

ADA 038320

P

AD

RCS MEDDH - 288 (RI)

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

E. C.
APR 18 1976
C

VOLUME II

WALTER REED ARMY INSTITUTE OF RESEARCH

WALTER REED ARMY MEDICAL CENTER

WASHINGTON, D.C. 20012

AD NO. _____
DDC FILE COPY

Approved for public release; distribution unlimited.
Destroy this report when no longer needed. Do not return it to the originator.

PII Redacted

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER RCS MEDDH-288 (R1)	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) RESEARCH IN BIOLOGICAL & MEDICAL SCIENCES, INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASE & IMMUNOLOGY, INTERNAL		5. TYPE OF REPORT & PERIOD COVERED Annual Progress Report-Vol II 1 July 1975 - 30 June 1976
		6. PERFORMING ORG. REPORT NUMBER NA
7. AUTHOR(s) Listed at beginning of each work unit report	8. CONTRACT OR GRANT NUMBER(s) NA	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington, D. C. 20012		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Listed at beginning of each report
11. CONTROLLING OFFICE NAME AND ADDRESS U. S. Army Medical Research & Development Command Washington, D. C. 20314		12. REPORT DATE July 1976
		13. NUMBER OF PAGES 852
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) NA		15. SECURITY CLASS. (of this report) UNCLASSIFIED
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE NA
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) NA		
18. SUPPLEMENTARY NOTES NA		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) BIOLOGICAL SCIENCES IMMUNOLOGY MEDICAL SCIENCES INTERNAL MEDICINE BIOCHEMISTRY NUCLEAR MEDICINE COMMUNICABLE DISEASES PHYSIOLOGY		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) 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.		

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 68 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

368450

YB

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

19. PSYCHIATRY
SURGERY
VETERINARY MEDICINE

2

A

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

AD

RCS MEDDH - 288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

INCLUDING

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

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

Annual Progress Report.
1 July 1975 - 30 June 1976.

11 Jul '76

12 848p.

Volume II

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

Approved for public release; distribution unlimited

Destroy this report when no longer needed. Do not return it to the originator.

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 77 report will be included in the FY 1977 Annual Progress 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.

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.

TABLE OF CONTENTS

VOLUME II

		<u>Page</u>
3A762760A806	MILITARY PREVENTIVE MEDICINE	
00	→ Military Preventive Medicine	
034	Epidemiologic studies of military diseases	781
3A162110A821	COMBAT SURGERY	
00	→ Combat Surgery	
121	Gastrointestinal responses to shock and trauma	793
124	Pathophysiology of systemic responses to shock and trauma	802
125	Control mechanisms of regional circulation	809
3A762760A822	MILITARY INTERNAL MEDICINE	
01	→ Military Internal Medicine	
120	Metabolic response to disease and injury	817
121	Pathogenesis of enteric diseases	832
123	Histopathologic manifestations of military diseases and injuries	843
126	Infectious hepatitis	897
128	Biochemical methodology and laboratory automation	901
129	Epidemiology of hepatitis in the military	906
130	Gastrointestinal diseases of military importance	913
3A762658A823	MILITARY PSYCHIATRY	
00	→ Military Psychiatry	
030	Military psychiatry	925

	<u>Page</u>
031 Military performance and stress: Factors leading to decrements of performance and disease	934
033 Anatomical and physiological correlates of brain function in stress and disease	958
034 Influence of stress on hormone response, performance and emotional breakdown in military personnel	965
 3A762758A824 RADIATION INJURY AND PROTECTION	
02 → Microwave Radiation	
057 Biological effects and hazards of microwave radiation	979
 3A762759A829 MALARIA PROPHYLAXIS	
00 → Malaria Investigations	
308 Biological evaluation of antimalarial drugs	992
309 Determination of pharmacological effects of antimalarial drugs	996
315 Blood level assays for antimalarial drugs	1066
318 Biological studies of insect infection and disease transmission	1073
324 Host responses to malaria	1097
336 Field studies on drug resistant malaria	1112
337 Syntheses of antimalaria drugs	1144
338 Protective immunity in malaria	1150
339 Studies of malaria parasitic metabolism and drug action	1156
 3A762759A831 TROPICAL MEDICINE	
00 → Tropical Medicine	
070 Antischistosomal drug development	1161
071 Field studies of rickettsiae and other tropical diseases	1166
073 Disease transmission in tropical populations	1214

	<u>Page</u>
074 Tropical and subtropical diseases in military medicine	1374
075 Rickettsial diseases of military personnel	1499
 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE	
00 > Biomedical Factors in Drug Abuse <i>J and</i>	
101 Assay methodology for drugs of abuse	1527
102 Military performance and drug abuse	1531
103 Drug abuse prevention in military personnel	1546
105 Cellular aspects of the metabolism of drugs of abuse	1553
106 Clinical and demographic studies of military drug abusers	1560
109 Neurophysiological localization of sites of actions of drugs of abuse	1562
110 Biorhythm studies in drug abuse	1566
111 Neuroendocrinology and neurochemistry of drugs of abuse	1568
113 Metabolism of drugs of abuse	1576
114 Pharmacokinetics of drugs of abuse	1581
 3A762760A837 MILITARY ANIMAL RESOURCES DEVELOPMENT	
00 > Military Animal Resources Development	
055 Development and evaluation of improved bio- logical sensor systems	1589
056 Diseases of military animals	1596
 DISTRIBUTION LIST	 1633

Project 3A762760A806
MILITARY PREVENTIVE MEDICINE

Task 00
Military Preventive Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
					DA OB 6489	76 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^c	6. WORK SECURITY ^d	7. REGRADING ^e	8A. ONE'S INSTR ⁿ	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 ^g	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER				
A. PRIMARY	62760A	3A762760A806	00	034				
B. CONTRIBUTING								
C. CONTRIBUTING	CARDS 114F							
11. TITLE (Precede with Security Classification Code) ^h								
(U) Epidemiologic Studies of Military Diseases								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁱ								
003500 Clinical Medicine 005900 Environmental Biology								
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
72 07		CONT		DA		C. In House		
17. CONTRACT / GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (In thousands)
A. DATES/EFFECTIVE: NA				PRECEDING				
B. NUMBER ^j				FISCAL		G		4
C. TYPE				YEAR		7		19
D. KIND OF AWARD:				E. AMOUNT:		F. CUM. AMT.		
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORG. ACRONYM				
NAME ^k : Walter Reed Army Institute of Research				NAME ^k : Walter Reed Army Institute of Research				
ADDRESS ^k : Washington, D.C. 20012				ADDRESS ^k : Washington, D. C. 20012				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Punish MAN if U.S. academic institution)				
NAME: Joy, COL Robert J.T.				NAME: Segal, Herbert E., LTC, MC				
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2480				
21. GENERAL USE				22. ASSOCIATE INVESTIGATORS				
Foreign intelligence not considered				NAME: Gaydos, Joel C., LTC, MC				
				NAME: Hodder, Richard A., LTC, MC				
23. KEYWORDS (Precede EACH with Security Classification Code)								
(U) Epidemiology; (U) Infectious Disease; (U) Congenital Malformation; (U) Renal Disease								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Punish individual paragraphs identified by number. Precede rest of each with Security Classification Code.)								
23. (U) To identify, define, and study known and potential causes of disability in military populations using relevant, existing epidemiologic techniques and developing appropriate new methodology. To apply this information to the prevention and control of disability in military populations.								
24. (U) Contemporary epidemiologic methods are applied to causes of disability in military populations. Multidisciplinary collaborative approaches are utilized and new methods developed as required.								
25. (U) 75 07-76 06 Analyses of data from investigations of salmonellosis and Group B streptococcus outbreaks at Fort Polk were completed. Analysis of data from an investigation of an A/swine influenza outbreak at Fort Dix is in progress. Analysis of data from an Adenovirus antibody prevalence study is in progress. (Influenza and Adenovirus studies are complementary to work described under DA OA 6441, Work Unit 166, entitled "Virus Infections of Man.") Computation of congenital malformation rates by time and post and correlation of these rates with demographic variables are in progress. Retrieval of renal disease admissions data has been requested. For technical reports see Walter Reed Army Institute of Research Annual Report 1 Jul 75-30 Jun 76. Support in the amount of \$2,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.								

PII Redacted

DD FORM 1498

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

Project 3A762760A806 MILITARY PREVENTIVE MEDICINE

Task 00 Military Preventive Medicine

Work Unit 034 Epidemiologic studies of military diseases

Investigators:

Principal: LTC Herbert E. Segal, MC
Associate: COL Taras Nowosiwsky, MC; COL Franklin H. Top, Jr., MC; LTC Joel C. Gaydos, MC; LTC Richard A. Hodder, MC; MAJ Michael W. Benenson, MC; MAJ Richard G. Kirchdoerfer, MC; CPT Virginia D. Soden, ANC; SSG Michael C. Callahan; L. Charlene Evans

1. Salmonella newport Outbreak Related to Milk Consumption

In early August 1975, the Fort Polk MEDDAC personnel noted an upsurge of salmonella isolations among pediatric patients. England AFB began to experience a similar increase. By mid-August, one to three cases were diagnosed daily at each installation as either Salmonella group C-2 or, more specifically, as S. newport. The source for these infections was not apparent. An Air Force epidemiology team (USAFSAM-EP) was first alerted to investigate the outbreak at England AFB with an invitation to conduct a similar inquiry at Fort Polk one or two days later. Because no obvious solution was apparent and Fort Polk was having the majority of cases, an EPICON team joined the investigation.

The initial investigation disclosed 23 culture confirmed cases of S. newport (or Salmonella C-2, partially typed) at Fort Polk and 13 confirmed cases at England AFB (the final counts were 25 and 17, respectively). There were no cases reported from the two adjacent civilian communities. All identified cases occurred in military families with children; unaccompanied service members and trainees were spared. There was no apparent association of cases by housing area, water or sanitary system, nor was there history of common social or recreational contacts. The initial case interviews failed to disclose an association between disease and usage of a typical salmonella vehicle.

The clinical picture in reported cases was a febrile, diarrheal illness of several days duration. In general, the illness was relatively uncomplicated and antibiotic or inpatient therapy was seldom necessary. Clinical findings are summarized for children and adults in table 1. Of note

is the 50% incidence of bloody stool and low occurrence of dehydration.

Table 1

Signs and Symptoms in Culture Confirmed Cases
Fort Polk, S. Newport

	<u>Adult (N = 6)</u>		<u>Children (N = 17)</u>	
Fever	3	(50%)	14	(82%)
Diarrhea	6	(100%)	16	(94%)
Cramps*	6	(100%)	7	(41%)
Nausea*	4	(67%)	6	(35%)
Vomiting	3	(50%)	8	(47%)
Dehydration	0		2	(12%)
Bloody stool	3	(50%)	9	(53%)

* Childhood cases difficult to interpret due to the subjective nature of the symptom. Infants are included.

The information obtained during the early phase of the investigation suggested this to be a food-borne outbreak propagated through a commissary-distributed food vehicle (both commissaries utilized the same procurement system). Initially, the contaminated food was sought by crude but time saving methods. When this approach failed, a more tedious but precise and systematic search was instituted.

First, a more accurate definition of the outbreak as to time, place and person was needed. Cases were reinterviewed to confirm onset dates and define contacts with other cases. Family contacts were cultured. Although no disease was reported in civilians or basic trainees, the evidence to support this was tenuous. Therefore, troop records were screened and dispensary personnel interviewed for a change in the incidence or severity of gastrointestinal complaints. The pediatric clinic, emergency room and troop dispensaries were requested to culture anyone with diarrhea. The State Epidemiologist, Louisiana Health and Human Resources Administration, Division of Health, was notified of the outbreak and was requested to intensify

disease surveillance in the nearby civilian communities. Concurrently, preparations were made to conduct a food preference survey. The first step was to reduce the quantity of separate line items handled by the two commissaries (over 5,000). By excluding those items not common to both a 10% reduction was achieved. A third commissary within the same procurement chain located at Barksdale AFB (no Salmonella cases), near Shreveport, LA was used to eliminate all line items that were common to all three. In this manner, it was possible to reduce the list of "suspect" foods to below 100. From it, approximately 20 items were chosen for more intensive scrutiny. These included foods which were believed to best fit the age and place characteristics of the outbreak. Particular weight was given to baby foods.

The food preference survey was conducted by phone. Pre-printed questionnaires, scoring sheets and detailed instructions for the survey were available to each interviewer. Only "primary" cases and controls were interviewed. A "primary" case was defined as the family's sole or index case who had no history of close contact with prior cases. The control group was selected randomly, by computer (last two digits of SSAN - 08, 15, 32, 36, 45, 64, 75, 91), and was matched to cases by age and family size.

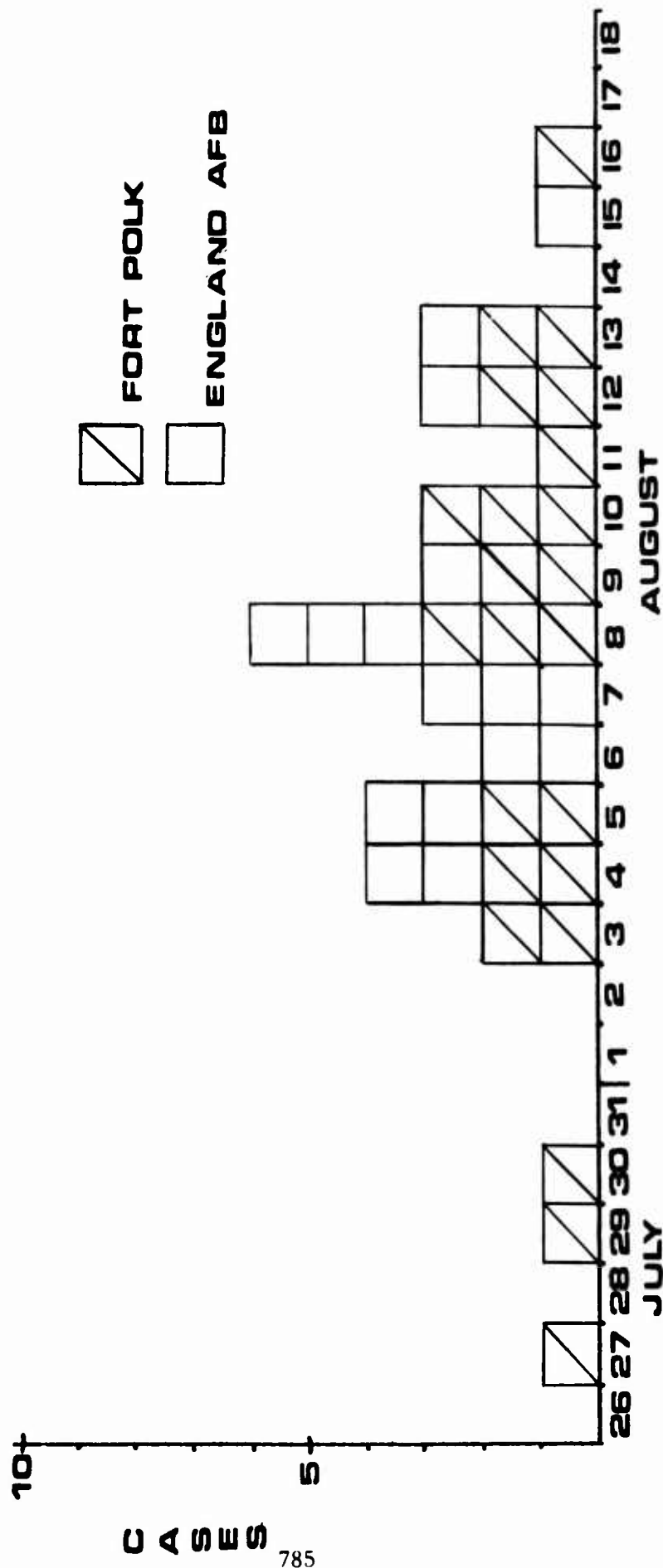
The initial preventive measures taken were those which would reduce the risk of person-to-person transmission. Children coming to the post nursery were screened for fever or a history of recent diarrhea and were refused admission if these symptoms were present. Primary medical care providers at dispensaries, clinics and the emergency room were asked to culture potential cases and exclude any symptomatic food handlers from duty until proven negative by culture.

Figure 1 shows the epidemic curve for culture positive cases at both installations. Noteworthy are the rather well circumscribed temporal limits of the outbreak over the period 27 July to 16 August. This is consistent with a continuous, common source exposure over a one to two week time interval. Some limited person-to-person spread probably had occurred, mainly within families. Cases ceased after 16 August and as of 1 October, there were still no further *S. newport* isolations. Although contact interviews and increased surveillance disclosed many other cases of gastroenteritis, none were positive for salmonella. Of the twenty-five (25) total cases, twelve (12) were in children less than 2 years old and no cases were in infants below the age of 5 months. Only a few cases were scattered

FIGURE ONE

CULTURE - CONFIRMED CASES OF SALMONELLA NEWPORT

BY DATE OF ONSET - FT. POLK & ENGLAND AFB, 1975



through the adult years.

Fort Polk cases were scattered randomly over several housing areas and were not linked through common social, recreational or occupational factors. The use of the post nursery or common babysitters were rare. In this setting, the striking predominance of young children as cases and the exclusion of troops and unaccompanied members from the illness provided strong presumptive evidence for a commissary food item as the source. The apparent sparing of the local community strengthened this possibility. A pediatrician at Fort Polk was interviewed about the feeding patterns of young children. Infants were changed from formula to milk at about the age of 6 months. The addition of baby foods and table foods was much more variable, the former was usually earlier than 6 months while the latter was usually later.

The distribution pattern of milk from the WR Dairy fit exactly the geographic distribution of cases. In addition, time, place and person characteristics of the outbreak were consistent with a milk vehicle. As the investigation progressed, it was noted that disease cases were associated with the WR brand of whole milk but only when it came in one gallon plastic containers (as opposed to WR whole milk in one half gallon cartons). A special phone interview was initiated to test this hypothesis. To assure minimum observer variance, only the two investigators interviewed at Fort Polk. Cases and the same controls used for the food preference survey were re-contacted and asked to specify the type (whole, skim, low-fat, etc.) brand and container size of milk they bought. Only after the individual expressed another brand and/or container preference were they asked specifically about the use of WR brand of milk and the type of container. In only one instance did specific questioning disclose a WR milk user who would have been called a non-user. The family, a devotee of another brand came back from vacation and borrowed a one gallon container of WR milk on 2 August. Their child became ill the next day. All other cases counted as "users" regularly bought WR milk in the one gallon containers.

Milk preference in cases at Fort Polk is tabulated in Table 2. Thirteen of 16 cases were positive for the use of WR milk while only eight out of 25 controls could be said to be users of WR milk.

Table 2

The Association of One Gallon WR Milk Use
and S. newport Gastroenteritis at Fort Polk, LA

S. newport Gastroenteritis

		YES	NO	
Use of one gallon WR milk	YES	13	8	21
	NO	3	17	20
		16	25	41

Chi-square (Yates) = 7.6
p = .006

The food preference survey did not identify an alternate suspect for the outbreak. Although the p value was significant for several items, either the item was actually "protective" or of such low usage level as to be easily rejected. Of note, there was no difference in milk usage between the controls and the cases. Since the survey did not specifically ask for milk brands (or container sizes), the WR milk hypothesis could not be tested. The major value of the survey was as a "pertinent negative" and a broad review of all other potential food vehicles.

The commissary was checked for containers of milk from the suspect period. There were three (3) soured 1 gallon containers and five (5) 1/2 gallon containers spanning the time period from 26 July until 7 August. Samples for culture were provided the laboratories at Fort Polk, Fort Sam Houston, Texas and the USAFSAM. Remaining milk samples were transmitted to the Enteric Disease Section, CDC for culture and fluorescent antibody studies. All test results were negative.

2. Group B Beta Hemolytic Streptococcal (GBBHS) Disease
Outbreak in a Nursery

During the period Feb to Oct 75 an increase in the GBBHS disease in the newborn nursery at Fort Polk was noted. Six of 527 liveborn infants in 1975 were thought to have had serious disease due to Lancefield GBBHS. One infant had early onset disease and his mother had GBBHS

isolated from the birth canal. The remaining five cases had late onset, two had septic arthritis, one multiple brain abscess, one meningitis, and one breast abscess. Additionally there were three other infants in the nursery with isolates of Beta Hemolytic Streptococci not groups A or D, one from an endotracheal tube, one an umbilical catheter tip, and the other from surface cultures. Six cases of GBBHS disease in 527 births represent a rate of 11.4 per 1000 live births. This is significantly more than expected given the rate of 2-3 per 1000 reported from other areas. In order to determine infant colonization rates in the nursery, all newborns from 17 November to 16 December were cultured. Using non-selective media a colonization rate of 26%, (14 of 53 infants) was obtained. An attempt was made to (retrospectively) culture the mothers of those infants that were found to be positive. These data did not allow differentiation between vertical and nosocomial transmission.

Two EPICON investigators arrived at Ft. Polk and, in order to define the problem, interviewed involved personnel and examined available data sources. Personnel interviewed included the Nursery Pediatrician, the Head Nurses in the Nursery and on the OB-Gyn Service, the Chief of Pediatrics, the Chief of Nursing Services, the Bacteriologist, and the Chief of the H&E Activity. Data sources reviewed were medical records of infants suspected of having GBBHS disease and their mother's obstetrical records, obstetrical ward log books, nursery daily census log books, and laboratory specimen log books.

Interviews provided background information about the hospital physical plant, nursery staffing practices, and other conditions with a possible relationship to GBBHS disease. The nursery, as is the entire hospital, is housed in World War II cantonment type buildings. Recently the nursery was enlarged to include a second room but prior to that time space and ventilation were inadequate. Complaints regarding the air conditioning in the OB-nursery wards during summer 1975 were common. Other findings regarding nursery staffing practices included the fact that during nursery staff shortages, nurses were pulled from other wards with perhaps insufficient regard for their prior experience or for their recent presence on infectious wards; and the nursing staff had to provide housekeeping services for the OB ward and the nursery.

The medical records provided investigators a means to make a determination whether a case was GBBHS disease or perhaps GBBHS colonization. Of the total of 10 suspected

cases, six were considered GBBHS disease occurring during 1957. Tabulations of maternal complications, premature rupture of the membranes (PROM), prematurity and number of births by month were obtained from review of the 1975 obstetrical log book. During the year there were 527 births. The first six months average was 38.3 per month compared to 49.5 for the balance of the year. The highest number of births was recorded in August. There were 56 "C" sections, with 44 as primary sections. Eleven of the total were performed in August. Twelve deliveries were noted as PROMS. All 12 were over 24 hours except for two of 16 and 18 hours and two with the duration not indicated. Four of the total occurred during August. Seventy-four premature infants were born evenly throughout the year.

Nursery log books were reviewed to find the daily nursery census and the number of infants on the seriously ill list (SI) as indicators of staff demand and efficiency. The greatest monthly nursery census average occurred in August, 1975. During the second week of that month there were three days with over 15 infants in the nursery (average 6.2). At this same time, there were four infants on the SI list. Bacteriology laboratory books for 1975 were reviewed to find positive culture reports pertaining to infants and mothers which might reveal possible cases that were overlooked. This led to the discovery of two additional possible cases, which on review of their medical records were determined not to be GBBHS disease.

The occurrence of six cases of GBBHS disease in this newborn population is more than would normally be expected. Three of the cases occurred during the months of August and September. Reviewing all data the following observations were made: (a) In August, there were 57 births, the largest monthly number for 1975; (b) The nursery census was higher in August than any other month of the year. During the second week, there was a 24 hour census of 19 infants in the nursery; (c) There were eleven "C" sections, the largest number for any one month. The "C" sections required more nursery care for the infants because post-op mothers are not as able or willing to care for their infants; (d) Ward B-1 was closed because of a potential fire/explosive hazard from 22 July to 27 August. All patients and infants were moved into ward B-2 within a few hours. This was an open ward and inadequate for use as a nursery and labor ward; (e) July, August, and September are "vacation" months and insufficient staffing and/or the necessity of pulling relatively inexperienced (for nursery techniques) personnel from other wards only compounded the problem; (f) On 4

August Case #5 was born and was considered a case of GBBHS sepsis. It is possible that this case was responsible for widespread contamination of the nursery environment.

The end of August 1975 Ward B-1 was reopened and the nursery was enlarged with another room added for intensive care. The drop in the nursery census, improved facilities, and patient care could explain the drop in the number of cases since that time.

