the unusual ability to ferment both lactose and sucrose was shown to be capable of transferring this ability, by conjugation, to Escherichia coli recipients. The E. coli exconjugants, in turn, were capable of transferring both fermentation properties together to other E. coli and S. typhi recipients. Genetic and molecular analysis of the E. coli exconjugants showed that the ability to ferment these sugars was determined by a conjugally transmissible plasmid, deoxyribonucleic acid molecular weight  $164 \times 10^6$ , bearing the genes for both fermentation characters.

2. A mouse virulent Salmonella typhimurium hybrid expressing the S. typhi antigens 9,12, Vi, and d, constructed by genetic crosses with an S. typhi Hfr, was employed as a challenge organism in Swiss white mice to test the ability of various typhoid vaccines to protect these animals against death. The animals were vaccinated with either purified Vi antigen (from Citrobacter freundii), acetic anhydride-treated S. typhi endotoxin, potassium methylate-treated S. typhi endotoxin, or an acetone-treated, whole cell vaccine prepared from S. typhi TY2. Only the whole cell vaccine was found to confer significant protection against death in this system.

3. A partially diploid Escherichia coli hybrid recovered from mating with a Salmonella typhimurium donor was converted to an Hfr strain, designated WR2080, to examine the manner in which the added Salmonella genetic material was conserved in it. The Salmonella argH<sup>+</sup>, metB<sup>+</sup> and rha<sup>+</sup> alleles contained as supernumerary genes in WR2080 were transferred together to E. coli recipients in interrupted mating experiments approximately 25 min after initial parental contact; transfer of the allelic E. coli genes by a haploid Hfr of the same transfer orientation occurred between 23.5 min (argH<sup>+</sup>) and 25 min (rha<sup>+</sup>) after initial contact. Entry of the E. coli ilv<sup>+</sup> marker of WR2080 in these experiments occurred at 29.5 min, 1.5 min later than the entry time of this marker from the haploid E. coli Hfr. When unselected inheritance of the recessive E. coli argH<sup>-</sup> and rha<sup>-</sup> alleles of WR2080 was examined among ilv<sup>+</sup> selected E. coli recipients in which unselected inheritance of the Salmonella donor genes was shown to be low (8%), inheritance of argH<sup>-</sup> was only 7%, whereas 51% inherited the neighboring rha<sup>-</sup> gene. In a comparative cross employing a haploid E. coli Hfr, in which rha in-

heritance was similar at 56%, argH inheritance was 41%. It was concluded that the Salmonella genes contained in WR2080 were conserved on a genetic segment about 1.5 min in length chromosomally inserted near the allelic E. coli genes, thus creating a duplication of that region within the hybrid chromosome.

Table 1. Protective activity of Vi, Acet-RE, Meth-RE, and AK vaccines in mice challenged with S. typhimurium hybrid H42

Vaccinated with: Vaccinating dose		No. of survivors/no. injected
Vi	250 $\mu$ g	1/20
Acet-RE	500 $\mu$ g	5/20
Meth-RE	250 $\mu$ g	2/20
AK	$5 \times 10^8$ cells	11/20*
Control-not vaccinated		2/20

\* Protected to a significant degree as compared with saline-injected controls ( $P < 0.01$ ).

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 092 Microbial genetics and taxonomy

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Project 3A161102B71R  
RESEARCH IN BIOMEDICAL SCIENCES

Task 03  
Psychiatry

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23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish 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 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) 75 07 - 76 06 Studies in the acquisition of complex behavioral chains by monkeys showed that immediate visual feedback on correctness of component behaviors in some cases can be more crucial to eventual successful performance than long term incentives like food and water. Experiments on choice indicated that the behavior of individual animals is predictable from the economic principles of substitutable and complementary commodities. Attempts to replicate and extend published work on behavioral effects of adrenocorticotrophic hormone have led to questions on generality of these phenomena. Reorganization of the Division necessitates termination of this unit. Further work in this vein will be pursued within work units 071 and 072 of task area 01, project 3A161102B71P. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.									

\* Available to contractors upon originator's approval

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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.