3. Influenza A/swine Outbreak at a Basic Training Center

In February 1976, throatwashings from 4 inpatients at Walson Army Hospital at Fort Dix were reported to contain a new strain of A Swine Influenza (A New Jersey). A fifth case was subsequently identified from post-mortem examination of a recruit who died of viral pneumonia. Documentation of the pattern and extent of the outbreak was undertaken. The index cases included 4 BCT recruits and one AIT cadre. An additional seven probable cases were identified from paired adenovirus surveillance sera. Case interviews failed to implicate a common source, suggesting infection from man-to-man transmission during a more widespread outbreak. Since recruit populations are grouped in discreet, isolated cohorts, antibody prevalence rates in units of cases could be compared to non-case units to test this hypothesis and define the pattern of spread. A ten percent sample of all BCT units on post was performed and a simple "case-control" comparison performed. Comparison showed that the seropositivity rate in companies with a case defined by virus isolation was 41.2%, in companies with a case defined by a four-fold titer rise was 20.0%, and in control companies was 10.2%.

The above preliminary data are being refined. Criteria for admission to the study, effects of vaccination and heterologous infection, and the association of selected demographic variables are being reviewed. Additionally, results in dependents, retirees and cadre will need adjustment by age, sex, vaccination history, etc. Additional preliminary serologic data are reported under DA Project 3A161102B71Q, Work Unit 166, Viral Infections of Man (DCD&I).

4. Prevalence of Antibodies to Adenoviruses 4, 7, and 21 in Male Basic Combat Trainees

A study was initiated to obtain current data on the prevalence of antibodies to adenoviruses in trainees arriving at BCT centers. These data were required to

estimate the potential impact of adenovirus 21 epidemics on BCT posts and to provide a basis for the design of adenovirus 21 vaccine trials. During the period 22-24 March 1976 a sample of incoming trainees at Forts Jackson, Knox, and L. Wood was selected for study. Questionnaires were administered to obtain demographic information and blood samples were drawn. For Dix trainees who participated in the investigation of the A/swine influenza outbreak (February 1976) and who had blood samples obtained within 7 days after arriving on post were also included in this study. Preliminary results from this study are reported under DA Project 3A161102B71Q, Work Unit 166, Viral Infections of Man (DCD&I).

5. Incidence of Congenital Malformations at Fort Hood, TX

During the study of an outbreak of viral hepatitis among active duty populations at Fort Hood, Texas (October 1972-March 1975) data were gathered pertaining to congenital malformations (CMs) reported by Darnall Army Hospital (Fort Hood). Compilation and analysis of data unexpectedly revealed marked increases in reported CM rates from 24.4 cases per 1,000 total births in 1971 to 41.6 in 1972, 51.2 in 1973 and 54.9 in 1974 (Jan-Jun). A more thorough investigation was then undertaken.

Computer tapes containing records for all births and all pediatric admissions (ages 1-14) from 1 Jan 1971 through 30 Jun 1975 for Fort Hood and six other CONUS installations were obtained from the U.S. Army Health and Information Systems and Biostatistical Agency, Fort Sam Houston, Texas. Data from these tapes were adapted for use in the Walter Reed Army Medical Computer System and an existing programming system was modified for data retrieval.

Currently, congenital malformation rates by time and installation are being computed and correlated with demographic variables.

6. Chronic Renal Disease Diagnoses Among Active Duty Personnel

In 1974, a joint committee established by the NIH and the National Kidney Foundation was charged with identifying resources by which renal disease information could be developed. The military was proposed as one key source of reliable statistics. However, their efforts to retrieve Army information were nonproductive. The need for Army collaborators was recognized, and a formal request for EPICON assistance was submitted and approved. The project is in its initial phases. The two major sources of error

in the preliminary study have been identified and corrected. An exhaustive list of ICDA renal codes has been submitted to the Individual Patient Data System (IPDS) to retrieve all records with renal disease codes in military treatment facilities from 1971-1975. Concurrently, the Division of Biometrics, WRAIR, is preparing a user-oriented program to analyze the tapes for the incidence, prevalence, morbidity, mortality, and demographic characteristics of patients with renal disease.

Project 3A162110A821
COMBAT SURGERY

Task 00
Combat Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
				DA OA 6467	76 07 01	DD-DR&E(A)1036	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^c	6 WORK SECURITY ^d	7 REGRADING ^e	8A DMS'N INST'N	8B SPECIFIC DATA - CONTRACTOR ACCESS	8C LEVEL OF SUM
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. MORE UNIT
9 NO / CODES ^f	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62110A	3A162110A821		00	121		
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						
11 TITLE (Precede with Security Classification Code) ^g							
(U) Gastrointestinal Responses to Shock and Trauma							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^h							
008800 Life Support 016200 Stress Physiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
63 09		CONT		DA		C. In-House	
17 CONTRACT/GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING			
b. NUMBER ⁱ				76		4	
c. TYPE:				CURRENT YEAR		118	
d. KIND OF AWARD:				77		3	
e. AMOUNT:						168	
f. CUM. AMT.							
20 RESPONSIBLE DDO ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL ^j				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R. J. T.				NAME: Reynolds, LTC D. G.			
TELEPHONE: 202 576-3551				TELEPHONE: 202 576-3791			
22 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Zinner, MAJ M. J.			
				NAME: Gurll, MAJ N. J. DA			
23 KEYWORDS (Precede EACH with Security Classification Code) (U) Splanchnic Hemodynamics; (U) Gastric Blood Flow; (U) Shock; (U) Vasoconstrictor Therapy							
23 TECHNICAL OBJECTIVE ^k , 24 APPROACH, 25 PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To elucidate the gastrointestinal response to shock and trauma. To evaluate the effects of epinephrine and norepinephrine on gastric blood flow. To evaluate the effects of these drugs and truncal vagotomy currently used to control gastrointestinal hemorrhage. To study the effect of hemorrhagic shock on the development of stress ulceration. These studies relate to the gastrointestinal pathology that frequently occurs in combat casualties.							
24 (U) To study the gastrointestinal hemodynamic response to experimental hemorrhagic shock in the primate and dogs. To study the functions of the gastric mucosal barrier during hemorrhagic shock. To evaluate the effects of adrenergic amines and vasopressin on the gastric circulation. To study the effect of vagotomy on gastric blood flow and AV shunting.							
25 (U) 75 07 - 76 06. 1. The primate gastrointestinal circulations do not seem to have significant amounts of ischemia during shock. During hemorrhagic shock, monkey GI blood flow fell only 20 to 30 per cent, and during endotoxic shock baboon GI blood flow fell only slightly. The regional GI exceptions are the stomach, pancreas, and spleen, which fell significantly during shock. 2. During endotoxic shock in the baboon there was no disruption of the normally defined gastric mucosal barrier. There was some increase in acid back diffusion. The presence of this acid back diffusion and the gastric ischemia resulted in stress ulcers. 3. Truncal gastric vagotomy does not appear to redistribute flow or open AV shunts in the primate stomach. Support in the amount of \$22,000 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.							

PII Redacted

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.

Task 00 Combat Surgery

Work Unit 121 Gastrointestinal responses to shock and trauma

Investigators.

Principal: LTC David G. Reynolds, MSC

Associate: MAJ Nelson J. Gurll, MC; MAJ Michael J. Zinner, MC

I. Gastrointestinal Blood Flow in Response to Shock

A. Background. In many previous clinical settings of shock the ischemic response of the GI tract has been held responsible for irreversibility of the shock state. This work was founded on early studies done in the dog. A study was conducted in primates to determine the effects of hemorrhagic and endotoxic shock in gastrointestinal blood flow.

B. Experimental Approach. Cynomolgus monkeys had left ventricular injections of radioactive microsphere (RMS) during a control period 4 hours following hemorrhagic shock and 2 hours following resuscitation. Baboons were used for the endotoxic shock study. These animals were injected with RMS during a control period, 4 hours after endotoxemia (LD25) and 12 hours following fluid resuscitation. Blood flow to all major GI organs was determined.

C. Results and Discussion. Blood flow to stomach, pancreas, and spleen are severely decreased during shock but blood flow to small and large bowel are not severely compromised. In the baboon during the endotoxic shock, a greater percentage of cardiac output went to the gut during shock and resuscitation than during the control period.

II. Regional Distribution and Arteriovenous (AV) Shunting in the Gastric Circulation

A. Background. Previous studies from this laboratory failed to demonstrate significant AV shunting in the gastric circulation. Even under the influence of intra-arterial epinephrine or vasopressin infusion, no increases in AV shunting were noted. The role of truncal vagotomy in control of stress ulcer bleeding has long been thought to be the result of opening AV shunts in the submucosa and diverting blood flow away from the mucosa. A study was conducted on the effect of vagotomy on regional blood flow and AV shunting in the gastric circulation of baboons.

B. Experimental Approach. Total gastric blood flow was measured electromagnetically by placing a flow transducer on the celiac artery of animals that had been splenectomized and had common hepatic artery ligation. The hepatic artery was used for intra-arterial delivery of radioactive microspheres ($15 \pm 5 \mu$) with 3 tags. These were injected

during a control period, immediately following truncal vagotomy and 2 hours following vagotomy. These were used to determine regional distribution of blood flow and AV shunting in the stomach.

C. Results and Discussion. At this time the study is not completed. Preliminary results would indicate that about 75% of gastric blood flow goes to the mucosa and this is not altered following vagotomy. Approximately 2% of the injected microspheres appear in the liver during control periods and this also does not appear to change following vagotomy. We would conclude the AV shunting plays a negligible role in the control of gastric blood flow.

III. Stress Ulceration

A. Background and Statement of the Problem. Acute superficial erosion of the gastric mucosa continues to be a frequent cause of life-threatening hemorrhage in the post-traumatic patient. Much attention has focused on the role of the gastric mucosal barrier (GMB) and its disruption in the pathogenesis of these stress ulcers. The traditional indications of a broken barrier are a fall in transmural electrical potential difference (PD) and an increase in mucosal permeability resulting in a greater luminal accumulation of sodium ion and back diffusion of hydrogen ion. Disruption of the GMB has been reported in critically ill patients thus implying a role in the development of stress ulcers.¹ We have investigated the effects of severe hemorrhagic hypotension in dogs and endotoxemia in baboons on the integrity of the GMB. A variety of factors have been implicated in the disruption of the GMB including bile salts, aspirin, alcohol, and urea. It has also been suggested that histamine release in the gastric mucosa is important in this disruption.² Recent work has focused on the metabolic state of the mucosal cell itself as a determinant of gastric mucosal integrity.³ In order to more fully elucidate the roles of histamine and metabolic state of the mucosa on the ability of the stomach to protect itself against erosion, we studied the effects of the histamine H₂ receptor blocker, metiamide, as well as alteration of acid-base balance on the ability of the gastric mucosa to deal with its own secreted acid.

B. Experimental Approach. Dogs and baboons were prepared with internally-drained Heidenhain pouches which had their blood supply based solely upon the splenic vessels. The net fluxes of volume, hydrogen, sodium, potassium, and chloride across these pouches were determined by instillation and recovery of an isotonic acid test solution containing a nonabsorbable volume marker. These tests were performed during a control period, for three hours of hypotension, and for one hour following resuscitation. The hypotension was induced either by hemorrhage to a blood pressure of 45 mm of mercury or intravenous injection of endotoxin 10 mg/kg. Resuscitation was with the shed blood, intravenous fluids, and correction of acid-base balance. Total pouch blood flow was measured by an electromagnetic flowmeter on the splenic artery. Mucosal blood flow was determined by an aminopyrine clearance method. Regional and fractional blood flows during control, shock, and post-resuscitation periods were determined by microsphere injections as described before.

Transmural electrical PD was monitored continuously. Cardiac output was measured by thermal dilution using a Swan-Ganz pulmonary artery catheter. Pouch and total-body oxygen consumptions could be calculated from the product of blood flows (pouch flow or cardiac output) and arteriovenous oxygen content differences (splenic artery to pulmonary vein or femoral artery to pulmonary artery).

Studies were also performed in monkeys in the attempt to produce a reliable primate stress ulcer model. Two species of monkeys were used - Rhesus and cynomolgus. Under light anesthesia, the stomach was cannulated after isolation of the duodenum and esophagus to prevent efflux of gastric contents. An isotonic saline solution containing sodium taurocholate (a known barrier breaker) was instilled into the stomach. Samples of this solution were periodically aspirated for measurement of non-absorbable volume marker and electrolytes. Fluxes were then calculated from changes in ion mass. The studies were performed during control, one to four hours of hemorrhagic shock, and following resuscitation with fluids. Blood flows were determined using microspheres.

The effect of histamine H₂ receptor blockade with metiamide on the gastric mucosa was assessed in rats and conscious dogs. The rats were divided into groups to receive either saline as a control or metiamide. Stress ulcers were induced by 24 hours of fasting and restraint in wire mesh. The metiamide was given in a dose of 5 mg/kg body weight intraperitoneally every eight hours for the 24 hours of restraint in one group; in another group this treatment was started four hours before restraint. Another control group was given metiamide without restraint or fasting. The stomachs were excised and graded by an impartial observer. The dogs were studied awake by perfusion of externally-drained Heidenhain pouches for three hours on each test day. The perfusion fluid was an isotonic acid test solution which contained sodium taurocholate in half of the tests. The dogs receive either saline or metiamide at 12 μ moles/kg/min as an intravenous infusion. Electrical potential difference was monitored continuously.

In anesthetized dogs, the effect of altered acid-base balance was investigated using isolated in vivo patches of full thickness gastric fundus. These patches were mounted in double lucite chambers one half of which was exposed to an isotonic acid test solution while the other half was exposed to a mannitol control solution. Fluxes of volume and ions were determined from changes in mass between instillation and recovery of the solutions with corrections for acid secretion on the control side. The studies were performed every fifteen minutes for one hour each of control, acidosis, and recovery. Acidosis was induced by decreasing the tidal volume from 10 to 5 ml/kg body weight and introducing 5% CO₂ with 95% oxygen into the ventilator.

C. Results and Discussion.

1. Systemic Hemodynamic Changes. Mean arterial pressure decreased from 122 \pm 5 to 79 \pm 9 mm of mercury ($p < .001$) and cardiac output fell

from 2.76 ± 0.31 to 1.43 ± 0.24 L/min ($p < .001$) by the fourth hour of endotoxic shock. The magnitudes of these changes were somewhat less than that induced in hemorrhagic shock which was reported previously. Endotoxemia resulted in an early decrease in total body oxygen consumption but a return to control values by the fourth hour of endotoxemia.

2. Regional Hemodynamic Changes. Endotoxemia resulted in the decrease in pouch mucosal blood flow from 5.2 ± 0.9 to 2.4 ± 0.7 ml/min ($p < .01$). This change was again smaller in magnitude than the change seen in canine hemorrhagic shock where total pouch blood flow fell from almost 40 ml/min to 5 ml/min. Endotoxic shock in the baboon did not result in a change in the fractional distribution of blood flow to the pouch. The mucosal blood flow, representing 65% of the total blood flow to the pouch, was unchanged by shock or resuscitation. In the monkey experiments, hemorrhagic shock resulted in dramatic decreases in gastric, pancreatic, and duodenal blood flow during shock which did not increase appreciably with resuscitation. In contrast, blood flow to the liver, distal small bowel, and colon was maintained fairly well during hemorrhagic shock.

3. Potential Difference and Ionic Permeability. Potential difference fell from 40 ± 3 to 28 ± 4 mV ($p < .01$) and remained low following resuscitation after endotoxemia. The magnitude of this fall was not as great as that seen in canine hemorrhagic shock. The ionic fluxes for potassium increased from $+5.9 \pm 2.1$ to $+11.1 \pm 2.5$ mEq/30 min/100 sq cm ($p < .01$) in shock and to $+19.5 \pm 7.0$ after resuscitation from endotoxic shock. This increase in the luminal gain of potassium ion was similar to that seen with hemorrhagic shock and indicates some damage to the gastric mucosa. With endotoxemia there was a moderate but not significant increase in back diffusion of hydrogen from -58 ± 26 to -131 ± 59 mEq/30 min/100 sq cm but no change in the luminal gain of sodium ion in the pouch from a control value of $+183 \pm 44$ mEq/30 min/100 sq cm. Following resuscitation from endotoxemia the luminal gain of sodium increased to $+257 \pm 74$ with hydrogen ion back diffusion decreasing to -52 ± 16 . As with hemorrhagic shock, these ionic fluxes do not indicate the traditional breaking of the gastric mucosal barrier.

4. Gross and Microscopic Examination. Despite the lack of physiological evidence to the disruption of the GMB, many of the Heidenhain pouches subjected to endotoxic shock showed superficial gastric erosions confirmed histologically. Most of these baboons had evidence of gross bleeding from the pouch sometime during the shock or resuscitation period. These findings are similar to those seen in hemorrhagic shock although the ulcerations were not as severe in endotoxemia.

5. Effect of Histamine H₂ Receptor Blockade. Metiamide did result in a slightly higher potential difference in both control and taurocholate-treated dogs but the fall in potential difference due to taurocholate was not attenuated by metiamide. The ionic fluxes of sodium, hydrogen, potassium, and chloride were unchanged by metiamide in animals exposed either to an acid test solution or to bile salts. In rats, metiamide protected against the development of stress ulcerations.

6. Effect of Acid-Base Balance on Hydrogen Ion Back Diffusion.

Respiratory acidosis resulted in a fall in arterial pH from 7.38 to 7.19 and an increase in calculated serum hydrogen ion concentration from 43 to 67 nM/L. Despite this change in the electrochemical gradient, hydrogen ion loss from the stomach was not significantly changed by acidosis, being 56 ± 23 before and 56 ± 24 mEq/15 min after acidosis. Recovery resulted in normalization of arterial pH and serum hydrogen ion concentration with no change in hydrogen ion loss from the pouch. However, during acidosis the hydrogen ion loss was inversely related to serum hydrogen ion concentration. In conclusion, respiratory acidosis does not seem to change the net loss of hydrogen ion. Any increase in the gastric mucosal permeability by acidosis might be offset by an increase in the adverse electrochemical gradient.

Even with severe ischemic insult to the gastric mucosa resulting from hemorrhage or endotoxemia there appears to be no increase in ionic permeability and no break in the gastric mucosal barrier in either the canine or baboon model. Acid in the lumen of the stomach is sufficient to cause acute superficial erosions of the gastric mucosa in the presence of severe ischemia. This probably results from an inability of the stressed mucosal cells to buffer the normal amount of acid that is back diffusing. This is made worse by the attendant acidosis in these shock states. Histamine H₂ receptor blockade with metiamide decreases the incidence of stress ulceration without affecting the changes in ionic permeability due to barrier-disrupting agents like bile salt.

IV. Cardiovascular Effects of Urokinase

A. Background and Statement of the Problem. Urokinase is an activator of plasminogen and has been used to treat pulmonary embolism.⁴ Urokinase was found to be more effective than heparin when used intravenously to treat pulmonary embolism. The chief problem associated with its use, however, was a high rate of bleeding complication which may have been due to the addition of heparin to the urokinase regimen. A variety of thrombo-embolic diseases are associated with vasospasm. While investigating the possibility of using urokinase intra-arterially, we discovered that this enzyme is also a vasodilator. This vasodilator property may be of value in the treatment of thrombo-embolic occlusions especially those associated with vasospasm. However, the relationships between vasodilation and the other properties of urokinase, namely fibrinolysis and thrombolysis, have not been elucidated. Such relationships may be important in the clinical application of intra-arterial urokinase therapy. The intra-arterial route may prove to be of more value than the intravenous route because of vasodilation and the requirements for a smaller dose.

B. Experimental Approach. Arterial blood flow in the superior mesenteric and femoral circulations of dogs was measured by electromagnetic flowmeters. Urokinase isolated from human urine was injected and infused intra-arterially over a dose range of several decades. The effects on arterial blood flow, arterial blood pressure, and venous blood pressure were recorded. These infusions then were repeated after blocking plasminogen activation with epsilon-amino caproic acid (EACA).

In another set of experiments on dogs, obstructing arterial thrombi were produced by a modified Wessler technique in the femoral and superior mesenteric arteries. The arteries were then either infused with saline or urokinase at several different dose levels. The results of these infusions were evaluated by arterial blood flow measurement (electromagnetic flowmeter), degree of thrombolysis (arteriogram), and fibrinolysis (fibrin plate assay and euglobulin lysis time). Systemic and regional blood samples were taken for determination of clotting-coagulation as well as fibrinolysis.

C. Results and Discussion. Intra-arterial urokinase infusion at 100 C.T.A. units/kg body weight/minute resulted in mild vasodilation in the femoral circulation. At this dose we were also able to achieve thrombolysis with complete dissolution of the clots in the femoral artery infused. No dissolution of the clot in the contralateral femoral artery was observed. At this dose level fibrinolysis was achieved by shortening of the euglobulin lysis time and prolongation of the fibrin plate lysis zone. We could regionalize this effect by concomitant treatment with EACA, and at this particular dose level, fibrinogen concentration was unchanged. In contrast, infusion of urokinase at 100 C.T.A. units/kg/min into the superior mesenteric arterial circulation produced no increase in blood flow. At this particular dose level there was thrombolysis as well as fibrinolysis similar to that seen in the femoral artery. However, serum fibrinogen began to fall at this particular dose level; and when 125 units was infused for 100 minutes serum fibrinogen fell to less than 50 mg %. Thrombolysis was also achieved at 75 units/kg/min at which point the fibrinogen level was normal. It would seem that in the superior mesenteric arterial circulation pushing the dose above 100 C.T.A. units/kg/min did not enhance thrombolysis, an approach too dangerously close to fibrinogenolysis. In other words, we were unable to use a dose in the superior mesenteric arterial circulation to produce vasodilation without untoward effects. In this circulation we were also not able to get a regionalization of effects as we did in the femoral circulation using EACA.

The intra-arterial administration of urokinase offers an exciting new way to treat thrombotic occlusions of major vessels without severe alterations in blood clotting. Dissolution of all thrombi was achieved in either the femoral or superior mesenteric circulation at a dose of approximately 100 C.T.A. units/kg/min for 100 minutes. These studies provide a firm background for the use of urokinase in the human clinical situation.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Gastrointestinal responses to shock and trauma

Literature Cited.

References:

1. Skillman, J. J., Gould, S. A., Chung, R. S. K., and Silen, W.: The gastric mucosal barrier: clinical and experimental studies in critically ill and normal man, and in the rabbit. *Ann. Surg.* 172: 564, 1970.
2. Davenport, H. W.: Gastric mucosal injury by fatty and acetylsalicylic acids. *Gastroenterology* 46: 245, 1964.
3. Menguy, R., Desbaillets, L., and Masters, Y. F.: Mechanism of stress ulcer: influence of hypovolemic shock on energy metabolism in the gastric mucosa. *Gastroenterology* 66: 46, 1974.
4. Urokinase Pulmonary Embolism Trial: A National Cooperative Study. *Circulation* 47 (Suppl. II): 1, 1973.
5. Reynolds, D. G., Zinner, M. J., Wickline, D. L., Locke, D., and Gurll, N. J.: The effect of endotoxic shock and fluid expansion on regional blood flow in the baboon. *The Physiologist* 19: in press, 1976.
6. Gurll, N. J., Callahan, W., and Hufnagel, H. V.: High-dose, short-term local urokinase for clearing femoral thrombi by vasodilation and thrombolysis. *J. Surg. Res.* 20: in press, 1976.
7. Gurll, N. J., and Harmon, J. W.: Canine duodenal mucosal neutralization of acid. *The Physiologist* 19: in press 1976.

Publications:

1. Jacques, D. P., Reynolds, D. G., Evans, L. W., and Swan, K. G.: Adrenergic mechanisms in the hindlimb circulation of baboons. *Surgery* 77: 577, 1975.
2. Zinner, M. J., Kerr, J. C., and Reynolds, D. G.: Hemodynamic effects of intra-arterial infusions of catecholamines on the canine gastric circulation. *Surgery* 78: 381, 1975.
3. Zinner, M. J., Kerr, J. C., and Reynolds, D. G.: Adrenergic mechanisms in canine gastric circulation. *Amer. J. Physiol.* 229: 977, 1975.

4. Fromm, D. Schwartz, J. H., and Quijano, R.: Effects of salicylate and bile salt on ion transport by isolated gastric mucosa of the rabbit. *Amer. J. Physiol.* 230: 319, 1976.
5. Zinner, M. J., Kerr, J. C., and Reynolds, D. G.: Primate gastric circulation: effects of catecholamines and adrenergic blockade. *Amer. J. Physiol.* 230: 346, 1976.
6. Zinner, M. J., Turtinen, L., and Gurll, N. J.: Stress ulceration and the gastric mucosal barrier. *Amer. Surg.* 41: 209, 1975.
7. Zinner, M. J., Turtinen, L., Gurll, N. J., and Reynolds, D. G.: The effect of metiamide on gastric mucosal injury in rat restraint. *Clin Res.* 23: 484A, 1975.
8. Zinner, M. J., Gurll, N. J., and Reynolds, D. G.: The effect of hemorrhagic shock and resuscitation on gastrointestinal blood flow in cynomolgus monkeys. *Fed. Proc.* 35: 450, 1976.
9. Zinner, M. J., Gurll, N. J., and Reynolds, D. G.: Gastric hemodynamic and mucosal responses to endotoxemia in the baboon. *Gastroenterology* 70: A97/955, 1976.
10. Gurll, N. J., and Callahan, W., II: Hydrogen ion back diffusion is not affected by acidosis. *Gastroenterology* 70: A34/892, 1976.
11. Gurll, N. J., and Callahan, W., II: The effect of metiamide on the gastric mucosal barrier and its response to topical bile salts. *Gastroenterology* 70: A34/892, 1976.
12. Gurll, N. J., Callahan, W., II, and Hufnagel, H. V.: Vasodilation and thrombolysis with intra-arterial urokinase infusion in the canine femoral circulation. *Clin. Res.* 24: 219A, 1976.
13. Gurll, N. J., Callahan, W., II, and Hufnagel, H. V.: Effect of intra-arterial urokinase infusion on blood flow, clotting and fibrinolysis in a canine model of superior mesenteric arterial thrombosis. *Clin. Res.* 24: 285A, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OB 6532	76 07 01	DD-DR&E(AR)636	
1 D-YE PRE / SUMRY	4 KIND OF SUMMARY	3 SUMMARY SCTY ³	4 WORK SECURITY ⁴	7 REGRADING ⁷	8A DDB'S INST'N	8B SPECIFIC DATA - CONTRACTOR ACCESS	9 LEVEL OF SW
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10 NO CODES ¹⁰	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62110A	3A162110A821	00	124			
B. CONTRIBUTING							
C. SECURITY	CARDS 114F						
11 TITLE (Precede with Security Classification Code) ¹¹							
(U) Pathophysiology of Systemic Responses to Shock and Trauma							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ¹²							
012600 Stress Physiology							
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	20 FUNDS (in thousands)	
A. DATES/EFFECTIVE. NA				PRECEDING			
B. NUMBER ¹⁷				FISCAL YEAR	76	2	90
C. TYPE				CURRENT YEAR	77	2	143
D. KIND OF AWARD				E. CUM. AMT.			
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME*Walter Reed Army Institute of Research				NAME* Walter Reed Army Institute of Research			
ADDRESS* Washington, DC 20012				Div of Surgery ADDRESS* Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR / (Furnish MAN II U.S. Academic Institution)			
NAME. Joy, COL R. J. T.				NAME* Giordano, LTC J. M.			
TELEPHONE 202 576-3551				TELEPHONE 202 576-3793			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				[REDACTED]			
ASSOCIATE INVESTIGATORS				NAME:			
				NAME			
22 KEYWORDS (Precede EACH with Security Classification Code) ²² (U) Microaggregates; (U) Pulmonary Hemodynamics; (U) Vein Grafts							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number Precede text of each with Security Classification Code) ²³							
<p>23 (U) To evaluate the effects of microaggregates in stored blood on pulmonary hemodynamics; to determine the mechanism of the pulmonary arterial pressor response to alveolar hypoxia. These studies are important in understanding the mechanism of the respiratory distress syndrome and in the resuscitation of patients in shock, an area of vital importance in the management of combat casualties. To evaluate various materials for use as venous prostheses. Such material is needed to improve treatment of peripheral vascular trauma.</p> <p>24 (U) Radioactive microspheres were used to determine change in pulmonary hemodynamics before and after the administration of stored blood with microaggregates. H1 and H2 histamine antagonists and alpha and beta blockers were administered in an attempt to block the pressor response to alveolar hypoxia. A "Teflon" type of graft material was subjected to patency tests in the femoral venous system of dogs.</p> <p>25 (U) 75 07 - 76 07. No change in pulmonary hemodynamics were demonstrated before or after the administration of microaggregates suggesting that microaggregates may not be responsible for the respiratory distress syndrome. The histamine antagonists and the catecholamine blockers did not influence the pressor response to alveolar hypoxia. The Teflon graft material did not prove to be a suitable venous prosthesis. Support in the amount of \$45,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>							
For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 75-30 Jun 76.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 66 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 124 Pathophysiology of systemic responses to shock and trauma

Investigators

Principal: LTC Joseph M. Giordano, MC

Associate: COL Norman M. Rich, MC*

I. Microaggregates

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

B. Experimental Approach

1. Evaluation of Microaggregates. Evaluation of microaggregates was undertaken using 3 techniques: A model T multichannel particle size analyzer, the screen filtration pressure (SFP) apparatus of Swank, and the weighing of filter grids before and after the passage of known volumes of blood. A comparison of these techniques revealed the Coulter counter to be the only reproducible quantitative method for evaluating microaggregates.

2. Effect of Microaggregates. To determine the effect of microaggregates on the pulmonary microcirculation, blood was withdrawn from dogs and stored for 5 days. The quantity of microaggregates was determined and the blood transfused back to the animal through a catheter placed in the left pulmonary artery. The measurement of pulmonary blood flow was determined before and after transfusion by the distribution of radioactive microspheres. Our hypothesis was that if blood is redistributed away from the left lung following transfusion, this would indicate an effect of the stored blood on the pulmonary microcirculation. Three

*Associate Investigator, Chief, Peripheral Vascular Surgery Service, WRAMC

groups were investigated: a) Hemorrhagic shock model in which the animal was resuscitated by transfusing stored blood with microaggregates; b) a non-shock preparation in which blood was transfused by stored blood with microaggregates; and c) a non-shocked preparation in which stored blood without microaggregates was transfused.

C. Results and Discussion

1. Microaggregates in Stored Human Blood. Strict quantitation of microaggregates and the time course of the appearance of microaggregates in human blood anticoagulated in both ACD and CPD was undertaken. No microaggregate formation was noted for the first 7 days. Between 7 and 21 days a great volume of microaggregate formation occurred. From 21 to 28 days of storage smaller increases occurred. The volume of microaggregates in both anticoagulants after 28 days of storage were equal, the value being approximately $1500 \pm 100 \mu^3 \times 10^3/\text{mm}^3$. The volume of microaggregates was noted to occur only in the buffy coat, with plasma and packed red blood cell fractions being virtually free of microaggregate concentrations. Additionally, packed red blood cells, glycerol frozen red blood cells, and commercially prepared plasma and albumin fractions were all noted to be low in microaggregate content.

2. Evaluation of Factors Involved in Microaggregate Formation. These studies suggest that the removal of platelets and white blood cells from fresh blood prior to storage prevents microaggregate formation. Additionally, the in vitro or in vivo treatment of blood with aspirin reduces by approximately 50% but does not prevent microaggregate formation. Treatment of blood, before or after storage, with activators of the fibrinolytic system (urokinase or streptokinase) likewise reduce but does not totally prevent microaggregate formation. The constant agitation of blood during storage reduces microaggregate formation by approximately 40% but causes significant red blood cell hemolysis and damage to blood components. After microaggregates have been formed, treatment with Arvin followed by routine filtration reduces microaggregate levels to immeasurable levels. Likewise, the centrifugation of blood for 5 minutes at 5000 rpm after storage followed by routine filtration completely removes all microaggregate volumes.

3. Effect of Microaggregates on Pulmonary Hemodynamics. No significant redistribution of blood flow away from the left lung occurred in any of the three groups studied. This suggests that microaggregates may not play a major role in the development of the respiratory distress syndrome.

II. Infectibility of Vascular Grafts

A. Statement of the Problem. This laboratory has documented the infection rate for dacron, bovine, and autogenous vein grafts by the intravenous infusion of bacteria 3 weeks after the graft was placed in the canine infra-renal abdominal aorta. An important clinical question

is how long following operation is the graft still susceptible to infection.

B. Experimental Approach. The canine infra-renal aorta was excised and reconstructed with dacron grafts. Six months following operation, the animals received 10^7 colony forming units of Staphylococcus aureus intravenously. Three weeks later the grafts were harvested and cultured.

C. Results and Discussion. Although the grafts were in the animal for 6 months, 3 out of 14 became infected. No correlation could be determined between pseudointima development and infection. This study suggests that 6 months following a vascular operation, the risks of infecting a graft are sufficiently great to warrant administration of antibiotics prior to a procedure that may be associated with bacteremia.

III. Pulmonary Arterial Pressor Response to Alveolar Hypoxia

A. Statement of the Problem. The mechanism that mediates the pulmonary arterial pressor response to alveolar hypoxia remains undetermined. Most investigators have implicated a humeral factor secreted by pulmonary alveolar cells following exposure to low oxygen tensions. A series of studies were instituted to (1) set up a non-invasive model to investigate this response; (2) to administer H_1 and H_2 histamine blockers in an attempt to prevent the pressor response; and (3) to administer alpha and beta blockers to determine the influence of catecholamines on this response.

B. Experimental Approach. Dogs were intubated with a double lumen endotracheal tube. The distribution of cardiac output was determined by the right atrial injection of radioactive microspheres. The right lung was ventilated with 100% O_2 and the left lung with 5% O_2 . Microspheres were again injected to document the redistribution of blood flow away from the hypoxic left lung. Histamine and catecholamine blockers were administered in an attempt to influence this response.

C. Results and Conclusions. The histamine, alpha and beta blockers did not attenuate the pressor response. These studies suggest that histamine or the catecholamines do not mediate the pressor response to alveolar hypoxia.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 124 Pathophysiology of systemic responses to shock and trauma

Literature Cited.

References:

1. Lamoy, R. E., and Hobson, R. W.: Indium chloride scanning for identification of experimental abscesses. *J. Surg. Res.* In press, 1976.
2. Giordano, J. M., Zinner, M., Hobson, R. W., and Gervin, A. S.: The effects of microaggregates in stored blood on canine pulmonary vascular resistance. *Surgery* 79:1976, in press.
3. Giordano, J. M., Lamoy, R., Wright, C. B., and Hobson, R. W.: A comparison of autographs and frozen irradiated homografts in canine femoral venous reconstruction. *Surgery* 79:1976, in press.
4. Giordano, J. M., Zinner, M., Guba, A., and Lynch, N.: A model for the investigation of the pulmonary arterial pressor response to alveolar hypoxia. *Clin. Res.*, 1976, in press.

Publications:

1. Hobson, R. W., Wright, C. B., Zinner, M., and Lamoy, R. E.: Cerebral blood flow determination by radioactive microspheres in subhuman primates: Influence of unilateral internal carotid ligation, hypercapnic acidosis and hypocapnic alkalosis. *Surgery* 80:176, 1976.
2. Collins, G. J., Hobson, R. W., Rich, N. M., and Levin, I.: Arterial dilator-shunt for use in carotid artery fibromuscular hyperplasia. *Am. J. Surg.* 130:381, 1975.
3. Giordano, J. M., Wright, C. B., Lamoy, R., and Hobson, R. W.: Recanalization of venous grafts: Comparison of homografts and autografts. *Clin. Res.* 23:470, 1975.
4. Lough, F., Giordano, J. M., and Hobson, R. W.: Regional hemodynamics of large and small arteriovenous fistulas in dogs. *Surgery* 79:346, 1976.
5. Gervin, A. S., Mason, K. G., and Buckman, R. F.: The source and removal of microaggregates in aged human blood and human blood components. *Surg. Gynecol. Obstet.* 141:582, 1975.

6. Hobson, R. W., Rich, N. M., Wright, C. B., and Fedde, C. W.: Operative assessment of carotid endarterectomy: internal carotid arterial back pressure, carotid arterial blood flow, and carotid arteriography. *Amer. Surg.* 41:603-610, Oct. 75.
7. Wright, C. B., and Hobson, R. W.: Prediction of intestinal viability using Doppler ultrasound technics. *Am. J. Surg.* 128:642-645, Jun 75.
8. Rich, N. M., and Hobson, R. W., II: Trauma to the venous system. In: *Vascular Surgery in the Lower Extremities*. Edited by K. G. Swan, D. G. Reynolds, N. M. Rich, R. W. Hobson, and C. B. Wright. St. Louis: W. H. Green, Inc., 1976, pp. 3-19.
9. Reynolds, D. G., Jaques, D. O., Boynton, W. A., Brungardt, J. M., and Swan, K. G.: Adrenergic control of femoral blood flow, pp.145-160, *ibid.*
10. Swan, K. G., Wright, C. B., Evans, L. W., Kerr, J. C., and Reynolds, D. G.: Cholinergic effects on femoral blood flow, pp.169-176, *ibid.*
11. Wright, C. B., Reynolds, D. G., and Swan, K. G.: Sympathectomy and femoral blood flow, pp.169-175, *ibid.*
12. Hobson, R. W., Howard, E. W., Wright, C. B., Swan, K. G., and Rich, N. M.: Hemodynamics of femoral venous ligation, pp.215-221, *ibid.*
13. Wright, C. B., Casterline, P. F., Sayre, J. T., and Swan, K. G.: Management of venous ligation, pp.223-238, *ibid.*
14. Rich, N. M., Hobson, R. W., Wright, C. B., and Swan, K. G.: Techniques of venous repair, pp.243-255, *ibid.*
15. Hobson, R. W., Wright, C. B., Rich, N. M., and Swan, K. G.: Evaluation of vein graft patency, pp.257-263, *ibid.*
16. Wright, C. B., Alix, E. C., Hobson, R. W., and Swan, K. G.: Results following reconstruction of the femoral vein, pp.265-273, *ibid.*
17. Gervin, A. S.: Complications of heparin therapy, pp.307-322, *ibid.*
18. Hobson, R. W.: Low molecular weight dextran and heparin in venous reconstruction, pp.337-341, *ibid.*
19. Rich, N. M., Levin, P. M., and Hutton, J. E.: Effect of distal arteriovenous fistulas on venous graft patency, pp.361-375, *ibid.*
20. Swan, K. G., and Wright, C. B.: Hemodynamic effects of the arteriovenous fistula, pp.377-383, *ibid.*

21. Hobson, R. W., and Wright, C. B.: The peripheral arterio-venous fistula, pp.385-389, *ibid.*
22. Perloff, L. J., Reckard, C. R., Rowlands, D. T., and Barker, C. F.: Immunology of the venous homograft, pp.395-406, *ibid.*
23. Wright, C. B., Hobson, R. W., and Swan, K. G.: Review of autografts and homografts in canine femoral venous reconstruction, pp.409-413, *ibid.*
24. Hobson, R. W., Popovic, N., Croom, R. D., Rich, N. M., and Bielke, S. R.: Fibrocollagenous tubes in experimental femoral venous reconstruction: functional and microscopic evaluation, pp.415-424, *ibid.*
25. Wright, C. B., Lamoy, R. E., and Hobson, R. W., II: Hemodynamic effects of intra-arterial injection of drugs of abuse. *Surgery* 79:425-431, 1976 (Apr).
26. Lough, F. C., Giordano, J. M., and Hobson, R. W.: Regional hemodynamics of large and small femoral arteriovenous fistulas in dogs. *Surgery* 79:346-349, 1976 (Mar).
27. Gervin, A. S., Mason, K. G., and Wright, C. B.: Microaggregate formation during regional hypoperfusion. *Surg. Gynec. Obstet.* 141:719-723, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3 DATE PREV SUMRY ^c	4 KIND OF SUMMARY ^d	5 SUMMARY SCTY ^e	6 WORK SECURITY ^f	7 REGRADING ^g	8 A. DMS/IN INSTR ^h	9. SPECIFIC DATA- CONTRACTOR ACCESS ⁱ	10 LEVEL OF SUM A. WORK UNIT ^j
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10 NO. CODES ^k	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62110A	3A162110A821	00	125			
b. CONTRIBUTING							
c. CONTRIBUTING							
11 TITLE (Precede with Security Classification Code) ^l							
(U) Control Mechanisms of Regional Circulation							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^m							
003500 Clinical Medicine 012900 Physiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
65 07		CONT		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES EST. RATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PREVIOUS		B. FUNDS (in thousands)	
B. NUMBER ⁿ				FISCAL		76	
C. TYPE				YEAR		77	
D. KIND OF AWARD				CURRENT		2	
E. AMOUNT						1	
F. CUM. AMT.						132	
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 Surgery			
				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R. J. T.				NAME: Harken, LTC A. H.			
TELEPHONE 202 576-3551				TELEPHONE 202 576-2355			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Woods, MAJ M.			
				NAME:			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Oxygen Consumption; (U) Tissue Respiration;							
(U) Organ Perfusion							
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 the mechanism of regulating regional circulation, the influence of oxygen delivery and oxyhemoglobin affinity on tissue respiration. These studies apply directly to respiratory support of soldiers with acute battle injuries or post-surgical stress.							
24 (U) Physiologic techniques have been employed to document the response of intact tissues to varieties of stress. Tissue respiration, organ metabolism and viability were evaluated.							
25 (U) 75 07 - 76 06. Control mechanisms of regional circulation. Completed studies in an isolated perfused canine hindlimb or heart indicate that tissue oxygen uptake is related to the pH and oxyhemoglobin affinity of the perfusing blood. Alkalosis is a profound stimulus to lactate production in an isolated canine limb. A rightward shift of the oxyhemoglobin dissociation curve (towards lower oxyhemoglobin affinity) permits increased oxygen off-loading at the tissue level and an increased oxygen uptake. Support in the amount of \$26,000 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.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 66 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project DA162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 125 Control mechanisms of regional circulation

Investigators

Principal: LTC Alden H. Harken, MC

Associate: MAJ Monty Woods, MC

I. Factors Influencing Tissue Respiration

A. Background and Statement of the Problem. Oxygen utilization by tissues is influenced by the chemical composition of the perfusing blood and cardiovascular reflexes. Variation in the chemical composition of the blood exerts influences both centrally and peripherally. Experiments were performed in isolated canine hindlimbs which were perfused under tightly controlled conditions. The effects of variation in pH, O₂ and CO₂ content, lactate, and endotoxin on respiration were determined.

B. Experimental Approach. A membrane lung perfusion system was devised with which a vascularly isolated but viable extremity could be perfused with blood of controlled composition. The chemical composition of the perfusing blood was changed by altering pH, PO₂, PCO₂, using stored rather than fresh blood, and adding endotoxin and steroid. The effects of these alterations on tissue respiration were measured.

C. Results and Discussion. Oxygen consumption (\dot{V}_{O_2}) was related to the blood's pH by the equation $\dot{V}_{O_2} = 100.1 \text{ pH} - 1643$ ($r = 0.86$); thus a change in acidity of the perfusing blood by 0.1 pH unit causes a 10% change in limb \dot{V}_{O_2} . When blood pH was above 7.3, lactic acid was produced and when pH was below 7.3, it was consumed. The relationship between the A-V lactate difference and blood pH is expressed by $\text{Lactate} = 22.5 \text{ pH} - 162$ ($r = 0.75$). Lactic acid production does not reflect tissue oxygenation during clinical alkalosis. In seventeen dogs the addition of 5 mg of endotoxin to the perfusate reduced \dot{V}_{O_2} significantly. The eventual addition of hemoglobin-oxygen affinity by perfusing the legs with 2-4 week old blood revealed reduced \dot{V}_{O_2} when the P₅₀ of the perfusate was lowered.

These studies demonstrate several relationships between the chemical composition of blood and tissue respiration that are relevant to anesthesia, surgery or blood banking. In addition, evidence is presented in support of steroids being of benefit in shock therapy.

II. In Vitro Studies of Liver Respiration

A. Background and Statement of the Problem. The relationship between extracellular fluid oxygen tension and hepatocyte oxygen uptake was evaluated in 70 rabbits. Normal extracellular fluid (ECF) oxygen tension is 30 torr. It was expected that hepatocyte oxygen uptake would decrease at ECF oxygen tensions below 30 torr - presumably due to inadequate diffusion of oxygen into cells. Liver cell oxygen uptake at ECF oxygen tensions above 30 torr was not expected to change.

B. Experimental Approach. Oxygen consumption of slices of rabbit liver was measured in chambers fitted with oxygen electrodes. The effects of variation of PO_2 in the electrolyte solution and addition of endotoxin on tissue respiration was determined.

C. Results and Discussion. Oxygen uptake ($\dot{V}O_2$) was maximal at an extracellular fluid PO_2 of 30 torr. At a PO_2 of 10 torr $\dot{V}O_2$ was significantly reduced. However, if PO_2 were increased to 90 torr, $\dot{V}O_2$ was also reduced. These observations indicate that hepatocyte respiration is optimal at low PO_2 s within a narrow range.

The action of endotoxin was evaluated on both slices and homogenates of liver. In both models, endotoxin induced a significant reduction of $\dot{V}O_2$. It would, therefore, appear that the hepatocyte cell membrane does not protect the cell from the detrimental effects of endotoxin.

III. The Clinical Use of a Pulmonary Artery Thermistor Catheter

A. Statement of the Problem. Much enthusiasm has been generated concerning the clinical value of a pulmonary artery thermistor catheter for the measurement of pulmonary artery wedge (left atrial) pressure and cardiac output.

B. Experimental Approach. The use of a pulmonary artery thermistor catheter for pressure measurement and thermodilution cardiac output determination was evaluated in eleven dogs. A pulmonary catheter was placed in the wedge position. A left atrial catheter was simultaneously placed via a superior pulmonary vein.

C. Results and Discussion. Pulmonary artery wedge pressure was a reliable index of left atrial pressure at end expiratory pressures less than 10 cm H_2O . Fluctuations in pulmonary artery temperature occurred at a frequency equal to the respiratory rate and an amplitude of $0.010^\circ C$ to $0.086^\circ C$. Changes in amplitude were associated with changes in ventilatory waveform, respiratory rate and level of anesthesia. Intermittent and continuous positive pressure ventilation generally dampened and reversed the pulmonary artery temperature pattern exhibited during spontaneous breathing. This suggested that when end expiration is used to time indicator injection, cardiac output will

be underestimated during spontaneous breathing and overestimated during continuous or intermittent positive pressure ventilation. When indicator was injected at the same point in the ventilatory cycle, successive values of cardiac output deviated from one another by 0.0 - 6.7%. Deviations as large as 14% resulted if sequential injections were out of phase by half a respiratory cycle. Deviations in measured cardiac output can be minimized by injecting indicator at the same point in the respiratory cycle if it is not feasible to measure cardiac output during apnea. The clinical utility of a pulmonary artery thermistor catheter can be optimized through appreciation of its specific strengths and limitations.

IV. Systemic Oxygen Delivery, Oxygen Uptake, and Surgical Stress

A. Statement of the Problem. Recent advances in respiratory physiology and technology have permitted and promoted endotracheo-bronchial maneuvering on a scale not previously considered safe. The ventilatory and hemodynamic sequelae of these diagnostic and therapeutic interventions are often not fully appreciated by the operating surgeon. Straight and fiberoptic bronchoscopes at best only partially obstruct large bronchi with resultant decrease in ventilation:perfusion ratio. Conversely, continuous positive pressure ventilation may decrease pulmonary blood flow with resultant increase in ventilation:perfusion ratio. The purpose of this study was to evaluate the relationship between systemic oxygen delivery and tissue oxygen uptake during surgical stress.

B. Experimental Approach. Ten, 25 kg, mongrel dogs were anesthetized with pentobarbital (25/mg/kg). The dogs were intubated and ventilated (15 ml/kg) at a rate of 16 breaths per minute. A femoral artery catheter was placed. Cardiac output was measured by thermal dilution. All parameters (cardiac output, arterial and venous blood gases, pulmonary artery pressure and oxygen consumption) were measured as soon as all catheters were in place (Period #1). Baseline values were again measured 15 minutes following the initial determination (Period #2). All parameters were subsequently measured under the following conditions:

- Period #3. 10 cm H₂O positive end expiratory pressure
- Period #4. Right thoracotomy with equilibration of intrapleural and atmospheric pressure
- Period #5. Right main stem bronchial occlusion with right chest up
- Period #6. Right main stem bronchial occlusion with right chest down
- Period #7. Right main stem bronchus released following several deep breaths, right chest up
- Period #8. 10 cm H₂O positive end expiratory pressure (pleural pressure at zero)

- Period #9. 10 cm H₂O positive end expiratory pressure and 100% oxygen (FiO₂ = 1.0)
- Period #10. Thoracotomy closed, intermittent positive pressure ventilation

Following the initial thoracotomy, a monitoring catheter (PE 240) was placed into the left atrium of each animal.

C. Results and Discussion. Systemic oxygen delivery (SOD) and oxygen uptake ($\dot{V}O_2$) decreased initially in parallel then SOD decreased more than $\dot{V}O_2$. Following closure of the thoracotomy, SOD was down by 60% and $\dot{V}O_2$ was 80% of control levels. SOD and $\dot{V}O_2$ did not relate to each other in a parallel fashion. Following moderate surgical stress, $\dot{V}O_2$ at the end of the procedure was 80% of control levels.