For the purposes of this report, research conducted in this unit may be seen as falling into two major subdivisions, the boundary of which might be said to consist of epithelial cells. One area of work is aimed at the analysis and development of laboratory models of complex behavior which allow specification of environmental contingencies leading to both effective and ineffective performance under stress. Experiments of this nature are thus primarily concerned with interactions of organisms with their external environment. A second area of work within this unit arises from cognizance of the fact that effectiveness, or lack of it, results from the combined effects of both internal and external factors. Experiments are thus directed at the behavioral effects of such factors as body chemistry, CNS function, hormonal balance, and disease. Reorganization of the Division of Neuropsychiatry requires that future work in both these areas be pursued within more comprehensive work units entitled "Behavioral Variables in Autonomic Function and Disease in Military Personnel" and "Biological Modulation of Military Performance," Work Units 072 and 071 of Project Number 3A161102B71P, Task Area 01.

LABORATORY MODELS OF COMPLEX BEHAVIOR

Studies reported in this section attempt to specify environmental factors or contingencies leading to both effective and ineffective performance in healthy, intact organisms under a range of physical and psychological stressors. Successful accomplishment of this task also provides general principles governing the acquisition and



maintenance of effective behavior, and technology and expertise necessary for objective assessment of other behavioral problems affecting the health and functioning of military personnel.

Principles of behavioral change-behavioral change controlled by visual signals for reward.

Three monkeys have completed this study and a report of the findings has been accepted for publication. The monkeys were trained to emit a chain of three responses on three separate levers in a set of six levers to obtain food. The chain producing food (correct chain) was changed each day. During a trial, a press on a correct lever produced a distinctive stimulus signalling possible reward; the third correct press produced a food pellet. Test sessions in which either the food or the distinctive stimuli were removed were interspersed with baseline sessions. In tests without food presentations but with the distinctive stimuli that previously signalled food reward, the subjects showed rapid acquisition of the correct chain with a level of accuracy comparable to baseline. Removing the distinctive stimuli signalling food for either the first or second member of the correct chain greatly retarded acquisition of that member of the chain. Removing all distinctive stimuli often reduced accuracy throughout the chain to the chance level even though food was presented following each correct chain. These results are interpreted as evidence that the distinctive stimuli presented after correct responses functioned as acquired or conditioned rewards. Furthermore, the order of acquisition of each member of the chain with signals was the reverse of the order when no signals occurred. Together, these results contribute new understanding to the process of behavioral change and demonstrate that informative feedback signalling the correctness of each appropriate response greatly facilitates the acquisition of an entirely new pattern of responses. This feedback can in some cases be more crucial to successful behavioral change than the eventual attainment of a long term goal such as the food pellets eventually earned by our monkeys. Thus, the process of behavior change requires more than long term incentives; it requires also attention by instructors to the immediate consequences of each correct approximation to the eventual target behavior in order to rapidly shape new behavior.

### The role of reinforcers in choice behavior.

Studies are being conducted on 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. 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 was a fundamental comparison of choice between similar and dissimilar reinforcers. The purpose was to discover those characteristics of choice which are unique to comparisons of dissimilar reinforcers. The results reported last year were that reductions in the availability of one food source increased responding for another food source but decreased responding for water. Two further experiments completed this year established that these changes in responding occur even when there are special provisions to insure constant daily intake of food and water. It was discovered, however, that when food intake was held constant, total food responding remained constant despite changes in the choice. This contrasted with the previous year's results indicating that total food responding increased with decreases in food availability, independent of total daily intake. As reported last year, 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 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). We have now made observation of one subject responding for postero-lateral hypothalamic and two responding for lateral-tuberal hypothalamic brain stimulation. The results from both electrode sites have been similar. As the availability of both reinforcers was decreased, the responding for food increased, maintaining constant intake, while responding for 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.