V. Development of a Thermal Diffusion Probe to Measure Blood Flow in Small Volumes of Tissue

A. Background and Statement of the Problem. The ability to measure tissue perfusion is currently limited by methods which are discontinuous, unacceptably invasive or both. Attempts at measuring tissue perfusion with the aid of thermal devices has met with limited success. Several methods provide qualitative data but quantification of tissue blood flow has not been realized. An improved technique for measuring thermal conductivity and diffusivity has been developed by engineers working at MIT and Northeastern University. A collaboration with this group has been established with the aim of modifying the technique for direct measurement of tissue blood flow.

B. Experimental Approach. The thermal diffusion probe was evaluated in an isolated hindlimb preparation which permitted control of flow, temperature and oxygen delivery. Thermal conductivity was measured over a physiologically relevant range of flows.

C. Results and Discussion. There is a direct and significant relationship between thermal conductivity and tissue blood flow. The initial evaluation of the probe suggests that it can be developed to quantify blood flow in 1 cm³ volumes of tissue.

VI. In Vitro Studies of Lung Slice Respiration

A. Background and Statement of the Problem. The relationship between extracellular fluid oxygen tension and lung slice oxygen uptake in the presence of varying concentrations of colloid was evaluated in 86 rabbits. The results described in "II" led to the question of an optimal PO₂ for lung slice oxygen uptake. Also, in vitro data from other labs suggest that the diffusivity of O₂ in solution is strongly influenced by protein concentration.

B. Experimental Approach. Oxygen consumption of slices of rabbit lung was measured in a Yellow Springs apparatus. The effects of varying PO_2 and the addition of varying concentrations of plasma on lung slice respiration were determined.

C. Results and Discussion. As with the liver slice experiments, oxygen uptake was maximal at a PO_2 of 30 torr and reduced at PO_2 's of 10 and 90 torr. There was a direct relationship between the degree of depression of lung slice oxygen uptake and the concentration of protein in the solution. Colloid inhibits oxygen uptake of lung slices. The relevance of these findings to the treatment of shock lung is speculative.

VII. Coronary Arterial pH as a Determinant of Myocardial Oxygen Uptake

A. Background and Statement of the Problem. Acidosis is known to weaken myocardial contraction, decrease heart rate and dilate coronary vessels. Previous work in this laboratory (see I) demonstrated a direct relationship between perfusate pH and skeletal muscle oxygen uptake. The purpose of this study was to examine the magnitude and mechanism of the influence of coronary arterial pH (pHa) on myocardial oxygen uptake ($M\dot{V}O_2$).

B. Experimental Approach. A perfused heart preparation was developed which permitted control of heart rate, pressure and flow work, coronary flow, oxygen delivery, pHa and temperature. $M\dot{V}O_2$ and ventricular contractility (dp/dt) could be measured under varying circumstances.

C. Results and Discussion. $M\dot{V}O_2$ was directly and significantly related to pHa in all animals studied: $M\dot{V}O_2\% = 109 \text{ pHa} - 743$ ($r = 0.823$). The mechanism of altered $M\dot{V}O_2$ was evaluated by relating dp/dt and $M\dot{V}O_2$ to pHa in control, beta-blocked, and potassium arrested hearts. Arterial pH did influence dp/dt and thus $M\dot{V}O_2$: $dp/dt\% = 199 \text{ pHa} - 1376$ ($r = 0.834$). This was mediated partially by the effect of catecholamines and pHa: after β -blockade $dp/dt\% = 139 - 929$ ($r = 0.832$). Basal $M\dot{V}O_2$ of the perfused potassium arrested heart was also sensitive to pHa: $M\dot{V}O_2 = 1.82 \text{ pHa} - 12.01$ ($r = 0.48, p < 0.001$).

VIII. The Isolated Influence of Temperature on the Ability of Red Blood Cells to Release Oxygen

A. Background and Statement of the Problem. Temperature influences every aspect of tissue respiration. It has thus been difficult to determine the influence of thermally induced shifts in the oxyhemoglobin dissociation curve because of the strong influence of temperature on tissue oxygen uptake.

B. Experimental Approach. A membrane lung perfusion system was devised which permitted perfusion of a second membrane lung. This

allowed the direct assessment of the influence of temperature on the ability of red blood cells to release oxygen in a metabolically inert system.

C. Results and Discussion. There is a direct and significant influence of temperature on the ability of red cells to release oxygen. This effect may render cooled tissues hypoxic in the face of adequate blood flow. It is also evident that the metabolic response of tissues to thermal challenge is difficult to assess when their metabolism is hemoglobin dependent.

IX. Alteration of Oxyhemoglobin Affinity of Canine Erythrocytes

A. Background and Statement of the Problem. There is considerable theoretical evidence that the position of the oxyhemoglobin dissociation curve (ODC) has an influence on oxygen delivery to tissues. Clinical and laboratory documentation has been sparse. Since the preferred animal for a large amount of surgical research is the dog, it would be of value to develop a technique for shifting the canine ODC.

B. Experimental Approach. Dihydroxyacetone, pyruvate, and phosphate were infused into 35 dogs at 1% of blood volume per hour for five hours. One week before or after, a saline solution was infused at the same rate. Hourly 2,3-DPG, ATP and P_{50} pre- and post-infusion were measured. Red cell triokinase was assayed.

C. Results and Discussion. Triokinase of canine RBC was 0.2190 IU/gm Hgb (150% normal human level). ATP rose from 1.9 to 2.45 $\mu\text{M/gm Hgb}$ after five hrs of infusion of dihydroxyacetone solution. Red cell 2,3-DPG rose from $16.19 \pm 0.4 \mu\text{M/gm Hgb}$ to $18.63 \pm 0.6 \mu\text{M/gm Hgb}$ after five hours infusion of dihydroxyacetone solution ($p < 0.01$). P_{50} changed from 27.57 ± 0.9 to 31.76 ± 0.6 over the same period ($p < 0.001$). Saline infusion did not alter 2,3-DPG or P_{50} . A simple method of shifting the ODC in awake, unanesthetized dogs has been developed.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 125 Control mechanisms of regional circulation

Literature Cited

Publications:

1. Harken, A. H., Woods, M., and Wright, C. B.: Influence of endotoxin on tissue respiration and oxygen dissociation in an isolated canine hindlimb. *Am. Surg.* 41:704-709, 1975.
2. Woods, M., Scott, R. N., and Harken, A. H.: Practical considerations for the use of a pulmonary artery thermistor catheter. *Surgery* 79:469-475, 1976.
3. Woods, M., Harken, A. H., Balasubramaniam, T. A., and Bowman, H. F., (Spon: R. J. T. Joy): An initial evaluation of a thermal diffusion probe (TDP) for measuring tissue blood flow. *The Physiologist* 18:457, 1975.
4. Harken, A. H.: Hydrogen ion concentration and oxygen uptake in an isolated canine hindlimb. *J. Appl. Physiol.* 40:1-5, Jan. 1976.
5. Harken, A. H., Woods, M.: The influence of oxyhemoglobin affinity on tissue oxygen consumption. *Ann. Surg.* 183:130-135, 1976.
6. Bell, W. A., Woods, M., and Harken, A. H.: Influence of extracellular fluid hyperoxia and protein concentration on lung slice oxygen uptake. *Clin. Res.* 24:32A, 1976.
7. Woods, M., Harken, A. H., Pringle, J. M., and Bell, W. A.: The influence of temperature (T) on the ability of blood to release oxygen. *Fed. Proc.* 35:723, 1976.
8. Harken, A. H.: Lactic acidosis. *Surg. Gynec. Obstet.* 142:593-606, 1976 (Apr).
9. Harken, A. H., and Woods, M.: The influence of oxyhemoglobin affinity on tissue oxygen consumption. *Ann. Surg.* 183:124-135, 1976.

Project 3A762760A822
MILITARY INTERNAL MEDICINE

Task 01
Military Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)1636		
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A ORG/INSTR ^a	9B SPECIFIC DATA CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		9C LEVEL OF SW A WORK UNIT
75 06 30	H. Term	U	U	NA	NL			
10 NO / CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER				
	62760A	3A76270A822	01	120				
11A PRIMARY		11B CONTRIBUTING		11C CONTRIBUTING				
		CARDS 114F						
11 TITLE (Precede with Security Classification Code) ^a								
(U) Metabolic Response to Disease and Injury								
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a								
012900 Physiology 003500 Clinical Medicine 002300 Biochemistry								
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD		
63 08		76 01		DA		C. In-House		
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS		20 FUNDS (in thousands)
A DATES/EFFECTIVE: NA				PRECEDING				
B NUMBER ^a				FISCAL YEAR				
C TYPE:				75		9		167
D KIND OF AWARD:				76		9		30
E AMOUNT:								
F CUM. AMT.								
21 RESPONSIBLE DOD ORGANIZATION				22 PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research				
ADDRESS: Washington, DC 20012				Div. of Medicine				
				ADDRESS: Washington, DC 20012				
RESPONSIBLE INDIVIDUAL Joy, R.J.T. COL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				
NAME:				NAME: Earll, COL, J.M.				
TELEPHONE: 202-576-3551				TELEPHONE 202-576-3529				
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]				
21 GENERAL USE				ASSOCIATE INVESTIGATORS				
Foreign intelligence not considered				NAME: Wartofsky, LTC, L.				
				NAME: Schaaf, MD, M.				
23. KEYWORDS (Precede EACH with Security Classification Code) ^a								
(U) Metabolic; (U) Stress; (U) Endocrine; (U) Hormone								
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number Precede text of each with Security Classification Code.)								
<p>23. (U) Investigation into basic mechanisms of diseases of military importance and the metabolic response of patients during stress of disease and injury to provide rational approach to therapy.</p> <p>24. (U) Metabolic balance studies with precise collection of biologic samples from patients under rigidly controlled diet, drugs, and activity. Development of techniques to measure alterations in homeostasis produced by disease or drugs. Provide clinical support and teaching for the Walter Reed Army Medical Center.</p> <p>25. (U) 75 07 - 76 01. Serum levels of prolactin, TSH, and growth hormone were found to increase in physiologically significant amounts in association with the stress of parachute jumping. Transsphenoidal microsurgery was found to be satisfactory and effective therapy for initial treatment of patients with pituitary tumors associated with acromegaly. Studies of iodothyronines in amniotic fluid revealed that it represents a readily dialyzable compartment for exchange of T₃, T₄, and iodine that must be considered in studies of the fetoplacental unit. Combined therapy of hyperthyroid subjects with lithium and/or iodine revealed that both agents were effective but that pre-treatment with lithium may blunt the effects of iodine studies of thyroid-pituitary axis during acute malaria suggested that infection appears to have a suppressive effect at the pituitary rather than hypothalamic level, and that alterations in T₃ and reverse T₃ support a shift in T₄ conversion from activating to inactivating pathways in this infection. A radioimmunoassay for 3,3'-diiodothyronine was developed and is being applied to studies of thyroid physiology. Studies were performed in collaboration with the Surgical Burn Unit at BAMC, and suggested that thermal injury (like infection) was associated with shifts in T₄ metabolism from T₃ to reverse T₃ generation. For technical report see WRAIR Annual Report 1 JULY 75-30 JUNE 1976.</p>								

PII Redacted

DD FORM 1498

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

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 120 Metabolic response to disease and injury

Investigators:

Principal: COL Jerry M. Earll, MC

Associates: Marcus Schaaf, M.D.; LTC Leonard Wartofsky, MC;
MAJ Richard C. Dimond, MC; Joseph Bruton, Ph.D.
MAJ Kenneth D. Burman, MC

Description

This work unit is concerned with investigations into basic mechanisms of diseases of military importance and the metabolic responses occurring during stress of disease and injury. To provide rational approaches to diagnosis and therapy, metabolic balance studies are utilized which require precise collections of biologic samples from patients during rigid control of diet, drugs, and activity. In addition, support is afforded Walter Reed Army Medical Center in training house staff, four endocrine fellows, diagnosis and treatment of endocrine patients, and technical laboratory support to other departments. The unit maintains the capability of mounting field studies.

Progress

1. Polypeptide Hormone Metabolism.

Plasma concentrations of prolactin in humans have been shown to rise in response to a number of stimuli which have in common some element of physical stress, among them surgery under general anesthesia, endoscopy, and vigorous exercise. Thyrotropin (TSH) secretion in human adults has not been found to be increased by the stress of surgery, by fever, or by exercise, but recently it has been reported that small increases in TSH secretion occurred in young men in anticipation of vigorous physical exercise. Growth hormone is known to respond to a wide variety of physical stresses, while growth hormone responses to psychic stimuli are less extensively documented. In this study, we have demonstrated that the stress of parachute jumping is associated with the release of prolactin and TSH as well as growth hormone. Prolactin, growth hormone, and thyrotropin (TSH) release during the stress of parachute jumping has been evaluated in 14 male subjects. Subjects were studied at several times before and immediately after their first military parachute jump. All three hormones had risen significantly 1 to 14 min after the jump, compared to mean levels measured immediately beforehand. Earlier studies of physical exercise by ourselves and others would suggest that emotional stress played a role in producing changes of this magnitude. We conclude that prolactin, TSH, and growth hormone are released in physiologically significant amounts in association with the stress of parachute jumping.

None of the numerous methods used for the treatment of acromegaly, including conventional x-ray irradiation, heavy-particle irradiation, transsphenoidal implantation of radioactive isotopes, cryohypophysectomy, and surgical hypophysectomy, have consistently achieved ideal therapeutic goals. All of these therapeutic modalities have a significant incidence of persistent disease, recurrent disease, or loss of normal pituitary function. Renewed interest in the surgical treatment of acromegaly and other pituitary neoplasms by the transsphenoidal route has been stimulated by technological advances in operating microscopes and image-intensifying x-ray equipment. Serum growth hormone levels, thyroid function, and adrenal function were measured before and after surgery in 16 of 17 acromegalic patients undergoing transnasal transsphenoidal microsurgery of the pituitary. Thirteen patients have been followed up for 12 to 24 months; three patients have been followed up for three to six months. Serum growth hormone levels decreased to less than 5 ng/ml in seven of nine previously untreated patients; thyroid and adrenal function were preserved in eight of these nine patients. In seven patients treated previously by other modes of therapy, growth hormone levels after transsphenoidal surgery decreased to less than 5 ng/ml in three, to between 5 and 10 ng/ml in three, and from 98 to 41 ng/ml in one. Preoperative adrenal function was normal in six of these seven patients and was preserved in four; thyroid function was normal in five patients preoperatively and was preserved in three. Transsphenoidal microsurgery appears to offer an effective means of lowering growth hormone levels and a possibility of preserving any remaining normal pituitary function. It may be considered for initial treatment in selected patients in whom more rapid arrest of acromegaly is indicated.

2. Thyroid Metabolism.

a. Thyroid function tests in amniotic fluid.

Because of recent advances in antenatal physiology, it has become relevant to quantitate thyroid function tests in human amniotic fluid. For this purpose, 21 sets of maternal sera, cord sera, and amniotic fluid were obtained from normal term labor and deliveries.

Total (T4) and dialyzable thyroxine (DT4), total (T3) and dialyzable triiodothyronine (DT3), total iodine (TI), thyroxine binding globulin (TBG), and resin T3 uptake (RT3U) were measured in amniotic fluid. In addition, T3, T4, TI, and TBG were measured in maternal and cord sera.

In 21 amniotic fluid samples, T3 and T4 concentrations were close to or below their lower limits of detectability (approximately < 25 ng/100 ml and < 1 ug/100 ml, respectively). However, when larger quantities of unknown samples were assayed, and when T3 and T4 content were increased per assay volume by lyophilization, the mean (+SEM) amniotic fluid content was 30 ± 2 ng/100 ml (n=5) and 0.54 ± 0.07 ug/100 ml (n=5), respectively. The mean (+SEM) percent DT3 in amniotic fluid was $1.76 \pm 0.349\%$ (n=3) and the mean (+SEM) percent DT4 was $0.225 \pm 0.02\%$ (n=4).

Amniotic fluid mean (+SEM) TBG, TI, and RT3U concentrations were 0.32 ± 0.04 mg/100 ml (n=5), 3.4 ± 0.2 ug/100 ml (n=9), and $0.55\% \pm 0.01$ (n=5) respectively. In maternal sera, the mean (+SEM) T3 concentration was

150 \pm 8 ng/100 ml (n=21), the mean (\pm SEM) T4 concentration was 10.8 \pm 0.4 ug/100 ml (n=12), the mean (\pm SEM) TI concentration was 7.2 \pm 0.7 ug/100 ml (n=9) and the mean (\pm SEM) TBG level was 8.56 \pm 0.96 mg/100 ml (n=5). In cord sera, the mean (\pm SEM) T3, T4, TI, and TBG concentrations were 30 \pm 3 ng/100 ml (n=21), 9.4 \pm 0.5 ug/100 ml (n=12), 7.6 \pm 0.4 ug/100 ml (n=10), and 5.12 \pm 0.39 mg/100 ml (n=5), respectively.

These data suggest that: (1) in the analysis of amniotic fluid by radio-immunoassay, different final T3 concentrations were obtained when hypothyroid amniotic fluid or hypothyroid sera were incorporated into the standard curve; (2) T3 and T4 are present in low concentrations in human amniotic fluid, but the percent DT3 and DT4 are relatively high; (3) because of their low concentrations it is not feasible to routinely measure T3 and T4 in amniotic fluid to aid in the prenatal detection of congenital hypothyroidism, although the utility of DT3 and DT4 in this regard remains speculative; and (4) amniotic fluid represents a readily dialyzable or transferable compartment of T3, T4, and iodine that must be considered in models investigating maternal and fetal transfer of these substances.

b. Synergistic effects of lithium (Li) and iodine (I) in the treatment of hyperthyroidism (HT).

Li and I have similar antithyroid effects and primarily act to inhibit thyroïdal release. We have attempted to compare the relative efficacy of these agents given singly and in combination in order to assess possible synergism of action which might be used advantageously in the therapy of either severe HT or for those patients in whom thioureas could not be employed. 18 patients with HT were studied utilizing a double isotope technique as an index of thyroïdal release (Wartofsky *et al.* *JCI* 49:78, 1970; Nicoloff *JCI* 49:1912, 1970), with ^{125}I serving as an intrathyroïdal label and ^{131}I -T4 as a marker of T4 disposal. Bidaily measurements of protein-bound (PB) ^{125}I , PB ^{131}I , and urinary (U) ^{125}I and ^{131}I were made during a 5 day control period and two subsequent 5 day treatment periods. Subjects were assigned to groups given either Li or I alone during the first treatment period, with the alternative drug added as combination therapy for the assessment of synergism during the second treatment period. Half of the patients in each treatment group were blocked with methimazole (MMI). Analysis of the changes with time in the slope of the ratios of $\text{U}^{125}\text{I}/\text{U}^{131}\text{I}$ and $\text{PB}^{125}\text{I}/\text{PB}^{131}\text{I}$ indicated that either I or Li given alone induced comparable and significant decreases in thyroïdal release. Further significant ($p < 0.05$) decreases in slopes during the second (combined) treatment period, occurred only in those patients who had received iodine initially. Results in all treatment periods were the same in the presence or absence of MMI blockade. Thus, I and Li may display synergism in the treatment of HT only if I is employed first, but the combination of Li + I, if Li is used first, is not significantly more effective than Li alone. These observations permit some further insight into the mechanism of action of these drugs. Since previous studies indicate that Li reduces thyroïd iodine uptake, pre-treatment with Li may prevent or attenuate accumulation of iodine, thus blunting the inhibitory effect of I on thyroïdal release, whereas administration of Li after I pre-treatment allows the antithyroid effects of both agents to be realized.

c. Alterations in T3, reverse T3 (rT3), and the response to thyrotropin releasing hormone (TRH) in acute malaria.

Previous studies from this laboratory on thyroid economy during malarial infection (JCI 51:2215, 1972) indicated that thyroid gland function was suppressed. Although never documented, it has been proposed that this suppression was related to a decline in TSH release during infection. The latter could be secondary to increases in serum T3 from enhanced T4 to T3 conversion in the periphery, or to inhibition by infection at a hypothalamic or pituitary level. To examine these possible mechanisms, we have performed serial measurements of T3, and have administered TRH to assess the integrity of pituitary function during infection. Basal and stimulated PRL levels were measured since this hormone is elevated in a variety of other stresses; and rT3 was measured since recent reports indicate that alterations in serum T3 may be accompanied by reciprocal changes in rT3. Two groups of 4 normal male volunteers were studied. In Group I, T4, T3, rT3, TSH, and PRL were measured in daily basal A.M. samples of blood before, during, and after induction of acute malaria. In Group II, TSH and PRL responses to TRH (500 meg i.v.) during infection were compared to those observed during the control period.

In Group I, TSH and PRL transiently decreased from mean control levels by days 1 and 3 of illness respectively, and returned toward control values after treatment. Serum T4 progressively increased during days 3-5 of infection, while a significant and marked decrease in serum T3 levels was observed on day 3 of infection which coincided precisely with significant increases in rT3 levels. In Group II, TSH responses to TRH during infection were unchanged while PRL release was increased.

These results suggest that in malaria: 1) decreases in TSH release are not related to increases in serum T3; 2) normal TSH and augmented PRL response to TRH are consistent with inhibition by infection at a hypothalamic but not pituitary level; and 3) the observed reciprocal alterations in T3 and rT3 in malaria indicate a shift in conversion of T4 from an activating to an inactivating pathway which may represent a homeostatic mechanism for decreasing thermogenesis in this illness.

d. Isolated thyrotropin (TSH) deficiency.

A 41 y.o. male presented with symptoms of hypothyroidism, low serum T4 and T3. Serum TSH was unmeasurable; a 500 ug i.v. bolus of TSH releasing hormone (TRH) before therapy with thyroid hormone revealed no increase in TSH but a normal increase in prolactin (PRL). The diagnosis of isolated TSH deficiency (ITSHD) was based on an absent TSH response to TRH and otherwise normal pituitary function. Prior to availability of TRH one could not distinguish between hypothyroidism due to hypothalamic disease (ITRHD) and ITSHD, and data from earlier cases did not permit differentiation between total and partial ITSHD. Our patient was given prolonged (6 hr) infusion of TRH (1 ug/min) to maximally stimulate the pituitary 2 mos. after withdrawal of thyroid therapy given for 5 mos. Sensitive assays for TSH (detecting 1.0 uU/ml) and PRL were employed. A 500 ug bolus TRH

test was repeated for comparison to the pre-treatment study, since a positive feedback of T3 on TRH production has been proposed. Simultaneously, to detect a thyroid response to unmeasurable TSH increases, an isotope technique was used as an index of thyroid secretion, with ^{125}I as a thyroid label and ^{131}I -T4 as a marker or peripheral T4 disposal. Bolus TRH increased TSH from 1.0 to 1.8 $\mu\text{U}/\text{ml}$. During TRH infusion, TSH increased to 1.5 $\mu\text{U}/\text{ml}$, PRL response was normal, and increases in the slopes of PB ^{125}I : PB ^{131}I (.004 to .02, $p < 0.001$) and urinary ^{125}I : ^{131}I reflected increased thyroid secretion secondary to rises in TSH.

We conclude that our patient had intact lactotroph but impaired thyrotroph response to TRH but did not have ITRHD; and further that some patients with ITSHD may have: 1) secretion of biologically active TSH; 2) a facilitated TSH response to TRH after prior treatment with thyroid; and 3) measurable secretion of TSH, and hence an incomplete form of the disease.

e. A radioimmunoassay for 3,3'Diiodothyronine (3,3'T2).

Because chromatographic studies had suggested that 3,3'T2 may be an important metabolite of T4, T3, and reverse T3, and in order to investigate its physiology in health and disease, we have developed a radioimmunoassay for its measurement. Utilizing a specific antiserum to L-3,3'T2-BSA conjugates developed in rabbits, cross reactivity was less than 0.5% with T3 and reverse T3 and less than 0.01% with T4. Intra-assay variation averaged 2.9%, and inter-assay variation was 7.8% and 18.5% when serum samples with 3,3'T2 concentrations of 8 ng/dl and 12 ng/dl, respectively, were analyzed. Assay sensitivity was considered to be 6 ng/dl by statistical criteria.

Although routine assays were performed on unextracted samples with ANS used to inhibit 3,3'T2 binding to serum proteins, comparable values were obtained if samples were extracted with ethanol prior to analysis. Exposure of serum samples to acid hydrolysis, a procedure which theoretically might inhibit binding of iodothyronines to other substances such as glucuronide or sulfate, did not alter measurable values of 3,3'T2. Preliminary studies do suggest, however, that the quantity of 3,3'T2 detected in amniotic fluid samples may be increased slightly by prior acid hydrolysis and ethanol extraction.