#### 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. For over a year we have been making observations in a setting in which an animal can be stressed and, by manipulating the duration and 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. In a study reported last year 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 a session length of 3 hr and R-S = 1 hr. the subject responded continuously and avoided all shock. When the session length was suddenly extended to 12 hrs to coincide with the day-night cycle, responding quickly deteriorated 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) represented an important psychological stress paradigm that was extended this year by investigating the role of abruptness in producing this disruption. In this experiment avoidance sessions were initially one hour long and the delay between responses and the next shock (R-S interval) was 10 sec. Sessions were gradually lengthened during a two month period to nine hours. Then the R-S interval was gradually lengthened to 50 minutes over an additional two month period. Finally, after four months with nine hr sessions and 50 minute R-S intervals, the session was lengthened to 12 hrs to coincide with the subject's day-night cycle. In contrast to the gross disruption of performance observed last year with a sudden increase in session duration, this gradual approximation to those same final parameters produced no gross disruption of eating or drinking and all shocks continued to be avoided. In fact, daily records of eating indicate that about half of the subject's ration of food is eaten in two or three daytime meals while responding to avoid shock. This study demonstrates the crucial importance of past experience in modulating the gross behavioral effects of an aversive situation. Stress as commonly understood is not simply a reflection of the current aversive situation but is a consequence of an aversive situation imposed without prior preparation to adjust to it behaviorally.

#### Behavioral rhythms

An aspect of military operations that is beginning to receive considerable attention is the requirement for continuous round-the-clock operations, or for operation at unusual times of day. While it is well established that performance under such requirements will be subject to rhythmic fluctuations (circadian and ultradian rhythms),

little is known of variables affecting such rhythmic processes, and little is known of dependent variables or behavioral processes most likely to show such variations. What literature does exist on behavioral rhythms deals almost totally with behavioral output, or the rates at which behavior is emitted. The studies in this section are initial investigations of rhythmic processes in aspects of behavior that are orthogonal to rate. Such aspects of behavior would include learning, differentiation, discrimination, and the effects of various stressors, including drugs on these dependent variables. The present studies are concerned with different types of discrimination behavior. Many studies of circadian rhythms have as a primary concern the nature of the entraining stimuli, and whether the observed rhythms are endogenously or exogenously controlled. The present studies are not particularly concerned with this facet of rhythms, since it is assumed that military operations to which the results of these studies may be applied would be carried on in the presence of normal (day/night; mealtimes) entraining stimuli.

#### Auditory duration discrimination rhythms in rats.

Each of four male albino rats lives in its own experimental chamber containing two levers, a "sonalert" auditory signal generator, a dispenser for delivering 45 mg food pellets, a continuously available water bottle, and overhead lights to produce general illumination. Eight experimental sessions are run daily, spaced three hours apart, four with the overhead lights on, and four with them off. Each session consists of a sequence of trials spaced 40 sec apart. Each trial lasts a maximum of 20 seconds and is signalled by the onset of an intermittent tone from the sonalert. Each tone burst consists of a train of pulses, one every two seconds, and it is the task of the rat to discriminate the duration of the pulses. If the pulses are short, 5 presses on the left lever produce a food pellet. If the pulses are long, 5 presses on the right lever produce food. The difficulty of the discrimination, then, can be varied by altering the relative duration of the short and long pulses. Half of the trials are of each type. The independent measures of behavior obtained for each session include the overall frequency of responding (percent of trials in which responding occurs), and the accuracy of those responses that do occur. In the present experiment, only the difficulty of the discrimination was altered. Three different difficulty levels were used, and each level was in effect for at least two months.

As expected, there was a considerable circadian rhythm evident in work rate at all levels of difficulty, with some reduction in the amplitude of the effect as the discrimination was made more difficult. There was also a small but consistent rhythm in accuracy of the discrimination, at all difficulty levels. This effect was most pronounced at the intermediate difficulty, since floor and ceiling effects were apparent at the extremes. The correlation between accuracy and work rate, though high, was not perfect, with some instances of high accuracy occurring with low work rates, and vice versa.

#### Interceptive discrimination rhythms in monkeys.

The rat experiment just described involved discrimination of stimuli in the external environment. The present experiment requires that the animals discriminate their own behavior by counting. Two rhesus monkeys each have two response keys available. During experimental sessions, which are one hour long, both keys are illuminated with white light. In order to get food, the monkey must press the right key exactly 8 times, then press the left key once. If the left key is pressed before 8 consecutive right key presses, or if the right key is pressed more than 8 times, the keys go dark for 5 sec, and the animal must try again.