The mean (\pm SE) serum 3,3'T2 concentration in 18 euthyroid subjects was 17 ± 1 ng/dl. Thyrotoxic patients generally had elevated 3,3'T2 levels (29 ± 2 ng/dl; $n=5$) and hypothyroid individuals tended to have decreased 3,3'T2 concentrations (range $< 6-22$ ng/dl $n=11$). Unextracted amniotic fluid, maternal sera, and cord sera samples had mean (\pm SE) 3,3'T2 concentrations of 20 ± 2 ng/dl ($n=10$), 27 ± 3 ng/dl ($n=11$) and 20 ± 1 ng/dl ($n=5$), respectively. In 8 normal thyroid glands the concentration of 3,3'T2 was $0.40 \pm .03$ ug/gram of tissue. TRH administration in 7 euthyroid subjects did not evoke an increase in serum 3,3'T2 levels when measured 90 minutes after injection. In contrast, serum 3,3'T2 concentrations did increase 90 minutes following TRH administration in two sheep, although it could not be discerned whether this rise in circulating 3,3'T2 was secondary to thyroidal secretion or to peripheral conversion.

In summary, these data suggest that 3,3'T₂ circulates in the serum of normal individuals and tends to parallel serum concentrations of T₃, T₄ and rT₃ in various states of thyroid function. Hence, the radioimmunoassay of 3,3,'T₂ should prove useful in further delineation of the peripheral metabolism of the iodothyronines. Moreover, the ability to detect 3,3,'T₂ in amniotic fluid indicates that further studies of its role in perinatal physiology are warranted.

f. Quantitative fluorescent thyroid scanning in thyrotoxic subacute thyroiditis.

The differential diagnosis between the hyperthyroidism associated with subacute thyroiditis (SAT) and that due to Graves' disease may be difficult. Hyperthyroninemia may accompany SAT in 10-30% of the patients, and when SAT is painless, the diagnosis may be erroneously ascribed to Graves' disease unless a radioiodine uptake (RAIU) is performed. In contrast to Graves' disease, the clinical course of the thyrotoxicosis of SAT is predictably self-limited. Serial fluorescent thyroid scanning as a means of quantitating thyroidal content of stable iodine was performed in 6 patients with thyrotoxic SAT in an attempt to differentiate this disorder from Graves' disease and to gain further insight into the temporal relationship between serum T₄, RAIU, and depletion and repletion of thyroidal iodine. Six previously untreated patients (4F, 2M; age range 36-61) presented with signs and symptoms of thyrotoxicosis. The syndrome was painless in 4 of the 6 patients. Initially, mean (\pm SE) T₄ averaged 18.9 ± 1.8 ug% and RAIU $0.7 \pm 0.4\%$. All were treated symptomatically with propranolol but no thiourea compounds.

Thyroidal iodine (TI) was quantified utilizing a modified source-detector for ²⁴¹Americium with two sets of single-channel analyzers and digital counters. Calibration of net counts versus mgms. of iodine was performed in phantoms. To validate the technique, the TI content of 12 cadaver thyroids as assessed by fluorescent scanning was compared to total iodine measurements by direct chemical analysis of anaerobic digests, and was found to correlate significantly ($r = 0.92$; $p < 0.01$). In clinical studies, normal subjects ($n=30$) had an average TI of 10.1 ± 3.9 mg, while patients with untreated Graves' disease and elevated RAIU ($n=28$) had mean TI of 24.4 ± 9.9 mg. In contrast, 6 patients with thyrotoxic SAT had a markedly decreased TI of 5.0 ± 1.8 mg, suggesting early depletion of hormonal iodine. During resolution of the thyroiditis (follow-up 1-6 mos.), serial measurements of TI remained low until after serum T₄ fell to below normal, with subsequent increases in TSH and RAIU.

Conclusions: 1) the fluorescent scan and RAIU allow discrimination between the thyrotoxicosis of painless SAT and Graves' disease; 2) TI is depleted early in SAT and repletion of glandular stores depends upon return of iodide trapping function; 3) fluorescent scanning permits accurate in vivo measurement of TI and promises to be a useful technique for study of the thyroid gland in health and disease.

g Alterations in serum triiodothyronine and reverse triiodothyronine in thermal injury.

Studies were conducted in collaboration on the surgical burn unit of the Brooke Army Medical Center. Hypermetabolism accompanies thermal injury (TI) and has been ascribed to increased catecholamines. The state of thyroid hormone economy in this circumstance has not been evaluated, however. Accordingly, in the 2 weeks following TI in 5 men (mean burn size, 66%), sequential measurements of serum free thyroxine index (FTI), thyrotropin (TSH), triiodothyronine (T₃), and reverse T₃ (rT₃) were made. FTI and TSH did not deviate from normal, but T₃ decreased greatly while rT₃ remained normal or increased.

Day Post-TI	3	6	8	10	13	15
T ₃ , ng/100ml (nl, 80-180)	52±12	26±5	36±7	31±7	16±4	44±12
rT ₃ , ng/100ml (nl, 36-84)	94±14	104±10	76±8	85±19	56±9	66±8

In 6 other men (mean burn size, 71%), rT₃ increased to 152±26 ng/100ml without further decline in T₃ in the 4 days prior to septic death. Despite the great decrease in T₃, the TSH response to thyrotropin-releasing hormone was not accentuated. The data suggest that TI is accompanied by a shift in the metabolism of T₄ from pathways that lead to T₃ generation to pathways that lead to rT₃ generation; this diversion may be conditioned by the hypermetabolism of TI.

h. The effect of varying serum T₄ concentrations on extrathyroidal production of T₃, reverse T₃, and 3-3'diiodothyronine (3-3'T₂).

To examine whether serum levels of T₄ are important in regulating its extrathyroidal conversion, 9 athyreotic subjects were studied during separate monthly periods of daily p.o. administration of .05 mg I-T₄ (period I) or 0.4 mg L-T₄ (period II). All parameters of thyroidal economy, including reverse T₃ (rT₃) and 3-3'T₂, were measured by specific radioimmunoassays during the last week of both study periods. The mean results obtained are expressed in relation to the treatment period (I/II): T₄, 4.1/15.4 µg%; T₃, 55/210 ng%; rT₃, 27/104 ng%; 3-3'T₂ 11/13 µg%; TBG, 4.3/3.4 mg%; TSH, 32/1.0 µU/ml. Mean Free T₄ (FT₄) and Free T₃ (FT₃) concentrations measured by equilibrium dialysis were 1.08/4.59 ng% and 224/1031 pg%; respectively. Each parameter increased during period II, except for 3-3'T₂ levels which did not change and serum TBG and TSH levels which decreased significantly. In addition, 4 subjects received ¹³¹I-T₄ and ¹²⁵I-T₃ i.v. to determine hormone production and conversion rates. Mean conversion rates, estimated both by concentration ratios of T₄/T₃, T₄/rT₃, and FT₄/FT₃ and by analysis of kinetic parameters, were the same during both study periods. In summary, these findings suggest that (1) the percentage conversion of T₄ to both rT₃ and T₃ are constant regardless of the serum levels of T₄, (2) serum TBG levels vary inversely with serum T₄ concentrations and, (3) peripheral conversion appears to represent one

source of circulating 3-3'T₂. The mechanism by which 3-3'T₂ levels remain constant during study period II while its presumed precursors increase requires further investigation.

i. Effect of T₃ on TSH and prolactin responses to TRH.

Previous investigators have shown that daily administration of TRH to normal individuals leads to diminishing TSH responses, which were believed due to rising serum T₃ with subsequent feedback inhibition of the pituitary. Patients with primary hypothyroidism were examined in the present study to test this hypothesis, since their T₃ cannot increase after TRH. Bi-daily TRH (100 µg) was given to 4 patients for 3 consecutive days, and repeated on a 4th day following oral T₃ (50 µg). TSH and PRL responses were unchanged during these 3 days of serial TRH and were unaltered by T₃ administration on the fourth day. In 5 other hypothyroid subjects studied before and during 3 consecutive days of T₃ administration, TSH but not PRL responses to TRH appeared to decrease slowly but progressively. These observations led us to re-examine TRH responses in normal subjects given T₃, since other workers had reported that 50 µg T₃ completely abolished TSH response. Responses to TRH in 11 normals on a control day were compared to those observed 1 hour after oral T₃. In spite of marked increases in serum T₃, there was no significant difference between the mean TSH responses of the two studies.

TRH was administered by continuous infusion (1 µg/min) for 6 hours in 5 normal subjects. PRL and TSH responses peaked within 60 and 180 minutes, respectively, and then plateaued. To ascertain whether this plateau effect was due to increases in T₃, similar infusions were given to 5 hypothyroid patients, and a similar pattern of response was observed, suggesting that the plateau phase was not due to increases in serum T₃. The total amount of TSH and PRL discharged during TRH infusion was estimated, and served as an index of pituitary content of these hormones. In the 5 normal subjects, total daily net TSH and PRL release averaged 123 mU and 197 µg, respectively. These estimates are consistent with direct analyses by others of PRL and TSH content in the human pituitary. In 5 hypothyroid patients, readily dischargeable TSH and PRL content averaged 640 mU and 307 µg, respectively.

We conclude that: 1) acute increases in serum T₃ do not suppress TRH-stimulated TSH or PRL secretion in either normal or hypothyroid subjects; 2) the decline and subsequent plateau in TSH and PRL levels during continuing TRH stimulation are related to factors governing *de novo* synthesis and release, rather than to rising T₃ levels; 3) integration of the early peak TSH and PRL responses to continuous TRH infusion may serve as a useful tool for the estimation of pituitary content of these hormones *in vivo*.

j. Hypothyroidism after X-irradiation to the neck.

Hypothyroidism caused by x-ray-induced radiation damage to the thyroid gland has been thought to be both a late and an uncommon complication of such treatment. Three patients who developed hypothyroidism after x irradiation

to the neck are presented. The first two cases demonstrate that patients can develop clinical and chemical hypothyroidism after a very short interval following radiotherapy. Hypothyroidism developed in the first patient in the absence of surgical manipulation of the neck, or a large iodine load 4 months after receiving 6800 rad of x-ray therapy to his neck for carcinoma of the larynx. The second patient developed hypothyroidism approximately 6 months after his radiotherapy for carcinoma of the esophagus. Both of these patients demonstrated high titers of serum antithyroid antibodies. A third patient with Hodgkin's disease did not manifest clinical symptoms and signs of hypothyroidism until 6 years after radiation therapy. These cases demonstrate the variability of onset of hypothyroidism after radiotherapy and emphasize the need for careful evaluation of thyroid function before and after neck irradiation.

k. Effect of propranolol on thyroid.

A dual isotope method allowing simultaneous analysis of both endogenous thyroidal release and peripheral thyroxine disposal was employed in four patients with thyrotoxicosis before and during propranolol therapy (160 mg/day) to determine whether beta adrenergic blockade with this agent affected the secretion or metabolism of thyroid hormone. Since catecholamines may be involved in the regulation of both thyrotropin (TSH) and prolactin (PRL) release from the pituitary, the effect of propranolol on the TSH and PRL responses to thyrotropin-releasing hormone (TRH) was also examined. In the dosage employed in these patients, propranolol had no demonstrable effect on either thyroid hormone secretion, the peripheral disposal of T_4 , or the TSH and PRL responses to TRH.

1. Thyroid function in Klinefelter's syndrome.

Thyroid function and prolactin (PRL) responsiveness to thyrotropin-releasing hormone (TRH) were examined in 6 patients with Klinefelter's syndrome prior to and after therapy with testosterone. The thyroid function tests, including serum triiodothyronine (T_3), thyroxine (T_4), thyroxine binding globulin (TBG), resin T_3 uptake (RT_3U), radioactive iodine uptake (RAIU), thyrotropin (TSH) stimulation and the TSH response to TRH were normal during both periods of study. Testosterone treatment had no significant effect on any of these parameters with the exception of the RT_3U which increased. PRL responses to TRH were significantly higher than those observed in normal men ($P < 0.05$). Despite the fact that mean plasma PRL responses to TRH were decreased when the patients were restudied during testosterone therapy, they remained greater than those of normal men. Mean serum estradiol concentrations were normal and did not increase significantly during testosterone therapy.

These studies suggest that: (1) thyroid function may be normal in patients with Klinefelter's syndrome more often than previously reported, and (2) patients with Klinefelter's syndrome may manifest PRL hyper-responsiveness to TRH that is decreased but not normalized during testosterone therapy. Because estradiol levels failed to increase despite a marked rise in testosterone, further studies are warranted to examine testosterone and estradiol clearance and conversion rates in patients with Klinefelter's syndrome.

m. Treatment of thyroid excess by resin hemoperfusion.

The ability of an extracorporeal hemoperfusion system employing neutral Amberlite^R resin to bind thyroid hormone and to decrease circulating levels of triiodothyronine (T3), thyroxine (T4), and free thyroxine (FT4) was evaluated in dogs made thyrotoxic by the intramuscular administration of thyroid hormone. Since the resin column and tubing were charged with saline, the effects of hemodilution from this source on serum T3 and T4 was assessed by control perfusion through a column which did not contain any resin. After correction for hemodilution, the mean serum T3, T4 and FT4 decreased during 2 hours of resin hemoperfusion by 39%, 35%, and 46%, respectively. Hormonal clearance rates were calculated in two experiments and the estimated net hormone removed averaged 60.4 μ g of T3 and 1990 μ g of T4. Hematologic indices and routine chemistries did not change significantly in these dogs during the procedure except for a decrease in mean serum albumin concentration and an increase in mean serum glucose concentration.

Hemoperfusion through this resin system seems to be a safe, effective means of decreasing serum T3, T4, and FT4 in thyrotoxic dogs and warrants evaluation for the treatment of thyroid storm in man.

n. Effect of water loading on TSH and prolactin responses to TRH.

Reports of suppression of plasma prolactin (PRL) in humans by water loading led us to examine the effect of a 20 cc/kg water load on serum TSH in 21 normal volunteers. In addition, the effects of a water load on basal and TRH-stimulated TSH and PRL levels were evaluated in seven patients with primary hypothyroidism. The water load had no effect on basal serum TSH levels in either normal or hypothyroid subjects, and did not alter the TSH response to TRH in hypothyroid subjects. Basal or TRH-stimulated plasma PRL was also unaffected by water loading in the hypothyroid subjects. These data suggest that a water load of 20 cc/kg does not significantly affect TSH release by the anterior pituitary, and also provide further evidence that water loading does not consistently suppress PRL secretion.

o. Effect of water and hypotonic saline on prolactin.

To test the effect of acute changes in plasma osmolality on plasma prolactin concentrations, the hormone was measured before and during oral water loading, hypotonic saline infusion, and hypertonic saline infusion in normal subjects. In 10 normal men there was a small but statistically significant rise in mean prolactin, from 7.6 to 12.3 ng/ml, occurring within half an hour after the ingestion of a water load of 20 ml/kg. A small but statistically insignificant rise in mean plasma prolactin was observed in 11 normal women, although there was a significant negative correlation between plasma prolactin and plasma osmolality in these subjects. No effect of the intravenous infusion of either hypotonic saline (0.45%) or hypertonic saline (5%) on mean plasma prolactin was noted in 5 normal men. These studies do not confirm a previously reported suppression of prolactin concentrations by oral water loading or hypotonic saline infusions in normal subjects.

While the data suggest a negative correlation between plasma osmolality and plasma prolactin, at least after water loading, they do not support a physiological role for prolactin in the short-term regulation of plasma osmolality in humans.

3. Metabolism

A comparison was made of the effectiveness of treatment with clofibrate and/or chlorpropamide in patients with diabetes insipidus. Six patients with vasopressin-responsive diabetes insipidus received clofibrate and chlorpropamide, singly and in combination. Decrease in urinary output averaged (mean \pm SE): Clofibrate, 2 gm/d, 44 \pm 7%; chlorpropamide, 250 mg/d, 50 \pm 8%; clofibrate, 2 gm/d and chlorpropamide, 125 mg/d, 48 \pm 6%; clofibrate, 2 gm/d and chlorpropamide, 25- mg/d, 59 \pm 3%. Water deprivation tests before and during treatment showed significantly higher basal, final and peak urinary osmolalities (U_{osm}) and lower free water clearance (C_{H_2O}) on chlorpropamide, singly and in combination; clofibrate raised U_{osm} less but significantly decreased C_{H_2O} . Water load tests before and during treatment showed that chlorpropamide, singly and in combination, markedly decreased maximal urinary flow, maximal C_{H_2O} , % water load excreted, and increased minimal U_{osm} ; clofibrate significantly decreased maximal urinary flow and C_{H_2O} only. One patient responded to combination therapy. Chlorpropamide caused serious hypoglycemia in 3 of 6 patients. Clofibrate had no significant side effects. We conclude that the oral agents are effective in treatment of patients with partial diabetes insipidus. In selecting an oral agent for treating diabetes insipidus we advise initial trial of clofibrate; if ineffective, low dose chlorpropamide may be tried with precautions to avoid hypoglycemia. An occasional patient who is unresponsive to chlorpropamide and clofibrate alone may respond to combination therapy.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 120 Metabolic response to disease and injury

Literature Cited.

Publications:

1. Noel, G. L., Dimond, R. C., Earll, J. M., and Frantz, A. G.: Prolactin, thyrotropin, and growth hormone release during stress associated with parachute jumping. *Aviat. Space Environ. Med.* 47(5):543-547, 1976.
2. Atkinson, R. L., Dimond, R. C., Becker, D. C., Martins, A. N., Schaaf, M., Wartofsky, L., and Earll, J. M.: Treatment of Acromegaly by Transsphenoidal Microsurgery. *J. Amer. Med Ass'n.* 233:1279-1283, 22 September 1975.
3. Burman, K. D., Read, J., Dimond, R. C., Strum, D., Wright, F. D., Patow, W., Earll, J. M., Wartofsky, L.: Measurements of 3,3',5'-Triiodothyronine (Reverse T3), 3,3'-Diiodothyronine, T3, and T4 in human amniotic fluid and in cord and maternal serum. *J Clin Endocrinol Metab.* 43:1976, In Press.
4. Wartofsky, L., Burman, K. D., Dimond, R. C., Noel, G. L., Frantz, A. G., and Earll, J. M.: Studies on the nature of thyroidal suppression during acute falciparum malaria: Integrity of pituitary response to TRH and alterations in serum T3 and reverse T3. Submitted for publication to *J Clin Endocrinol Metab.* 1976.
5. Boehm, T. M., Dimond, R. C., and Wartofsky, L.: Isolated thyrotropin deficiency with thyrotropin-releasing-hormone induced TSH secretion and thyroidal release. *J Clin Endocrinol Meta.* 43:1976 in Press (Article).
6. A Radioimmunoassay for 3,3'-Diiodothyronine (3,3'T2). Burman, K. D., Strum, D., Dimond, R. C., Djuh, Y. Y., Wright, F. D., Earll, J. M., and Wartofsky, L.: Submitted for publication, *J. Clin. Investigation.* 1976.
7. Becker, R. A., Johnson, D. W., Wilmore, D. W., Burman, K. D., Wartofsky, L., and Woeber, K. A.: Alterations in serum T3 and reverse T3 in thermal injury. *Clin. REsearch.* 24:269, 1976 (Abstract).
8. Burman, K. D., Dimond, R. C., McGuire, R. A., Earll, J. M., Strum, D., and Wartofsky, L.: The effect of varying serum T4 concentrations on extrathyroid production of T3, reverse T3, and 3-3' Diiodothyronine. *Clin Research.* 24:270, 1976 (Abstract).

9. Wartofsky, L., Dimond, R. C., Noel, G. L., Frantz, A. G., and Earll, J. M.: Effect of acute increases in serum triiodothyronine on TSH and Prolactin responses to TRH, and estimates of pituitary stores of TSH and PRL in normal subjects and patients with primary hypothyroidism. *J Clin Endocrinol Metab.* 42:451-466, 1976.
10. Adler, R. A., Corrigan, D. F., and Wartofsky, L.: Hypothyroidism after X-irradiation to the neck. *Johns Hopkins Medical Journal.* 138:180-184, 1976.
11. Wartofsky, L., Dimond, R. C., Noel, G. L., Frantz, A. G., and Earll, J. M.: Failure of propranolol to alter thyroid iodine release, thyroxine turnover, or the TSH and PRL responses to TRH in patients with thyrotoxicosis. *J Clin Endocrinol Metab.* 41:427-432, Sept. 1975.
12. Burman, K. D., Dimond, R. C., Noel, G. L., Earll, J. M., Frantz, A. G., and Wartofsky, L.: Klinefelter's Syndrome: Examination of thyroid function, and the TSH and PRL responses to TRH prior to and after Testosterone Administration. *J Clin Endocrinol Metab.* 41:1161-1166, December 1975.
13. Burman, K. D., Yeager, H. C., Briggs, W. A., Earll, J. M., and Wartofsky, L.: Resin Hemoperfusion: A method of removing circulating thyroid hormones. *J Clin Endocrinol Metab.* 42:70-78, January 1976.
14. Wartofsky, L., Dimond, R. C., Noel, G. L., Adler, R. A., Frantz, A. G., and Earll, J. M.: The effect of an oral water load on serum TSH in normal subjects, and on TSH and PRL responses to TRH in patients with primary hypothyroidism. *J Clin Endocrinol Metab.* 41:725-728, October 1975.
15. Adler, R. A., Noel, G. L., Wartofsky, L., and Frantz, A. G.: The effects of oral water loading and intravenous hypotonic and hypertonic saline on plasma prolactin in man. *J Clin Endocrinol Metab.* 41:383-389, August 1975.
16. Burman, K. D., Monchik, J. M., Earll, J. M., and Wartofsky, L.: Ionized and total serum calcium and parathyroid hormone in hyperthyroidism. *Annals Int Med.* 84:668-671, June 1976.
17. Burman, K. D., Dimond, R. C., Earll, J. M., Wright, F. D., and Wartofsky, L.: Sensitivity to lithium in treated Graves' Disease: Effects on serum T4, T3, and reverse T3. *J Clin Endocrinol Metab.* 43:1976 in Press.
18. Wartofsky, L., Dimond, R. C., Noel, G. L., Earll, J. M., and Frantz, A. G.: Estimates of pituitary stores of TSH and PRL in normal subjects and patients with primary hypothyroidism by the use of continuous TRH infusion, In Proceedings VII International Thyroid Conference, Boston 9-13 June 1975, Excerpta Medica, Amsterdam, pp. 268-271.

19. Burman, K. D., Dimond, R. C., Wright, F. D., Earll, J. M., Burton, J., and Wartofsky, L.: A radioimmunoassay for 3,3',5'-L-Triiodothyronine (reverse T3): Assessment of thyroid gland content and serum measurements in conditions of normal and altered thyroidal economy and following administration of TRH and TSH. Submitted for publication to J Clin Endocrinol Metab. 1976.

20. Wartofsky, L.: A Therapeutic Approach to Hyperthyroidism. Medical Opinion. 5:46-51, June 1976.

21. Dimond, R. C.: Diagnosis of Thyroid Disease Based upon Radioimmunoassay of TSH. Medical Opinion. 5:14-17, June 1976.

22. Thompson, P., Earll, J. M., and Schaaf, M. S.: Comparison of Clofibrate and Chlorpropanide in Vasopressin Responsive Diabetes Insipidus. Submitted to Archives Int Medicine. 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OA 6445	76 07 01	DD DR&E(A)/636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ³	6 WORK SECURITY ⁴	7 REGRADING ⁵	8A DMS ⁶ INSTR ⁷	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 ⁸	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	62760A	3A762760A822	01	121			
	XXXXXXXXXX CARDS 114F						
11 TITLE (Precede with Security Classification Code) ⁹							
(U) Pathogenesis of Enteric Diseases							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ¹⁰							
010100 Microbiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
59 05		CONT		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A DATES/EFFECTIVE NA				PRECEDING		B FUNDS (in thousands)	
D NUMBER*				FISCAL 76		3 269	
E TYPE				CURRENT 77		3 373	
F KIND OF AWARD				F. CUM. AMT.			
20 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME* Walter Reed Army Institute of Research				NAME* Walter Reed Army Institute of Research			
ADDRESS* Washington, DC 20012				ADDRESS* Washington, DC 20012			
RESPONSIBLE INDIVIDUAL JOY, COL R. J. T.				PRINCIPAL INVESTIGATOR (Furnish NAME // U.S. Academic Institution)			
TITLE:				NAME* FORMAL, S. B.			
TELEPHONE 202-576-3551				TELEPHONE 202-576-3344			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Gemski, P. DA			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Diarrhea; (U) Dysentery; (U) Bacillary; (U) Salmonellosis; (U) Immunity; (U) Immunization							
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 study the pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised. Diarrhea is a significant problem in military personnel operating overseas.</p> <p>24. (U) The genetic control of O-antigen specificity of enteric pathogens is being studied since such cell envelope components are of importance in disease and its prevention through vaccination.</p> <p>25. (U) 75 07-76 06 Monkeys with high circulating levels of S. dysenteriae 1 antitoxin (anti-neurotoxin) are not resistant to oral challenge with S. dysenteriae 1. Form II S. sonnei fails to regain its virulence after acquiring the ability to synthesize E. coli O-25 polysaccharide. Mini cells from strains of S. dysenteriae 1 and S. flexneri 2a have been prepared and characterized. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$105,000 from FY 76 funds is programmed for the period 1 Jul-30 Sep 76.</p>							

PII Redacted

DD FORM 1498
1 MAR 68

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

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 121 Pathogenesis of Enteric Diseases

Investigators

Principal: Samuel B. Formal, Ph.D.