A consistent problem in the investigation of cyclic processes in non-rate measures of behavior is the maintenance of enough behavior during the animal's normally inactive period of the day to measure non-rate processes. This problem is particularly severe when food is the reinforcer being used to maintain the behavior. One solution to the problem would be to run only a single session each day, thus assuring adequate food deprivation at session time. However, this creates a situation wherein the animal could possibly adapt to the altered work schedule, thus attenuating effects due to the unusual time of day. The present experiment attempts to deal with this problem by using inter-session intervals different from 24 hours. In particular, intersession intervals of 28, 20, and 15 hours have been investigated. The animals are housed in a room in which the overhead lights are on for 12 hours daily, and off for the other 12.

Preliminary results show that as the interval between sessions is decreased, work rate of animals begins to show a more pronounced rhythm. Despite the unusual feeding pattern, however, the work rate remains entrained

to the light-dark cycle. One of the animals also shows a consistent rhythm in accuracy of the counting task, while the other does not. Several parameters of this procedure will be manipulated in the future, including the difficulty of the task, which now seems to be too easy, and the amount of effort required to terminate the response chain.

#### Estrus-correlated cyclicity in operant performance

The steady increase in the number of women in the armed forces over the past few years (current plans count on a 25% female Army by 1980) and their relative concentration in high technology, high skill jobs has called attention to a second type of behavior rhythm--one with a cycle length approximating 28 days rather than 24 hours. Although the complex interplay of hormones underlying the menstrual cycle has been well studied, and related to sexual behavior in a variety of species, much less is known about effects of this cycle on behavior which is not obviously sexual. Complaints of premenstrual irritability, fatigue and nausea have been reported to be paralleled by alterations in sensory acuity, time estimation, and autonomic responsivity. Others have reported a disproportionate incidence of arrests, accidents, and psychiatric admissions during the premenstrual phase. In an effort to extend these clinical observations, we have begun an attempt to document the existence and assess the generality of estrus-related changes in well-studied, well-controlled laboratory models of non-sexual behavior. Thus far we have examined the lever-press performances of female rats (whose estrus cycle is only 4 days in length) under two schedules of food reinforcement. While our survey of performance will ultimately include aversively controlled lever pressing and respondent conditioning as well, preliminary results seem to indicate that under some conditions at least, response rate under a variable-interval (VI) schedule of food reinforcement is reliably lower during the estrus stage of the cycle (vaginal smears consist almost exclusively of cornified epithelial cells). However, pressing for food pellets under identical conditions, but on a schedule (DRL 20-sec) which maintains much lower rates, is apparently unaffected by estrus. In addition, no cyclicity in VI performance is observed if behavioral testing takes place during daylight hours and/or under conditions of mild food deprivation. Estrus effects thus appear to be highly situation-specific, and considerable further research will be required to discern critical features of estrus-sensitive situations.



## INTERNAL DETERMINANTS OF EFFECTIVE PERFORMANCE

The ability of soldiers to perform effectively in the presence of the demands imposed upon them is wholly dependent on the interplay of two major classes of factors. External factors such as the tactical situation, the quality of leadership and training, the terrain and the environment can either support or oppose effective performance. Similarly, internal factors such as body chemistry, fatigue, hormone levels and disease also impact on the soldier's effectiveness. Effectiveness, or lack thereof, results from the combined effects of both internal and external factors operating in the combat environment. The purpose of this program is to continue to develop and refine a base of laboratory data to explain how these two classes of variables interact to maintain the effectiveness of soldiers and their units. Animal models of performance are created using the techniques of operant and respondent conditioning, and the role of internal factors in performance variability assessed by measuring concomitant changes in physiology and, conversely, by directly effecting major changes in specified physiological systems and measuring resulting changes in aspects of the subject's behavior. By doing so we identify and attack potential problems, provide solutions to problems already identified from previous combat operations, and develop and maintain the analytical technology and the fundamental knowledge necessary to answer questions which will arise the next time our personnel are committed to combat.

### Behavioral effects of adrenocorticotrophic hormone

Adrenocorticotrophic hormone (ACTH), a pituitary hormone implicated in stress research since the field emerged, has recently been discovered to have effects on the central nervous system quite independent of its stimulation of the adrenal cortex. This has been determined by using adrenalectomized subjects or synthetic ACTH analogs without adrenal effects. Such CNS effects are inferred from altered performance in stressful situations or tasks. We expect to determine the boundary conditions for these effects, examine the extent to which these effects might be common to other hormones, and determine whether ACTH and/or other pituitary or adrenal hormones might also have behavioral effects in situations not ordinarily deemed stressful by human observers. In



the past year we have collected data on the effects of exogenous ACTH on a wide variety of performances by albino rats. Statistically significant alterations in behavior attributable to ACTH have been observed in three experiments, all conducted sequentially with the same subjects. First, although the amount and temporal pattern of locomotor activity during a daily 15-min test in a dark, sound attenuated chamber was unchanged by 16 I.U. ACTH, animals given such injections each day for 10 days showed a significantly greater increase in activity in response to food restriction during this period. The same animals were subsequently trained to lever press for food reward. Although no statistically significant ACTH effects were observed in the acquisition of bar pressing under a schedule of continuous reinforcement (each press produces a pellet), a subsequent change to a schedule reinforcing only presses spaced 20-sec apart (DRL-20 schedule) revealed a significantly quicker adaptation by rats receiving injections of ACTH shortly before each daily testing session. However, subsequent shifts to increasingly more demanding fixed-ratio x schedules (pellet only after X responses, where  $2 < X < 256$ ) and extinction (no pellets) schedules were ineffective in differentiating ACTH from saline-treated subjects. The final testing procedure which did yield a significant ACTH effect was a passive avoidance test, in which the latencies of water-deprived rats to enter and drink in a small chamber were recorded each day, both before and after application of an aversive electric shock to their feet during drinking. Chronic ACTH treatment led to significantly lower latencies to enter and drink in the ten days following the shock. A parallel taste aversion experiment, using a high dose of the antineoplastic agent cyclophosphamide as the aversive stimulus rather than electric shock, has been attempted with several variations (e.g., in number of bottles; number, timing, and dose of ACTH injections, etc) with results each time resembling those of the passive avoidance study, but never quite reaching conventional levels of statistical significance. In this context it should also be mentioned that attempts to replicate the effects of ACTH on lever pressing and passive avoidance with new groups of naive subjects have thus far been unsuccessful. We see several possible courses of future action in response to these negative findings. First, we may attempt to replicate in every detail, rather than merely in general design, some of the more successful published studies in this area. Second, we may extend the work we have reported here in a parametric fashion, trusting that ACTH effects are confined to certain as yet untried

doses, times courses, shock levels, schedule values, etc. Third, we may turn to the use of ACTH analogs without adrenal effects (adrenal cortex hormones have occasionally been reported to have effects opposite to those of ACTH) and/or the use of the ACTH blocker dexamethasone rather than saline as the control injection (to preclude the possibility that our "control" animals are sufficiently stressed by the injection procedure to produce brain levels of ACTH similar to those of the ACTH "experimental" subjects).

Taste aversion conditioning in olfactory bulbectomized rats as a function of duration of exposure to ionizing Radiation

Exposure to ionizing radiation can motivate in one trial the acquisition of a powerful and long-lasting avoidance response in many species of animals. This avoidance conditioning results in the conditioned avoidance of a normally palatable taste stimulus that has been paired with radiation. It has been frequently reported that olfactory bulbectomy attenuates taste aversion conditioning in rats. The present study investigated the bulbectomy-produced deficit in taste aversion conditioning as a function of intensity of the unconditioned stimulus. Groups of olfactory bulbectomized and sham-operated rats were exposed to 0, 25, 50, or 100 roentgens of X-Rays after consuming a 0.1% saccharin solution. Taste aversion conditioning was subsequently assessed using both one-bottle and two-bottle testing techniques. Both methods of testing revealed that taste aversion conditioning varied directly with the level of radiation received, in bulbectomized as well as sham-operated animals, but that the bulbectomized rats required higher levels of radiation to produce aversion levels comparable to the sham-operated controls. Thus bulbectomized rats can learn taste aversions, but generally show weaker aversions than controls at any given radiation level. These results suggest that either bulbectomized rats are less sensitive than intact rats to the effects of ionizing radiation, are impaired in making the associative corrections required in taste aversion learning, or do not develop the same affective response to internal stimuli as do normals. The latter interpretation is preferred on the basis of the results of other studies of the effects of bulbectomies.