Associate: Peter Gemski, Ph.D.

Description

The pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised.

Progress

By employing an integrated, immunologic, cytologic and genetic approach (see previous annual reports) studies in this department are concentrating on further elucidation of: (I) virulence factors and mechanisms involved in intestinal penetration and toxin elaboration by pathogens, key mechanisms by which enteric diseases are provoked; (II) the genetic control of O antigen specificity of enteric pathogens since such cell envelope components are of importance both in disease and its prevention through vaccination; and (III) the application of genetic techniques for development of live, oral vaccines against Shigellosis.

1. Monkeys were immunized with crude native "exotoxin" produced by Shigella dysenteriae 1 and developed high levels of circulating anti-toxin. These animals were not protected against oral challenge with virulent S. dysenteriae 1.

2. Guinea pigs immunized with a "rough E" S. minnesota strain or a galactose epimeraseless E. coli 0111 strain were protected against a penicillin-induced gram-negative bacteremia.

3. A significantly higher proportion of Escherichia coli strains isolated from the blood or urine of human beings elaborates a heat-labile material which is toxic for mice than do similar organisms isolated from the feces.

4. Form II Shigella sonnei fails to regain its virulence after acquiring the ability to synthesize E. coli 0-25 polysaccharide.

5. Minicells from strains of S. dysenteriae 1 and S. flexneri 2a have been prepared and characterized.

Materials and Methods, and Results in 5 Areas of Research Named Above

1. Studies have been conducted to determine if antibodies to Shigella dysenteriae 1 toxin is effective in protecting against disease caused by this organism. Six monkeys were injected subcutaneously - first with three weekly doses of crude toxoid, followed by eight doses of crude native toxin spaced at 3 to 5 day intervals. The preimmunization sera from these animals contained less than 4 HeLa cell neutralizing units of antitoxin; following the 3 doses of toxoid the titer rose to 500 neutralizing units; and after the 8 doses of toxin it rose to greater than 4,000 units. These animals, together with 5 controls, were orally challenged with virulent S. dysenteriae 1 cells 2 weeks after the last dose of toxin was administered. Three of the six animals injected with the toxin suffered severe dysentery with blood, mucous and inflammatory cells in the diarrheal stool. Two of the five control animals experienced a watery diarrhea. The vaccination procedure failed to confer protection.

2. A significant number of guinea pigs die after being injected with 50,000 units of penicillin. Deaths follow an overwhelming overgrowth of gram-negative organisms in the intestine which results in a severe cecitis, a moderate ileitis and a high incidence of bacteremia. In our animals, Klebsiella are the most common organisms which are isolated at the time of death. We have employed this model to assess the protective capacities of three bacterial strains which others have suggested might confer some degree of non-specific protection against gram-negative infections. These agents were E. coli strain J-5 which is a galactose epimeraseless mutant of an 0-111 parent strain; strain R595, which is a "rough E" (Re) mutant of S. minnesota; and E. coli 0-14, which evokes antibody against the Common Enterobacterial Antigen. Guinea pigs received subcutaneously 6 graded doses of heat killed cells followed by 5 graded doses of living bacteria. The animals were given penicillin two weeks after the last vaccine dose. Significant protection against penicillin-induced bacteremia was observed in those animals which were inoculated with Re mutant S. minnesota (R595) strain (Table 1).

3. Work in domestic animals and fowls has indicated that extra-intestinal infections with E. coli has been associated with the organism's ability to elaborate a heat-labile toxin. Sterile ultrasonic filtrates of overnight broth cultures of these organisms kill mice within 24 hours of intravenous injection. The incidence of this heat-labile toxin has been compared in strains of E. coli isolated from the stools of normal adult human beings, from the urine of individuals with

cystitis or pyelonephritis, and from the blood of bacteremic patients. The results are summarized in Table 2. The heat-labile toxin from human *E. coli* strains is inactivated by both trypsin and pronase. While the toxin in strains isolated from domestic animals has been demonstrated to be plasmid-controlled, we have not yet been able to demonstrate that this is the case with strains from human beings. Eighty-five percent of the toxin-producing strains also elaborated a hemolysin. On the other hand forty-one percent of the toxin-negative cultures were hemolytic. The hemolysin of strains from animal sources have been found to be plasmid-determined. Preliminary studies with strains from human sources indicate that this hemolysin is under chromosomal control.

4. *Shigella sonnei* is responsible for most of the Shigellosis in developed countries. The virulent form (FI) of this organism may dissociate at a high frequency both in the human intestine and on laboratory media to an avirulent mutant (FII). The FI and FII forms have different colony types and also differ serologically. The serologic difference is attributable to the loss of the FI O-specific polysaccharide side chain by the FII mutants and this loss is considered also to be responsible for the lack of virulence of the mutant.

Previous studies had demonstrated that transferring the O-25 antigen from *E. coli* to a virulent strain of *S. flexneri* 2A (using microbial recombination techniques) did not alter the virulence of the dysentery strain. Similar experiments were performed with the FI and FII *S. sonnei*. Hybrids of the FI strain expressing O-25 polysaccharide side chains were isolated which retained their virulence. However, similarly constructed hybrids of the FII mutant failed to regain the ability to cause disease even though they now elaborate a side chain compatible with virulence. The possibility exists that the length of the O-25 side chain of the FII avirulent hybrids is less than that of the FI hybrid and that this may account for the fact that these organisms did not regain virulence.

5. A mutant strain of *Escherichia coli* K-12 has been shown by Adler to express an aberrant cell division cycle which causes the production of small anucleate cells termed minicells. Studies have revealed that suspensions of minicells with less than one contaminating parental cell per 10^6 minicells can be prepared by a combination of differential and rate zonal sucrose density gradient centrifugation. Purified minicells, although lacking chromosomal DNA, contain normal levels of protein and RNA. While they are incapable of cell division, minicells possess functional cell wall, ribosomal and metabolic systems.

The uniqueness of the minicell system has been utilized as a model for studying the cell division cycle and the function and replication of plasmids. Their use as a model for studying factors related to pathogenesis of enteric diseases has not been exploited. Thus we have initiated studies to prepare and characterize minicell producing mutants

of various pathogens known to cause enteric diseases. Minicells producing mutants of S. typhimurium, S. typhosa, toxogenic E. coli, Proteus and Shigella have now been isolated. We describe the isolation and characterization of minicell producing strains of Shigella flexneri 2a and Shigella dysenteriae 1 which retain their property of virulence.

The S. flexneri 2a from which a minicell producing mutant was isolated is strain M42-43, previously shown to elicit classical dysentery in man and other primates. S. dysenteriae 1 strain 1617, isolated during the recent epidemic of Shiga dysentery in Central America was employed as the parent for constructing a minicell producing mutant of toxigenic S. dysenteriae 1.

Minicell producing mutants were isolated after mutagenesis of parental strains with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Cells surviving mutagenesis were plated on TSA and after 18 hours incubation at 37C, the colonies were examined microscopically for an aberrant colonial morphology. Saline suspensions of such clones were then screened for the presence of minicells by examination with light phase microscopy. Isolates which were filamentous or which produced small spherical cells were recloned and further tested to establish their minicell producing capacity. A minicell producing mutant of S. flexneri 2a, designated M42-43MCI, and one of S. dysenteriae 1 designated 1617-MCV were so recovered.

Broth cultures of both mutants yield similar findings with respect to cell morphology. When examined with light phase microscope, such cultures were heterogeneous in that they contained cells either smaller or larger than wild type, some cells which were filamentous and free minicells. In addition to free minicells, cells undergoing asymmetrical division to yield a minicell are also seen.

Purified suspensions of minicells were prepared by the following procedure. Strains M4243-MCI and 1617-MCV were grown in BHI broth for 24-36 hours at 37C with aeration. After a preliminary low speed centrifugation (2000 xg, 10 min) to remove a large proportion of the whole cells, the minicell-rich supernatant was centrifuged for 15 minutes at 20,000 xg. The resulting pellets, which contained both minicells and whole cells were resuspended in physiological saline to yield a cell concentration 50-100 fold greater than the original culture. Samples (about 2.0 ml) of this suspension were then layered on 50 ml preformed sucrose density gradients (5-30 w/v) and centrifuged for 25 minutes at 3000 RPM in a Beckman L3 ultracentrifuge equipped with a SW 25.2 swinging bucket rotor. This procedure resulted in the separation of a large dense band of minicells which was distinct from a secondary diffuse band found to consist of whole cells. The minicells were withdrawn from the gradients with a syringe, suspended in saline and sedimented at 20,000 xg for 15 minutes. A second and on occasion third sucrose density gradient separation was employed to obtain purified preparations of minicells. The level of contaminating whole cells was determined by plating minicell preparations on TSA. The number of

minicells in purified suspensions were estimated by counting in a Petroff-Hauser Chamber. Purified minicell suspensions, prepared as described, routinely contained less than one whole cell per 10^6 minicells.

As summarised in Table 1, purified MCI and MCV minicell suspensions contain very low levels of DNA. Protein concentrations were determined by using the method of Lowery - with crystalline bovine serum albumin as the standard. DNA concentrations were established by employing the method of Burton with herring DNA as a standard. The small amounts of DNA detected in minicell suspensions can be attributed to contaminating parental cells and to the possibility that MCI and MCV minicells contain plasmid DNA.

Our studies indicate that minicell producing strains, M4243 MCI and 1617 MCV retain properties related to virulence of Shigellae. Although both strains yield a colonial morphology typical of rough strains and tend to sediment when grown in liquid medium, they agglutinate to titer in respective antisera and are insensitive to phages specific for strains defective in lipopolysaccharide structure. Both strains evoke a positive Sereney test for keratonconjunctivitis which reflects the capacity of Shigellae to penetrate colonic epithelial cells. This invasive property is further illustrated by their penetration of HeLa cells. The ligated ileal loop model was also employed to assess the virulence of these minicell producing mutants. Both strains evoke a positive ileal loop in rabbits when cells are employed in the test, thus indicating mucosal invasion. In addition, cell free sonicates of *S. dysenteriae* 1617 MCV produce a positive ileal loop indicating that this strain has retained its enterotoxin producing property. Thus it is evident that their aberrant cell division cycle has not significantly affected penetration of a multiplication within the epithelial cells of the cornea and gut mucosa. The recovery of such virulent minicell producing mutants provides an opportunity to study the effectiveness of purified minicells as a nonreplicating oral vaccine against Shigellosis. Moreover, investigation of the ability of purified *Shigella* minicells to penetrate epithelial cells and in the case of *S. dysenteriae* to produce enterotoxin, may provide some insight into our understanding of these factors in the pathogenesis of Shigellosis. Such studies are now underway.

Table 1

Protection of guinea pigs from penicillin* induced bacteremic death

<u>Vaccine</u>	<u>Characterization</u>	<u>Deaths Total</u>	<u>Percent death</u>
1. <u>S. minnesota R595</u>	Re	17/59	29+11.6**
2. <u>F. coli J-5</u>	galactose epimeraseless	19/49	39+14.5
3. <u>E. coli 0-14</u>	common antigen	14/24	58+19.5
4. Saline		39/69	57+11.8

* 50,000 units injected subcutaneously 14 days after the last vaccine dose

** 95 percent confidence limits; group 1 vs group 4 P= <.001

Table 2

Heat labile toxin lethal for mice in E. Coli strains from various sources

Source	<u>Number with toxin</u> <u>Total</u>	Percent positive	χ^2 (group I vs group 3)
1. Blood	61/201	30	P= .001
2. Urine	72/62	35	
3. Stool	6/64	9	

Table 3

Characteristics of Minicell Producing Mutants *S. flexneri* 2a
M42-43 MC-I and *S. dysenteriae* I 1617 MC-V

Strain	Agglutination in specific antiserum	Rough phage sensitivity	Sereny test	Hela cell penetration	Rabbitileal loop reaction	Bacteria Sonication
M4243						
MC-I	+	-	+	+	+	-
1617						
MC-V	+	-	+	+	+	+

Table 4

Characteristics of minicells preparations of S. flexneri 2a
(M42-43) and S. dysenteriae 1 (1617)

Strain	Whole cells (WC)	Mini cells (MC)	Protein mg/g wet wt cells	DNA mg/g wet wt cells	DNA mg/mg Protein	Ratio MC DNA WC DNA
<u>S. flexneri 2a</u> M4243 MCI			139.3	3.26	0.023	0.095
<u>S. dysenteriae 1</u> 1617 MCV			113.3	0.248	0.0022	
	Whole cells (WC)	Whole cells (WC)	137.5	3.17	0.023	
	Mini cells (MC)	Mini cells (MC)	117.6	0.175	0.0014	0.061

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 121 Pathogenesis of enteric diseases

Literature Cited.

Publications:

1. Genski, P. and S. B. Formal. 1975. Shigellosis: An invasive infection of the gastrointestinal tract. Microbiology. Ed. by D. Schlessinger.
2. Takeuchi, A., H. R. Jervis and S. B. Formal. 1975. Animal model of human disease: Monkey shigellosis or dysentery. Am. J. Path. 81: 251-254.
3. Giannella, R. A., R. E. Gots, A. N. Charney, W. B. Greenough III, and S. B. Formal. 1975. Pathogenesis of salmonella-mediated intestinal fluid secretion: Activation of adenylate cyclase and inhibition by indomethacin. Gastroenterology, 69: 1238-1245.
4. Giannella, R. A., W. R. Rout, S. B. Formal and H. Collins. 1976. Role of plasma filtration in the intestinal fluid secretion mediated by infection with Salmonella typhimurium. Infection and Immunity, 13: 470-474.
5. Wohlhieter, J. A., P. Genski, and L. S. Baron. 1975. Extensive segments of the Escherichia coli K12 chromosome in Proteus mirabilis diploids. Molec. gen. Genet. 139: 93-101.

Abstracts:

1. MacDermott, R. P., P. F. Summers, A. A. Reeder, G. H. Lowell, and S. B. Formal. 1976. Antibody dependent, cell-mediated cytotoxicity of shigella. Gastroenterology 70: 910.
2. Kinsey, M. D., S. B. Formal, G. J. Dammin and R. A. Giannella. 1976. The importance of oral inoculation in the pathogenesis of shigella diarrhea. Gastroenterology 70: 902.
3. Thompson, M. R., S. B. Formal and P. Genski. 1976. Toxins of Shigella dysenteriae 1 and Shigella flexneri 2a. Fed. Proc. 35: 1394.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OA 6446	76 07 01	DD DR&E(A)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ⁵	6 WORK SECURITY ⁶	7 REGRADING ⁷	8A DISPN INSTR ^{8A}	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 ¹⁰	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62760A	3A762760A822	01	123			
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114E						
11 TITLE (Precede with Security Classification Code) ¹¹							
(U) Histopathologic Manifestations of Military Diseases and Injuries							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ¹²							
002600 Biology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
63 08		CONT		DA		C. In-House	
17 CONTRACT / GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING			
B. NUMBER ^{17B}				FISCAL YEAR		FUND (\$ in thousands)	
C. TYPE				76		5	
D. KIND OF AWARD				77		35.0	
20 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME ²⁰ Walter Reed Army Institute of Research				NAME ²⁰ Walter Reed Army Institute of Research			
ADDRESS ²⁰ Washington, DC 20012				ADDRESS ²⁰ Division of Pathology			
				ADDRESS ²⁰ Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: JOY, ROBERT J. T., COL				NAME ²¹ Takeuchi, A.			
TELEPHONE: (202) 576-3552				TELEPHONE (202) 576-2024			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Jervis, H., Hase, T., Ewing, E.,			
				NAME: Henley, G., Colvin, R.			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Immune responses; (U) Enteroviruses; (U) Intestine; (U) Electron Microscopy; (U)							
23 TECHNICAL OBJECTIVE ²³ , 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code) ²³ Dysentery							
<p>23(U) To define histopathologic manifestations of injuries and diseases which have current or potential problems in military personnel. The current effort is directed toward studies of enteric diseases and immunologic responses with infections. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in enteric infectious diseases of military personnel.</p> <p>24(U) Various morphologic techniques including histology, histo- and cytochemistry, autoradiography, immunofluorescent microscopy, transmission and scanning electron microscopy are employed. Various immunologic techniques have also been utilized.</p> <p>24(U) 75 07-76 06 Further studies on experimental Entamoeba histolytica infections have clarified pathogenesis of the histolysis of bowel, the hallmark of amoebic dysentery; cytolytic enzymes released from granulocytes caused by invading amoeba and concomitant vascular thrombotic events result in histolysis and ulceration of the bowel. Immunopathologic studies of responses of the gut mucosa to parenteral immunization with shigella bacilli are being carried out in rabbits and guinea pigs; parenteral immunization fails to prevent mucosal invasion by orally administered shigellae but appears to prevent clinical bacillary dysentery. A new experimental model was established in rabbits and guinea pigs in order to obtain basic information on acute immune response in guts. EM studies on experimental canine coronavirus have established characteristic cytopathic effects <u>in vivo</u>; the virions penetrate from gut lumen into enterocytes by pinocytosis, replicate exclusively on smooth membranes and release from host cells thru disrupted luminal plasma membrane into gut lumen. Support in the amount of \$90,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p> <p>For technical reports, see Walter Reed Army Institute of Research Annual Reports, 1 Jul 75 - 30 June 76.</p>							

PII Redacted

DD FORM 1498
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1488A 1 NOV 65
AND 1488-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

843

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Diseases
and Injuries

Investigators.

Principal: Akio Takeuchi, M.D.

Associates: MAJ Edwin Ewing, MC; Tatsuo Hase, M.D.; Helen R.
Jervis, Dr. Nat. Sc.; SSG Garnett Henley, MS;
LTC Paul K. Hildebrandt, VC

Description:

To define histopathologic manifestations of injuries experimentally produced and diseases which present current or potential problems in military personnel. The current effort is directed toward studies of diseases of the digestive tract and immune responses due to infections and injuries. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in diseases and injuries in military personnel.

Approach to the Problem

A multi-disciplinary approach including conventional histology, histo- and cytochemistry, autoradiography, radio-tracer methods, various immunologic techniques, immunofluorescent microscopy, transmission and scanning electron microscopy is employed.

Progress

In the past, this work unit was primarily concerned with studies of histopathologic manifestations of acute diarrheal diseases of infectious origin. In the past year, we have expanded this work unit and included collaborative studies of experimental rickettsial and trypanosomal infections with other departments of WRAIR and WRGH.

I. STUDIES OF HOST-INDIGENEOUS MICROBE RELATIONSHIP IN THE DIGESTIVE TRACT

Medical microbiology has been concerned primarily with the potentially pathogenic members of indigenous microflora. Symbiotic species are of at least equal importance because they maintain essential anatomical and physiological function with the host (Dubos 1967). The lumen of the digestive tract is now acknowledged as the site of a dynamic ecological system composed of extremely large populations of different microbes maintained in balanced proportions. Studies have indicated that indigenous microbes in the gastrointestinal flora of mammals predominantly populate within certain anatomical and

histological divisions of the digestive tract (Dubos et al. 1962). Some of these microbes are preferentially localized in a close proximity to the surface of gastrointestinal mucosa, while others are predominantly found in the glandular lumen of the crypt (Savage, Dubos & Schaedler 1968). A study indicated that the large concentration of epithelial-associated indigeneous microbes resist access of pathogenic bacteria to the intestinal epithelial surface (Savage 1972). Since the adherence of indigeneous bacteria is required for colonization of epithelial cells of the buccal mucosa of man, it has been suggested that the bacterial adherence may be inhibited by the presence of abundant secretory immunoglobulin (Williams & Gibbons 1972, 1974).

Little is known about "gastrointestinal epithelial cell-associated indigeneous microbes" in mammals including monkey and man (Nelson & Mata 1970; Takeuchi & Jervis et al. 1974). For this reason, we have continuously studied these problems; current efforts have been directed toward studies of gastric spirilla in the stomach of rhesus monkeys and man. (See 1974-75 Annual Report, Dept. of Experimental Pathology, WRAIR).

A. Studies on Structure and Host-Relationship of Gastric Spirilla of Monkeys

The presence of spirilla (GS) in gastric mucosa has long been recognized in various animal species including monkey and man. Yet, little is known about the entity and the condition is often overlooked. Using histology, thin sectioning, negative staining and shadow-casting techniques by electron microscopy, studies on structure of GS and their host relationship in healthy rhesus monkeys are being completed.

In collaboration with the Department of Pathology, WRGH and Laboratory Service, Kansas City VA Hospital, we have been studying GS in human patients. Our preliminary findings indicate that the structure of GS and their host-cell relationship in gastric mucosa appears to be indistinguishable from those of monkeys.

In paraffin section stained by H&E, GS may be mistaken for strands of mucus (Fig. 1 & 2). In thick sections of Epon-embedded tissue, GS are recognized as "corkscrews" with up to 12 coils. They are 8 μ m long and 0.8 μ m wide. GS have characteristic polar flagella. They concentrate in isthmus and are found less frequently in neck and base of gastric glands, while they are rare or totally absent in gastric lumen. GS are closely associated with parietal cells (Fig. 3) and are capable of penetrating into parietal cytoplasm. In either intra- or extra-cellular locations, GS elicit neither changes of host cyto-components nor inflammatory response.

Further studies on GS in human subjects will clarify the structural characteristics of GS and its clinical entity in human subjects.