## Lesions of central noradrenergic pathways and behavioral arousal

As part of an overall program of research investigating the relationships between central neurotransmitter pathways and behavioral arousal, studies are being conducted involving monitoring the behavior of rats after specific brain lesions that alter the neurochemical balance within the central nervous system. One such study involved an attempt at gaining information concerning the functions of the dorsal noradrenergic fiber bundle that originates in the nucleus locus coeruleus in the dorsal pons and innervates various forebrain structures, including the cerebral cortex, with the putative neurotransmitter norepinephrine. The pontine area of the brain has been repeatedly implicated in the neuro-anatomical control of behavioral arousal, alertness, sleep and waking, and recently a great deal of interest has been generated in the dorsal bundle as one of the potentially critical pathways involved in the control of arousal.

Mating behaviors provide the basis for a convenient animal model for the study of behavioral arousal. Mating behaviors in animals, especially the laboratory rat, are extremely stereotyped and well documented, thus providing an ideal tool for the laboratory study of arousal. Standard recording techniques for quantifying the sexual behaviors of laboratory animals provide several indices of arousal, including latencies to initiate and complete copulation, and latencies required for rearousal after the completion of a copulatory bout. It was reasoned that if the dorsal bundle was functionally related to behavioral arousal, then the integrity of this fiber system may well be necessary for the proper execution or patterning of the behaviors involved in mating. The present study examined the copulatory behavior of rats in which the DNB was discretely lesioned at its source in the nucleus coeruleus (LC) in the dorsal pons. Male Wistar-derived rats were given three copulatory tests before receiving bilateral electrolytic lesions of the LC or sham-lesion operations. Standard measures of copulatory behavior were recorded for two copulatory series on each test: mount and intromission latencies; ejaculation latency; mount and intromission frequencies; main interintromission interval; and postejaculatory interval. After a two-week recovery period testing was resumed and three post-operative tests were conducted,

separated by one week each. Cortical NE was reduced by 51-91% (mean = 65%) in the lesioned animals, but no lesion-related changes in mating behaviors were observed. PEI measures were normal as compared with the Shams. These results suggest that increased sexual vigor resulting from rostral midbrain lesions may be unrelated to the interruption of the DNB and depletion of telencephalic NE. The role of the locus coeruleus in the control of sleep and, thus, arousal has long been appreciated. This experiment demonstrates that the LC may not generally influence all types of arousal and in particular is not critical for the development and maintenance of behavioral arousal required for mating behavior in the male rat.

A second study is presently underway which utilizes wheel-running activity as an index of behavioral arousal and which is aimed at determining if lesions of the dorsal noradrenergic pathways alter the amount of patterns of activity in rats. Lesions of the dorsal bundle at the locus coeruleus reportedly abolish REM or dream-state sleep patterns in rats. In humans, deprivation of such REM sleep patterns produces restlessness, irritability, and poor performance on memory consolidation tasks. In rats, disrupted sleep patterns may be reflected in changes in total levels of general activity, or, more likely, in changes in the circadian patterning of general activity. Six male rats were adapted to living in activity cages which had an attached running wheel. Preoperative activity in the wheels was recorded every quarter-hour round the clock for two weeks, then the rats were subjected to bilateral lesions of the nucleus locus coeruleus and post-operative measures were begun, also round the clock. Preliminary results indicate that the lesions produce reduced activity during the first week post-operatively, but as yet it remains to be seen if this effect is permanent, or if the circadian distribution of activity is affected. Future studies will be designed to evaluate stress-induced wheel running in lesioned and non-lesioned rats, to further investigate possible changes in arousal mechanisms as a function of adrenergic depletions.

#### Effects of alpha-adrenergic agonist clonidine on locomotor activity in the rat.

Clonidine, a new anti-hypertensive agent, may exert a general depression of behavior at higher doses.

It is not clear, however, whether or not the facilitating effects of the clonidine on responding for hypothalamic electrical stimulation, food, and water at lower dose levels are the result of a non-specific behavioral excitation or are due to the activation of specific adrenergic systems modulating these behaviors. To examine this question, the general activity of rats was recorded before and after the injection of clonidine hydrochloride over a wide range of doses: 0, 6.25, 12.5, 25, 50, 100, 200 mcg/kg.

Activity testing was conducted in two circular photocell activity chambers (Lehigh Valley, Inc) 61 cm in diameter with wire mesh floors and metal walls 41.3 cm high, painted flat black. Two rows of three photo-beams aligned perpendicularly to each other illuminated photocells which detected movements along the grid floor. The animals were habituated to the chambers for 1 h prior to drug injection. After the habituation hour they were removed from the chambers, injected i.p. with the appropriate drug dose, then replaced in the chamber for three more hours. Activity counts were totaled at the end of each hour.

Clonidine had no stimulating effect on locomotor activity within the dose range that increased responding for food, water, and ESB. Rather, clonidine significantly depressed activity at doses of 50 mcg/kg or higher. In order to rule out the possibility that the one-hour habituation period was too short for the demonstration of any stimulating effects of clonidine, another experiment was conducted using a four-hour habituation period before drug injections. A clonidine dose of 12.5 mcg/kg was chosen for study, as this dose had been found to have facilitatory effects on operant behaviors without depressing locomotor activity. Student's *t*-test comparisons of the mean activity by hour revealed no significant differences between the drug and control groups. Clonidine-induced increases in operant responding for food, water, and EBS hypothalamic electrical stimulation thus cannot be attributed to a general stimulation of locomotor activity. Indeed, clonidine significantly depresses activity at doses of 50 mcg/kg or higher. Such a depressant effect could be expected to adversely interact with performance of many behavioral tasks and may reflect a general depression of physiological functions.

### Dissociation of visual deficits following partial inferior temporal lesions in monkeys

Severe impairment on a delayed matching-to-sample task was found after lesions of the anterior inferior temporal cortex. The finding is consonant with earlier suggestions that anterior temporal lesions yield visual deficits that are dissociable from the visual discriminative deficits produced by posterior temporal lesions. In the delayed matching study, however, reliable impairment was found only with anterior temporal removals that were larger than in any previous study. They included the rostral two-thirds of the inferior temporal convexity (area TEO) rather than just the middle third, while the posterior temporal removals were limited, as in earlier studies, to the caudal third of the convexity (area TEO). In order to determine whether the dissociation of deficits found earlier could be obtained despite the increased size of the anterior removal, monkeys with TE and TEO lesions were compared on automated versions of both delayed matching with a pair of grossly dissimilar stimuli and simultaneous discrimination learning with pairs of highly similar patterns. The TE lesion produced the greater effect on delayed matching, yet the smaller TEO lesions still had the greater effect on pattern discrimination learning. The results thus support the original proposal that the posterior part of the inferior temporal cortex participates mainly in visual discriminative functions while the anterior part contributes primarily to visual processes involving the mnemonic or affective domains.

### Interactions of limbic system with neocortex in monkeys

Previous studies done in collaboration with Dr. Mortimer Mishkin of NIMH have shown that damage to the ventral frontal lobe (the orbital cortex, considered to be the major cortical component of the limbic system so intimately involved in emotional behavior), produces a severe and long-lasting deficit in the performance of a well-learned delayed match-to-sample problem. We have also found severe deficits following removal of the neocortex on the inferior temporal pole. Current experiments indicate that these lesions produce equivalent impairments because they interfere with different portions of a common system. The number of subjects is still quite small, but it appears

that although unilateral orbital or temporal pole ablation in one hemisphere and an orbital ablation in the other leaves the monkey unable to regain his pre-operative level of performance, unlike monkeys with bilateral orbital or bilateral temporal pole lesions, he does ultimately perform at better than chance levels. Our initial feeling that these above-chance scores were due to interhemispheric communication via the corpus callosum and/or anterior commissure has gone unsupported thus far, since severing these connection in these subjects has been without effect.

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Task 03 Psychiatry

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