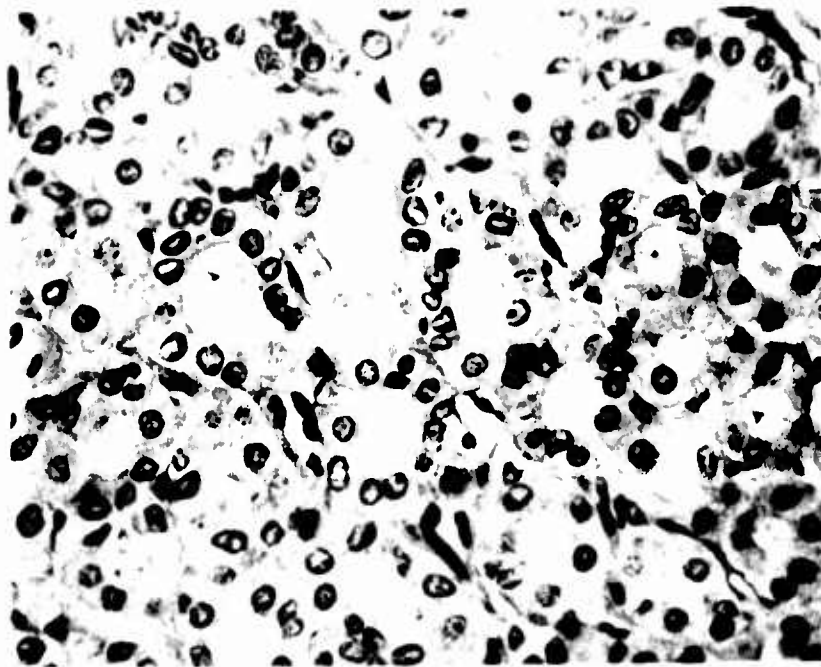


Fig. 1 - Gastric mucosa of isthmus portion of pylorus gastrectomy specimen. The patient had a gastrectomy for performance of a peptic ulcer at the duodenum. The glandular contents containing numerous gastric spirilla are mistaken for strands of mucus in paraffine sections routinely stained by hematoxylin-eosin. X440

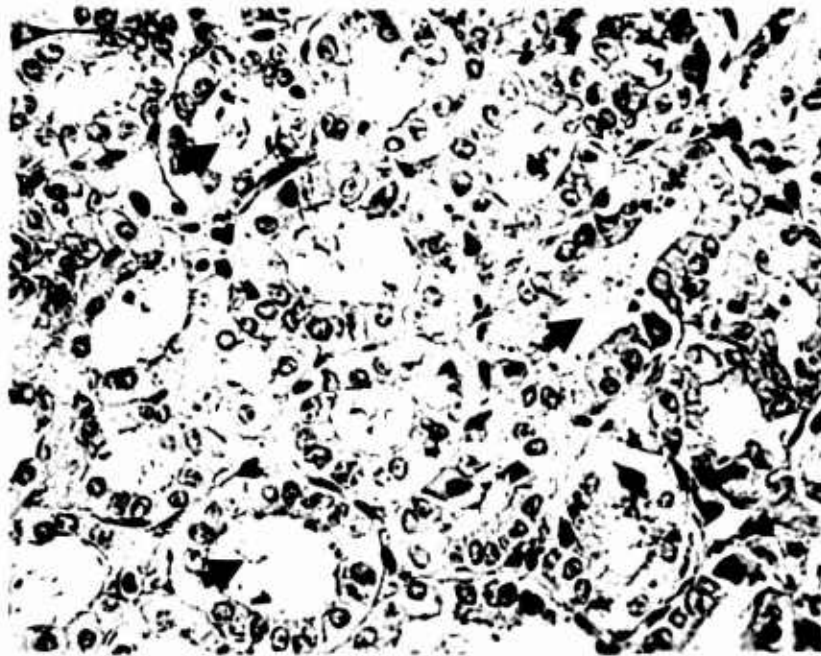


Fig. 2 - Gastric mucosa of isthmus portion of pylorus. This was prepared from a section adjacent to that shown in Fig. 1 and stained by Walthin-Starry (silver). Numerous silver stained gastric spirilla are aggregated and seen as slender and slightly curved microbes in the glandular lumen (arrows). X440

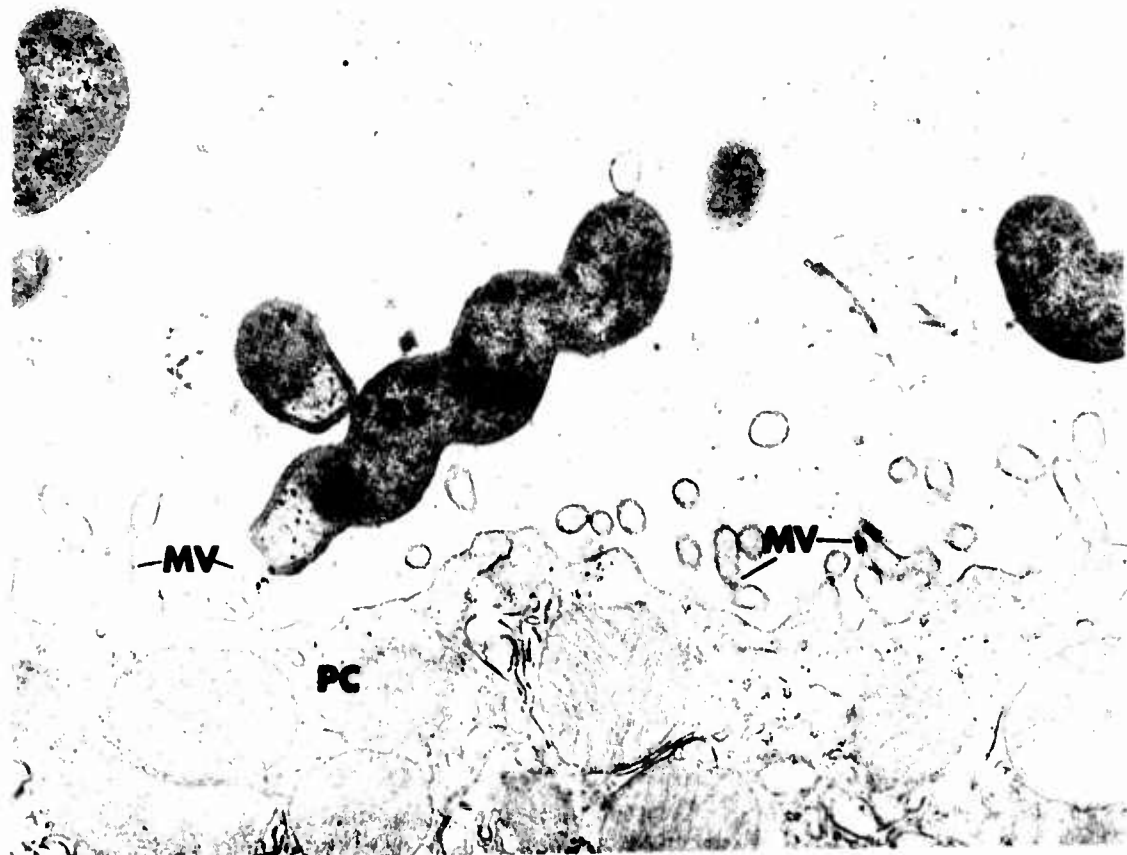


Fig. 3 - Gastric spirilla (GS) in glandular lumen of isthmus, stomach, healthy rhesus monkey. Flagella of spirillum are in a direct contact of microvilli (MV) of a parietal cell (PC). X52,000

II. STUDIES OF HOST-PATHOGENIC MICROBE RELATIONSHIP IN THE DIGESTIVE TRACT

A. Studies on Histolysis and Ulceration of the Cecum Experimental in Entamoeba Histolytica Infections

Background

In man, acute diarrhea caused by *E. histolytica* is attributable to colonic lesions associated with invading amebae. The most common colonic lesions in human patients are acute ulceration which initially developed in the cecum. Cecal ulcers often complicate the prognosis of patients because they develop frequently into perforation of the bowel wall followed by peritonitis and also because they are an initial source of extra-enteric amebic lesions. Yet the pathogenesis of acute amebic ulceration has never been satisfactorily clarified. Some believe that ulcers develop from necrosis of bowel tissue by "lytic enzyme" produced by invading amebae, while others postulate that secondary invasion of bacteria is responsible for ulcer formation in the colon. This discrepancy had been mainly related to as yet unclarified mechanism of initial penetration of the gut mucosa by amebae and the subsequent early changes of the mucosal tissue surrounding invading amebae. Some believe that the ameba penetrated the epithelium by mechanical means. Others postulate that necrosis of gut mucosa by cytolytic enzymes produced by the ameba is responsible for penetration and establishing tissue infections.

By electron microscopy, Griffin (1972) and Pittman et al. (1973) studied rectal biopsy specimens from human patients with *E. histolytica* infections. Although these studies clarified several aspects of amebic-colonic mucosa interactions, they did not demonstrate penetration of colonic epithelium by amebae and their effect on the epithelial cell. This may be due to the limitations inherent in rectal biopsy material and the fact that human cases clinically encountered represent a relatively advanced stage of the disease.

It has been found that young germfree guinea pigs, inoculated intracecally with cultured *E. histolytica* and the enteric flora from a patient with acute amebic colitis, develop lesions similar to those of human amebiasis and thus provide a good experimental model for studies on the pathogenesis of amebic disease. Using this model at an early stage of infection when trophozoites invade the cecal mucosa, we have demonstrated for the first time, ultrastructurally, how amebae penetrate from the gut lumen to the lamina propria through the cecal epithelium and how cytoplasmic components of epithelial cells respond to

penetrating amebae. (See Annual Report, 1973-74, Department of Experimental Pathology, WRAIR) and also early responses of mucosal cells and vasculatures to invasion of amebae (See Annual Report, 1974-75, Department of Experimental Pathology, WRAIR).

We have continued to study these lesions. The present study is concerned with the subsequent changes of the cecum during tissue invasion by amebae. Our special attention is paid to analyze the pathogenesis of histolysis and ulceration of the cecum in this experimental amebae infection.

EXPERIMENTAL INFECTION AND MORPHOLOGIC METHODS

NIH Hartley strain germfree guinea pigs were used as experimental hosts. The animals were obtained by Caesarean section, maintained in Reynier's series 500 stainless steel isolators on dietary regimen L-445 and monitored at weekly intervals by procedures described (Phillips & Gorstein 1966). All animals were inoculated at the age of 12-17 days and each received a 1.0 ml inoculum containing 200,000 *E. histolytica*. CDC J-190 strain amebae were injected directly into the cecum during laparotomy under sodium pentothal anesthesia. They were maintained in vitro in Locke's egg-rice flour medium with enteric flora from the patient, incubated at 37° C and transferred thrice weekly. Inocula were prepared by pooling the sediment from 48 hour cultures and quantitating with a hemocytometer. Control animals were treated just as the experimental group except they were inoculated with only the enteric flora without amebae.

Guinea pigs were killed at post-inoculation intervals of 7-12 days by ether anesthesia and autopsied in a conventional manner. The cecum was removed immediately and immersed in chilled physiological saline wherein the cecal wall was opened and the luminal contents carefully removed. Multiple sections were taken from mucosa of the cecum. Each section was divided into three pieces and processed for histochemistry (HC), light (LM) and electron microscopy (EM).

PRELIMINARY OBSERVATIONS

Light microscope observations on microerosions and the margin of ulceration at the bowel mucosa showed that areas of the histolysis is characterized by amorphous material, cell debris and red blood cells (Fig. 4 & 5). When the ulcerated lesions of the mucosa were stained by Alkaline phosphatase, the reaction products are limited to the ulceration (Fig. 6). In order to clarify the nature of the localized activity of the phosphatase activity, microerosions and ulceration at the bowel mucosa have been studied by electron microscopy. At ultrastructural level, lysis of granulocytes would correspond to the areas of histolysis.



Fig. 4 - Interglandular (surface) portion of cecal mucosa infected with E. histolytica. Amebae (*) are in process of invading through area of histolysis consisting of amorphous material, cell debris and red blood cells. Epithelial cells arrows show severe degeneration and cell death while those away from invading amebae are unaltered. Few granulocytes are seen in the lamina propria. X650



Fig. 5 - Glandular portion, cecal mucosa with *E. histolytica* infection. Multiple ameba (*) are in process of mucosal invasion. Severe degeneration is only limited to epithelial cells (between arrows) and mesenchymal cells close to invading amebae. Epithelial cells distal to ameba appear unaltered. X650



Fig. 6 - Cecum infected with *E. histolytica*. Frozen processed for the demonstration of Alkaline phosphatase shows reaction products distributed throughout the area of histolysis in the center and normal reaction at the brush border of the gut epithelium. X50

III. STUDIES OF INTESTINAL INFECTIONS WITH ENTEROVIRUSES

Background

Abundant information is available concerning the physico-chemical, infective and pathogenic properties of enteroviruses. Most of these data have been obtained from studies of virus-cell interactions in tissue culture system rather than observations on enteric infections in vivo.

In natural virus infections the precise role of the gastrointestinal tract either as the portal of entry or as the initial site of virus replication has not been well established. It is generally considered, however, that it is an initial site of replication for many enteroviruses (Dowie, 1963; Stenhouse, 1970). For instance, Enders and his colleagues demonstrated that the Lansing strain of the poliomyelitis virus multiplied in suspension cultures in human embryo intestinal tissue more than 25 years ago (Enders et al. 1949). Yet, little is known about host cell-virus relationship in the gastrointestinal tract in vivo.

In collaboration with two groups of investigators, light and electron microscope studies on enteric infections by enteroviruses have been completed. In these studies we used a murine adenovirus K87 and a canine coronavirus 1-71. Special attention has been given to site of viral replication and cytopathic effects of host cells in the gut mucosa.

A. Study of Experimental Enteric Infection of Mice with a Mouse Adenovirus K87

The physicochemical, pathogenic and oncogenic aspects of adenovirus-host cell interaction have been well studied in in vitro systems. Yet, little is known about adenovirus-host cell interaction in vivo (Margolis & Kilham et al. 1975). In particular, we lack information concerning virus tissue tropisms and the histopathologic aspects of adenovirus infections in the digestive tract.

Adenovirus strain K87, (Hashimoto & Onishi et al. 1973, Hashimoto & Sugiyama et al. 1966 and Hashimoto & Sugiyama et al. 1970) originally isolated and identified by Hashimoto and his colleagues from the feces of a healthy inbred mouse, strain DK1 (Ushiba & Kitasato et al. 1962) grows well in mouse kidney tissue culture (Hashimoto & Sugiyama et al. 1966). Either oral or parenteral administration of K87 to mice results in replication of the viral antigens exclusively in the bowel but produces no symptoms in the infected animals (Sugiyama & Hashimoto et al. 1967). When DK1 mice are orally infected with suspension of K87 grown in mouse kidney culture cells, viral antigens start to increase at 3 days and reach a maximum between 7 and 14 days after infection (Hashimoto & Sugiyama et al. 1970). At the height of the viral replication in the gut, numbers of virally infected cells are found in the epithelial lining of the distal small intestine; this has made feasible electron microscope observations on the adenovirus-host cell interaction in vivo.

The present work describes morphologic feature of an experimental enteric infection of the DK1 mouse, inoculated orally with the mouse adenovirus strain K87, as observed by fluorescent antibody technique and by light and electron microscopy. In particular, it establishes the small intestinal epithelial cells as the site of replication of the structural antigen of K87 adenovirus.

MATERIALS AND METHODS

Dr. Hashimoto, Prof. of Microbiology, Keio University, Tokyo, Japan, has infected mice with his adenovirus in his laboratory and supplied us with all necessary virological data and infected intestinal tissues.

Mice. Fifty-eight mice used in this experiment were four-week-old inbred strain DK1 white mice (Ushiba & Kitasato et al. 1962), caged individually and allowed access to food and water *ad libitum*. Of these, 46 animals were experimentally infected and the remaining 12 were used as noninfected controls.

Virus. Mouse adenovirus, strain K87, isolated by Hashimoto was used (Hashimoto & Sugiyama et al. 1966). The virus suspensions were prepared from freeze-and-thawed mouse kidney tissue cultures (MsKTC) infected with the virus after 5-9 passages through MsKTC, as described in detail elsewhere (Sugiyama & Hashimoto et al. 1967).

Viral challenge. The inoculum, of 0.4 ml containing 4×10^5 TCD₅₀, was administered orally to each mouse, through a metal tube inserted into the stomach under ether anesthesia. The control inoculum was 0.4 ml of physiologic saline (Hashimoto & Sugiyama et al. 1970).

Tissue preparation. At 5, 7, and 14 days after oral inoculation groups of infected and control mice were anesthetized by ether, sacrificed by cervical dislocation and necropsied in a conventional manner. A segment of the ileum was removed, divided into four portions and processed as follows:

Viral isolation and titration. For virus isolation, a 10% suspension of feces and a 5% suspension of the homogenized first portion of the ileum were made and titrated by procedures described in detail elsewhere (Hashimoto & Sugiyama et al. 1970).

Direct fluorescent antibody technique (FA). A second portion of the ileum was opened longitudinally, rolled and placed on a strip of paper in a test tube which was held for 10 minutes in a mixture of ethanol and dry ice, to freeze the tissue at -73 C. The frozen tissues were sectioned in a cryostat, fixed and treated with fluorescein labeled mouse K87 virus antiserum. Preparations of the antiserum and the method of conjugation with fluorescein isothiocyanate have been

described previously (Hashimoto & Sugiyama et al. 1970). Sections were examined under a Nikon Fluorescence Microscope, Type SUR-F. The specificity of the FA staining was established by treating in the same fashion sections from uninoculated mice or by blocking the reaction in inoculated mice with a gamma globulin solution prepared from K87 immune serum. In both cases no fluorescence was seen.

Light Microscopy (LM). A third portion of the ileum was fixed in chilled 10% buffered neutral formalin and routinely processed for histologic study. Paraffin sections were stained with one of the following stains: hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Giemsa alcian blue, or Feulgen. Thick 1-2 μ m sections of Epon embedded material were stained with azure II-methylene blue.

Electron Microscopy (EM). A fourth portion of ileum was minced, fixed in 2% glutaraldehyde in phosphate buffer for 2 hours and washed with phosphate buffer. The tissues were further fixed with 3.3% osmium tetroxide in S-collidine buffer and processed for EM examinations.

OBSERVATIONS

FA microscopy observations on frozen sections of the ileum from infected mice, stained by FA techniques with serum immunized with K87 adenovirus were essentially the same as those reported previously. Cells containing viral antigen were localized in the intestinal in vitro (Hashimoto et al. 1966) epithelial lining and absent in the lamina propria. They appeared singly and were randomly distributed in both villi and crypts. The viral antigen was seen as a round or sometimes as an angular or crescent-shaped mass, usually located at the base but occasionally close to the luminal surface of the epithelial lining. In many instances, the fluorescence appeared to be confined to the nucleus of epithelial cells.

Light Microscopy. The ileum of control noninfected DK1 mice was essentially the same as that of mice of other strains. The ileal mucosa of infected DK1 mice examined at 5 and 7 days after oral challenge was undistinguishable from its counterpart in the control mice. In contrast, the ileum at 14 days post-infection showed significant changes which included a decreased villus-crypt ratio and a moderately increased cellularity in the lamina propria. However, there was no acute inflammation in the mucosa. The crypts were slightly distended and contained cellular debris.

Paraffin sections stained with H&E and carefully examined at magnifications of more than 300 times revealed large, bizarre shaped, homogeneously basophilic nuclei which were sparsely distributed in the epithelial lining of both villi and crypts. Some were oval or round; others were angular. Under oil immersion (X1,200) they often showed several smaller, dense inclusions surrounded by less dense nucleoplasm. These inclusions were consistently Feulgen positive but PAS negative, indicating the presence of DNA.

The enlarged nuclei in the infected ileal tissues were identified by LM more easily on Epon preparations than on paraffin sections; moreover, Epon sections revealed better structural details. The nuclei were round or oval or had an irregular perimeter. The most common shape was oval, outlined by a distinct nuclear membrane with a lightly stained nucleoplasm, containing small dense granules often concentrated at the periphery. The distribution of these granules, however, varied from one enlarged nucleus to another, and they could be also evenly distributed throughout the neoplasm.

Some enlarged nuclei were clearly located in columnar cells of the villus epithelium. It was not possible, however, at the LM level to recognize with certainty all the cell types in which these enlarged nuclei were located, especially in the crypt epithelium, which is closely populated by different types of cells.

Electron Microscopy. Ultrastructural studies confirmed the LM observations and provided further details. The fine structure of the small intestine of mice was similar to that of other mammals and has been described in detail (Takeuchi & Sprinz et al. 1965). On the basis of their ultrastructural characteristics, intestinal epithelial cells could be divided into four types (Trier, 1963); columnar, goblet, Paneth, and argentaffine cells. Between the epithelial cells of both villi and crypts were thelial lymphocytes (Takeuchi & Sprinz, 1967). The villi were covered predominantly by columnar cells between which mucus secreting goblet cells were sparsely located, while the crypt epithelium consisted of immature columnar cells, Paneth cells, goblet cells and argentaffine cells. Cellular elements in the ileal lamina propria were similar to those previously described in guinea pigs (Takeuchi & Sprinz, 1967).

In general, the ileal mucosa of infected animals at 5 and 7 days after oral challenge was similar to that of control animals. Enlarged nuclei were occasionally identified in cells within the epithelium, but their presence was extremely rare and appeared to have no effect upon the surrounding cellular organelles and on the other components of the gut mucosa; therefore, the following ultrastructural observations were mostly made on ileal tissues obtained 14 days after oral infection. Enlarged nuclei were then easily identified in both crypt and villus epithelium at low magnification and corresponded to those observed by FA and LM. They were confined to three types of epithelial cells - columnar, goblet and Paneth cells. Nuclei containing large viral masses belonged to columnar and goblet cells on the villi, while in the crypts they were found preferentially in the Paneth cells and more rarely in goblet cells.

At high magnification, fully formed virions were identified in the infected nuclei (Fig. 7). They were strikingly uniform in size, being 75 ± 5 nm in diameter and appeared hexagonal (Fig. 7).

In favorable sections, two types of virions were identified, some with dense central cores and others with electron lucent centers (Figs. 7 & 8). Occasionally, capsids could be resolved in some virions (Fig. 7). Most virions were arranged in typical arrays (Figs. 7-9) while others were free and randomly scattered in the nucleoplasm (Fig. 10), or close to arrays in dense homogeneous matrix, or abutted to less dense and granular matrix (Fig. 10). The number of virions in each infected nucleus varied from one cell to another; in some nuclei they aggregated in small numbers (Fig. 10) while in others they formed large crystals (Fig. 7), often exhibiting alternate light and dark bands (Figs. 8 & 10). In some infected nuclei there were several ill-defined electron dense spherical masses (Figs. 8 & 10), while in others also containing homogeneously dark spheres, portions of the peripheral nucleoplasm, including virus aggregates, projected toward the cytoplasmic matrix (Figs. 10 & 13). These nuclear projections resembled the nuclear projections filled with virus aggregates which have been described by Fong and colleagues (Fong & Bensch et al. 1968) as well as the "protrusions of the nuclear membrane" of Yamamoto (Yamamoto, 1969). Occasionally the nuclear membrane topographically close to viral particles were partially dissolved, resulting in free communication between the nucleoplasm and cytoplasmic matrix and in the release of virions from the nucleus.

In the cytoplasm of infected cells, virions might be enclosed in membrane-bound vacuoles together with dense amorphous material and fragments of membranes (Fig. 10), or might lie free, without enclosing membranes (Fig. 12). Other cytopathic changes in infected columnar cells included the emergence of slit-like translucent spaces enclosed by a single membrane (Fig. 8) and swelling of mitochondria, while other cytoplasmic organelles such as endoplasmic reticulum remained unchanged. In contrast, the cytoplasmic components, including the granules of the goblet and Paneth cells, showed no significant changes, even though they contained a large number of virions.

Both columnar and goblet infected cells were seen in the process of shedding from the mid-villus epithelium into the intestinal lumen (Fig. 12), while the Paneth cells were seen shedding into the crypt lumen (Fig. 11). Extruded cells containing virions, showed a variety of degenerating cytoplasmic organelles as described above. Sometimes virions were identified free in the gut lumen.

Neither viral attachment to the microvillus or viral penetration through the microvillus and nuclear membrane were observed. Mesenchymal cells and the extracellular space of the lamina propria were free of virions.

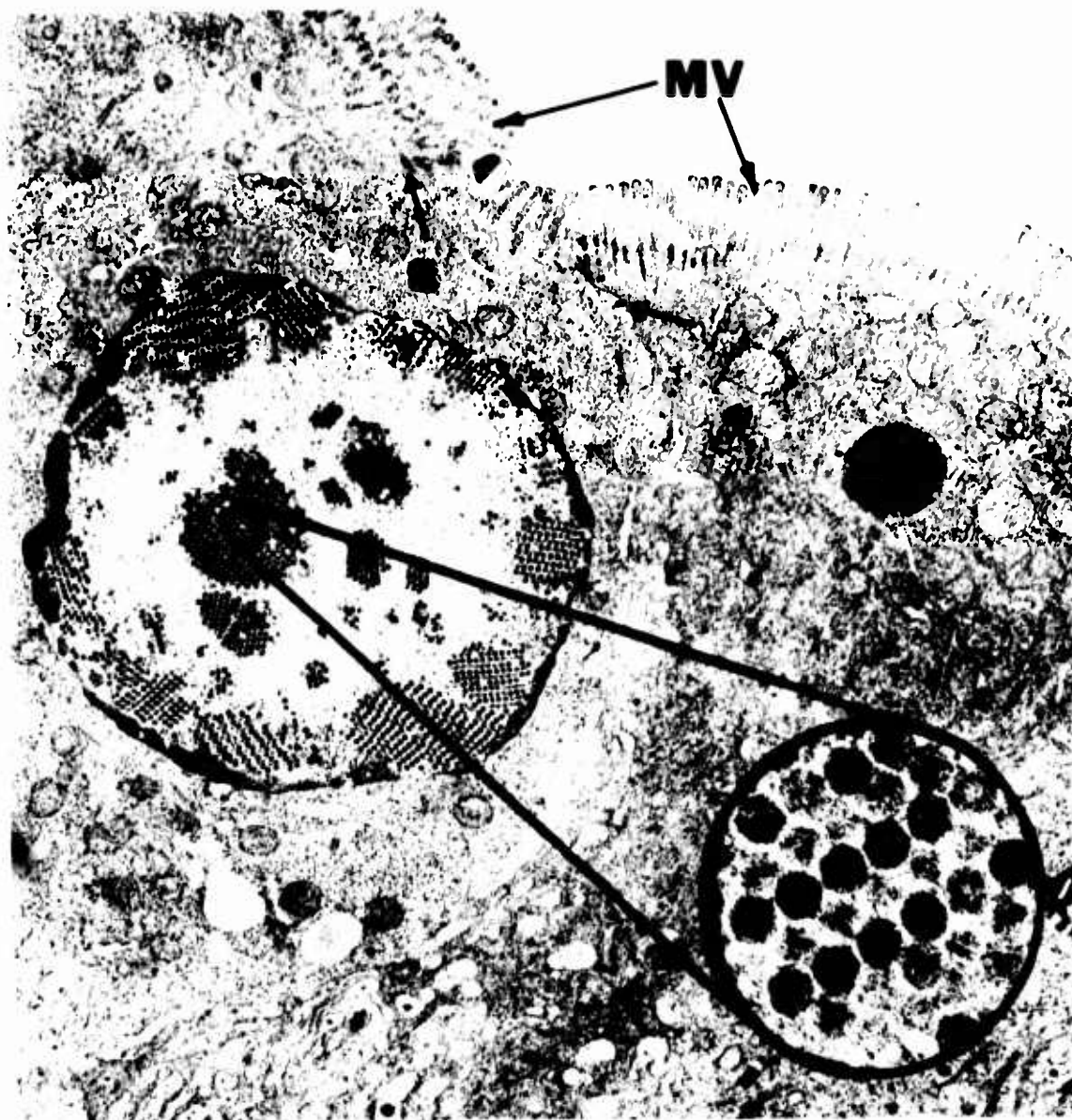


Fig. 7 - Apical portion of infected ileal villus epithelium. The columnar cell shows a large nucleus containing crystalline arrays of virions. Note the difference between the infected nucleus and noninfected nucleus on the left (N). The other cytoplasmic components, including microvilli (MV), and the intercellular junctions (arrows) remain unaltered. Basal lamina of epithelial cells is marked by arrowheads; below it is a capillary (C). X8,200. The subunit structure of the capsid is seen in some virions (Inset) X110,000.



Fig. 8 - Paneth cell in infected ileal crypt epithelium. Numerous virions form crystalline arrays. The nuclear membrane shows the typical double or parallel membranes. The Paneth granules are synthesized in the Golgi system (GI) and accumulate in the cisterna (arrow) developing into larger and denser mature granules (*). In the cytoplasm, there are slit-like translucent structures (SLT). Other cytoplasmic components are not remarkable. X13,000.

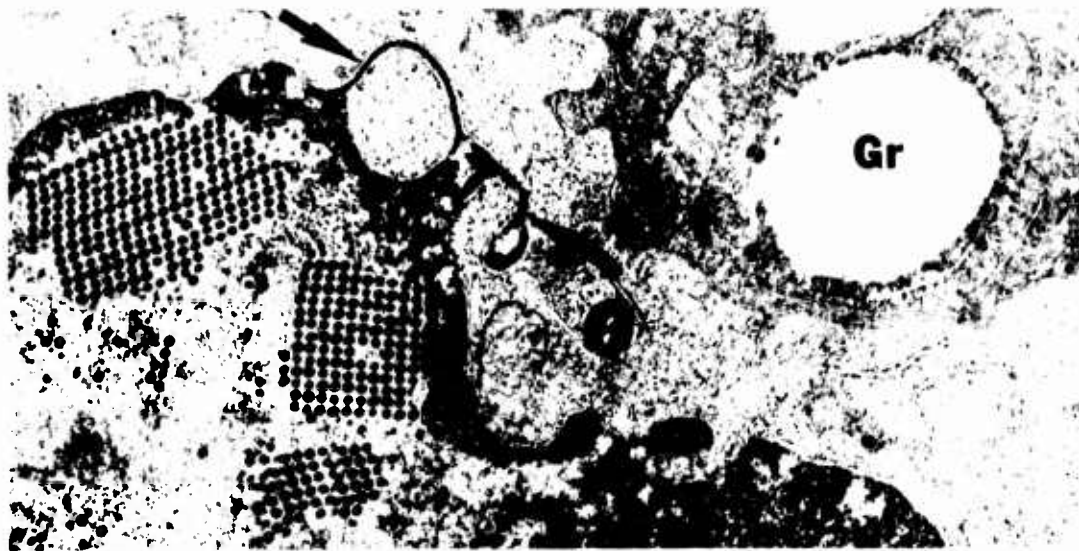


Fig. 9 - Portion of nucleus of villus columnar cell showing peculiar projections of nucleoplasm into the cytoplasm, which focally enfold the cytoplasmic matrix (arrows). Note crystalline arrays of virions in nucleus and mucus granule (G) of adjacent goblet cell. X19,000.

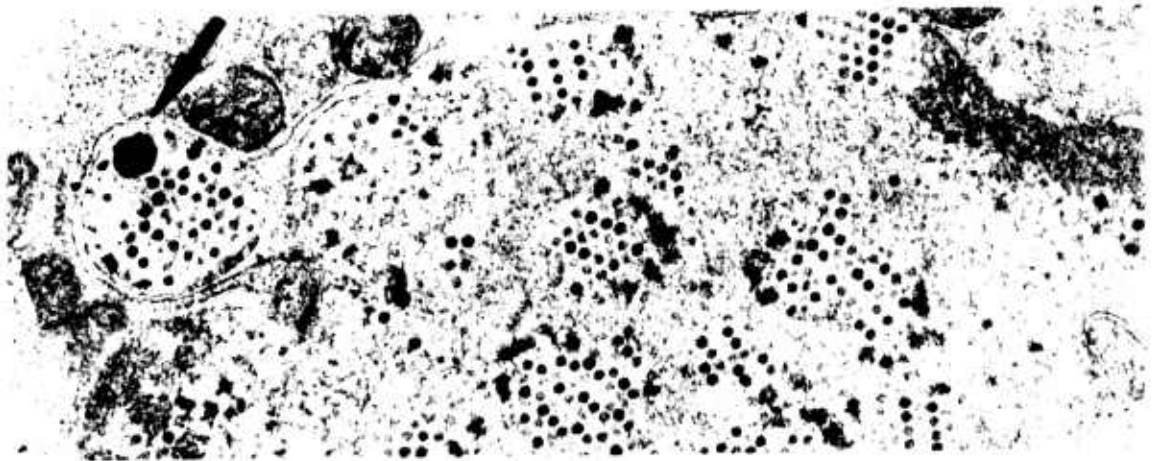


Fig. 10 - Part of infected nucleus of villus columnar cell. Nuclear protrusions are developing at both ends (Pr); the left one, separated from the main portion of the nucleus and enclosed by the inner nuclear membrane (arrow), contains virus particles and a dark round inclusion. X22,000.

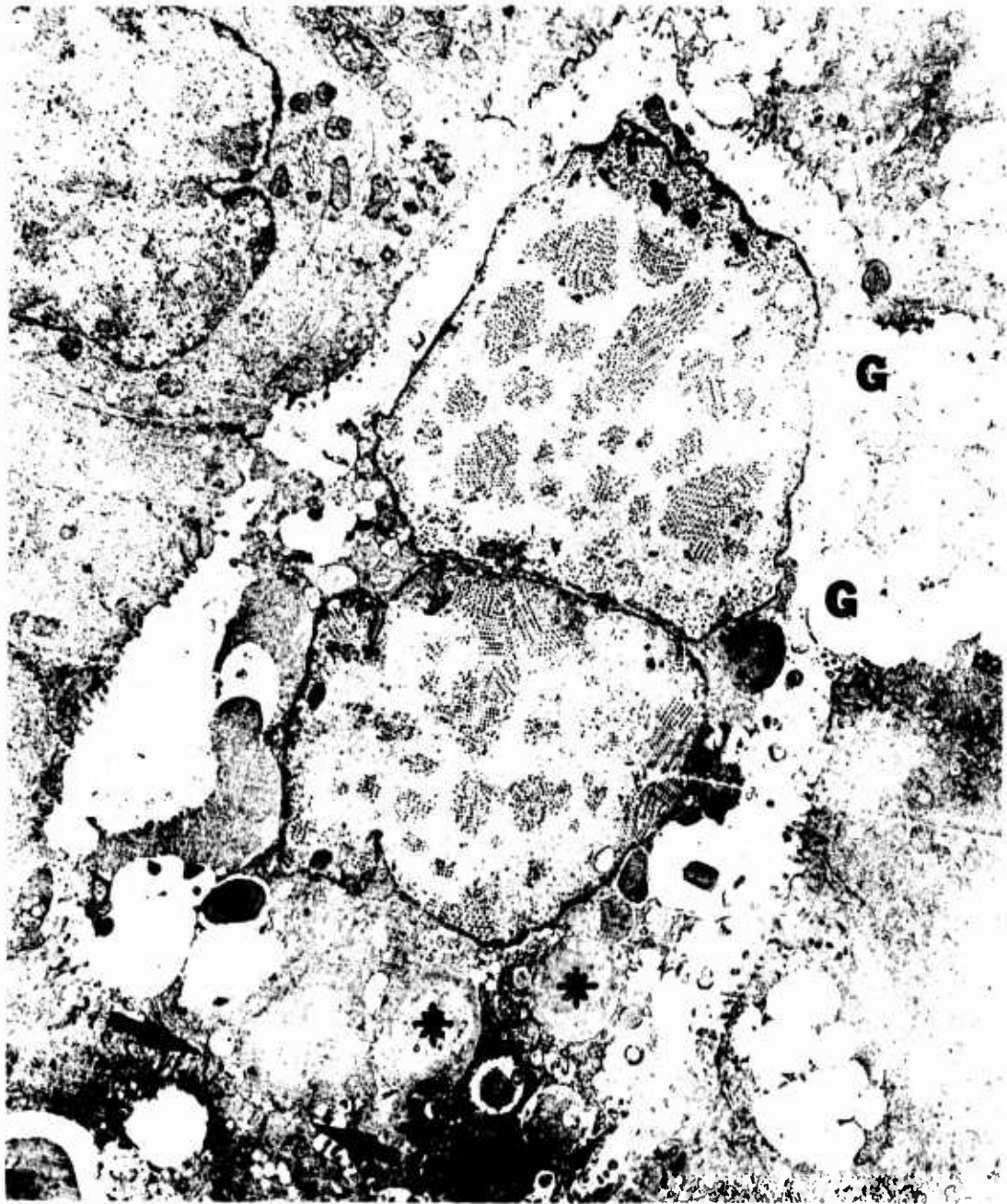


Fig. 11 - Crypt of infected ileum. The nuclei of two cells, apparently Paneth cells, are in process of shedding into the crypt lumen. A portion of shedding cell is still attached to epithelial cells (arrows). Note Paneth granules (*). Goblet cell is discharging mucus into crypt lumen (G). X6,800.

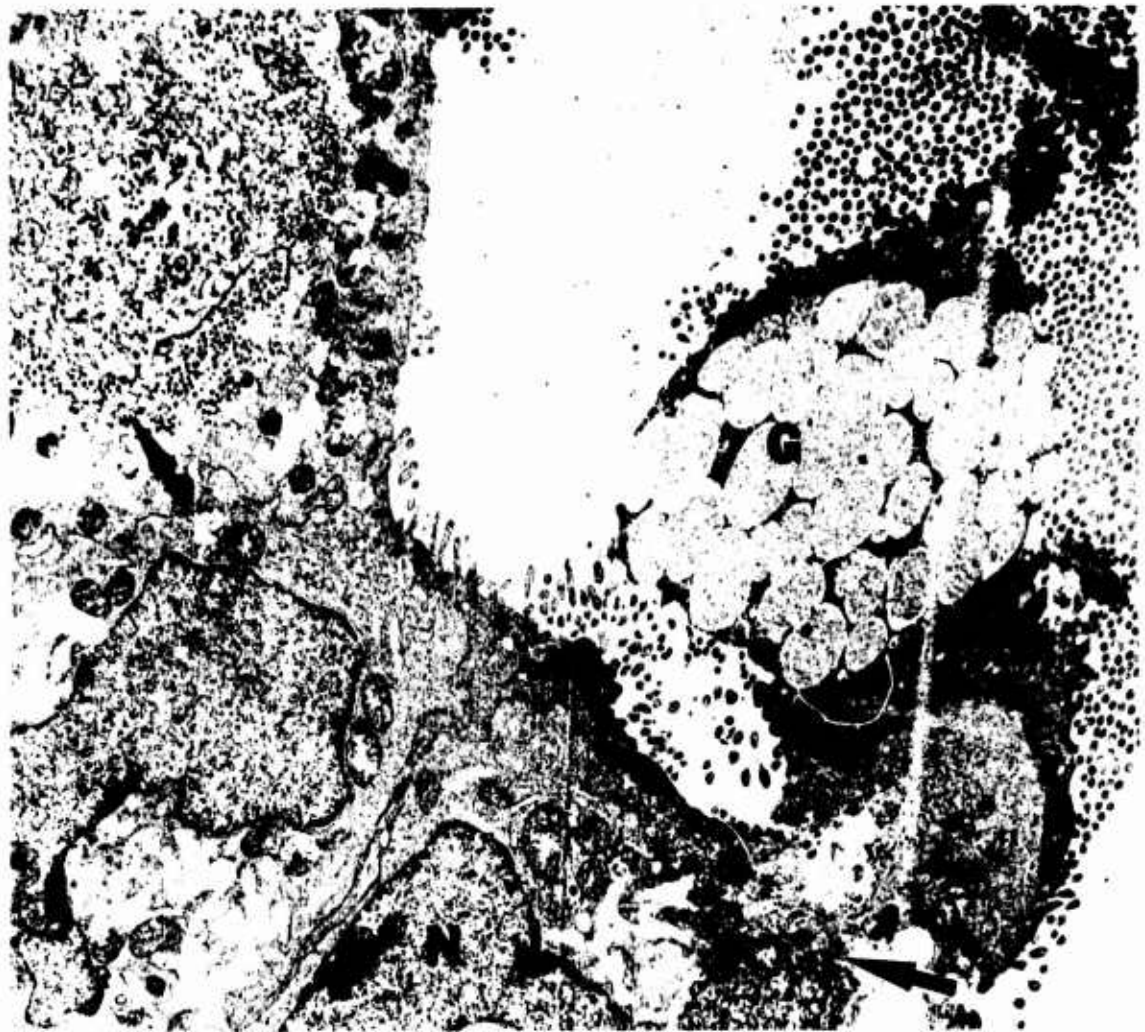


Fig. 12 - Mid-villus portion of infected ileal epithelium. Infected columnar cells together with goblet cell (G) are in process of shedding into lumen. Aggregates of virions are present in the cytoplasm (arrows). The nuclei (N) of these infected cells are free of virions, as confirmed when observed at a higher magnification. X5,800.

COMMENTS

The present observations have demonstrated viral replication of mouse adenovirus K87 in the epithelial cells of the small gut mucosa. By FA technique, the specifically labeled fluorescent viral antigen has been clearly localized within the intestinal epithelial layer of both villus and crypt, and LM has identified it with nuclear inclusions of cells in the epithelium. Since the epithelium of the small bowel of the normal mouse consists of four kinds of epithelial cells, that is columnar (absorptive), goblet, Paneth and argentaffine cells, and includes also migrating cells of mesenchymal origin (Takeuchi & Jervis et al. 1969) such as lymphocytes, neutrophilic leukocytes and globular leukocytes, it has not been possible to determine by FA and LM the specific cell type in which the adenovirus replicates.

The present EM evidence has ruled out the possibility of mesenchymal cells as the replication site of the K87 viral antigen and has established without a doubt that K87 adenovirus replicates only in columnar, goblet and Paneth cells. Adenovirus K87, therefore, demonstrates a specific tissue tropism, as virions are absent from other intraepithelial cells such as argentaffine cells and lymphocytes. Characteristically, the columnar, goblet and Paneth cells are the only cells that face the gut lumen with a microvillus surface. It is, therefore, possible that specific receptor sites such as those shown in cultured cells for other adenoviruses (Dales, 1973, Longberg-Holm & Philipson, 1969, Philipson & Longberg-Holm et al. 1968), might be present on the microvilli. The adenovirus K87 might absorb to such a receptor site and penetrate the cells through the microvilli. The demonstration of such penetration, however, has not been possible during the present in vivo study.

A tissue tropism similar to that seen in the mouse enteric adenovirus K87 has been reported by Clemmer in the small bowel of the chicken infected orally with adenovirus strain 93 (Clemmer 1965). In their FA and LM observations, Clemmer and Ichinose have found that both the viral antigen and nuclear inclusions appear only in the villus epithelium but are totally absent in the crypts (Clemmer & Ichinose, 1968). They have stated that, in their in vitro system, the viral antigen Strain 93, is first revealed between 8 and 10 hrs after infection (Clemmer & Ichinose, 1968). Since in the normal chicken the intestinal epithelial cells migrate from the crypts to the villus in 8 hrs, they have concluded that adenovirus 93 probably infects the epithelial cells in the crypts and replicates during their migration into the villi where it becomes visible. In this context it must be remembered that chickens lack Paneth cells (Windisch, 1966).

As in the chicken, the distribution of virus infected cells in the mouse ileum, may well be explained on the basis of intestinal cell kinetics. The columnar cells of the epithelium of the small intestine of the mouse, undergo continuous renewal and rapid migration out of the crypts (Merzel & Leblond 1969, Thrasher & Greulich 1966)

as do, if albeit more slowly, the mucus secreting goblet cells (Cairnie 1970, Merzel & Leblond 1969). In contrast, the Paneth cells are renewed at a much slower rate and do not migrate out of the crypts (Cairnie 1970, Cheng & Merzel et al. 1969). It is conceivable that, in the mouse adenovirus K87 infection reported here, all cells may be infected in the crypts, but that, in the time necessary for viral replication and visualization, the columnar and most goblet cells would have migrated out of the crypts and onto the villi, while the Paneth cells, which remain in the crypts and whose turnover is much slower, would allow replication to occur in the crypts to such an extent as to be easily visualized.

In the normal mucosa, epithelial and goblet cells are not extruded until they reach the tip of the villi, and the structural integrity of the gut epithelium is maintained by characteristic interdigitation and invagination of the lateral plasmalemma of epithelial cells which are tightly connected through intercellular junctional complexes. During the adenovirus K87 infection, extrusion of infected columnar and goblet cells may occur at the mid-villus level while an accelerated extrusion of Paneth cells has been observed in the crypts. The premature shedding of adenovirus infected cells, we believe, represents a morphologic expression of adenovirus induced cytopathic effect (CPE). A similar excessive loss of villus epithelial cells is commonly associated with infections by pathogenic organisms such as *Shigellae* (Takeuchi & Formal et al. 1968, Takeuchi & Sprinz et al. 1967), *Salmonellae* (Takeuchi & Sprinz, 1967), and *Entamoeba histolytica* (Takeuchi & Phillips, 1975). It has been suggested that under these conditions, accelerated shedding of epithelial cells results from failure of intercellular junctions which may be related to loss of calcium due to changes in calcium metabolism (Takeuchi & Phillips, 1975). It may be possible that a similar mechanism is at work in the present virus infection.

Intracytoplasmic membrane-enclosed vacuoles bearing viral particles resemble the phagocytic inclusions containing adenovirus 7, described by Chardonnet and Dales in HeLa cells infected with that agent (Chardonnet & Dales, 1970). They have shown that viral particles, after penetrating the cell membrane, are sequestered in membrane-bound phagocytic vacuoles which contain lysosomal enzymes. The virus-containing vacuoles found in our material, bear also a close resemblance to the autophagic vacuoles or phagolysosomes, containing pathogenic bacteria which are found in intestinal epithelial cells (Takeuchi & Sprinz et al. 1965).

In contrast to the columnar cells, the cytoplasm of the secretory goblet and Paneth cells appear unaltered even when infected with virus. Under normal conditions, in both goblet and Paneth cells, granular contents are synthesized in the endoplasmic reticulum and packaged in the Golgi complex where they accumulate and develop into membrane-bound mature granules. Synthesis and

secretion continue in the infected cells, indicating that they are capable of functioning in spite of the presence of large numbers of virions.

In neither columnar nor secretory epithelial cells, we have seen those other early changes observed in adenovirus-infected cell cultures, such as intranuclear fibers (Kalnius & Stich et al. 1966), and paracrystalline formations (Henry & Slifkin et al. 1971). These morphologic differences between in vivo and in vitro response to viral infection may be due to difficulty of sampling, but may be also related to differences in the strain of adenovirus or to host cellular factors - in this case, the biological nature of intestinal epithelial cells or perhaps the specific environment conditions in the gut (Hashimoto & Onishi et al. 1973).

Nor has cell necrosis been observed even in the prematurely shedding cells of the gut, such as has been reported by Margolis and colleagues (Hoenig & Margolis et al. 1974, Margolis & Kilham et al. 1974) in their study on the interaction between host cells and the adeno-tropic mouse adenovirus. Multiplication of this adenovirus produces severe degeneration and necrosis of the infected adrenal cortical cells and an acute inflammatory response; the latter, possibly, secondary to cell necrosis. It is possible that the premature extrusion of the mouse intestinal epithelial cells infected with K87 adenovirus may prevent the severe degeneration and death seen in the adeno-tropic adenovirus infection, and therefore may also be responsible for the lack of inflammation noted in the viral enteric infection.

In conclusion, the present FA, LM and EM in vivo studies of adenovirus K87 in the mouse have provided new information on several aspects of the interaction between virus and host cells as yet undisclosed by in vitro observations.

B. Studies of Enteric Infection of Neonatal Dogs with Canine Coronavirus 1-71

The coronavirus-host cell interaction has been well studied in in vitro systems (Becker & McIntosh et al. 1967). Yet, comparatively little is known about coronavirus-host cell interaction in vivo, and especially about intracellular sites of replication and cytopathic effects (CPE) of coronavirus in the small intestine (Pensaert & Haelterman et al. 1970).

A coronavirus, designated coronavirus 1-71, was recovered by Binn and his colleagues from US military dogs during an epizootic of diarrheal disease in Germany (Binn & Lazar et al. 1975). Neonatal dogs, when challenged orally with this coronavirus, developed an acute diarrhea. Keenan et al. have reported clinical, histologic, histochemical, and immunofluorescent findings in this experimental infection (Keenan & Jervis et al. 1976). At the peak of infection (4 days post-infection) mucosal changes were most striking in the ileum where the viral antigen was easily identified in the epithelial lining. This made feasible ultrastructural studies on canine coronavirus-host cell interactions in vivo.

Using this experimental model, we have studied by electron microscopy changes in the ileal mucosa. In particular we have defined the intracellular site of virus replication and described the CPE in the intestinal epithelial cells during such replication. The results are discussed in comparison with other enteric viral infections.

MATERIALS AND METHODS

The tissue utilized in this study was obtained from pups born of bitches free of canine coronavirus 1-71 antibodies. These were some of the same pups (Litter 1700) whose course of infection and histologic findings have been described in detail previously (See Annual Report, 1974-75, Department of Veterinary Microbiology).

Viral inocula were prepared in primary dog kidney cell cultures maintained in serum-free medium 199, the 50% tissue infective dose (TCID₅₀) being determined by standard methods (Binn & Lazar et al. 1975).

Three pups were challenged orally with a 3×10^4 TCID₅₀ dose when 4 day-old; one control pup was killed with phenobarbital on day 0 just prior to challenge of the infected pups, one infected pup was similarly killed on day 2 post-infection (PI) and the remaining two were killed on day 4 PI, since histologic and immunofluorescent antibody (IFA) observations suggested that these stages might show virions penetration and the most severe histologic changes (Keenan & Jervis et al. 1976). A small sample of the terminal ileum adjacent to those used for histologic, histochemical and IFA studies was collected at necropsy for electron microscopy. These tissues were fixed and processed by EM techniques which have been described in detail elsewhere (Takeuchi & Jervis et al. 1969, Takeuchi &

Zeller, 1972). Sections 2 μm thick for Epon embedded tissues were stained with toluidine blue-pyronin Y for light microscopy.

RESULTS

LIGHT MICROSCOPY

The histologic appearance, based on the study of paraffin sections, of noninoculated control and of 1-71 canine coronavirus inoculated pups, has been described in detail elsewhere (Keenan & Jervis et al. 1976). In brief, at 2 days PI the mucosa of the inoculated pup appeared normal, although IFA showed the presence of antigen in some cells of the villous epithelium. At 4 days PI, it showed distinct structural differences from that of the neonatal control, such as a shortening, blunting, and fusion of the villi and lengthening of the crypts, with a marked increase in the cellularity of the lamina propria.

Although the epithelial cells of the crypts appeared normal, except for a higher than normal rate of regeneration, the epithelial cells on the villi were flattened and occasionally vacuolated due to the presence of fat inclusions. IFA showed the presence of antigen in increasing amounts from the base to the tips of the villi in practically all the epithelial cells on the villi. In this report will be described only the LM observations, made on 2 μm thick sections of Epon embedded tissue that added significantly to the information obtained from paraffin sections. At magnifications below $\times 1000$ the appearance of the ileal mucosa in Epon sections was comparable to that seen in paraffin sections, but at higher magnifications the details of the ileal mucosa were better resolved. When compared to those in the noninfected ileum, (Fig. 13) the epithelial cells of the villi of the ileum in the infected animals at 2 days PI, showed only occasional, abnormal lipid inclusions, while at 4 days PI they were all irregular, not as tall, with disrupted brush border. They contained also numerous vacuoles and lipid droplets which increased in number and size proceeding toward the apex of the villi. These droplets were spherical or oval and occasionally occupied most of the epithelial cell cytoplasm (Fig. 14). In occasional epithelial cells, the brush border was particularly attenuated and the cytoplasm showed a distinct abnormal vacuolation. Mucus secreting goblet cells were completely discharged. In contrast the epithelial cells lining the crypts appeared no different from those seen in the control pup mucosa. The lamina propria contained increased numbers of lymphocytes, macrophages, and focally, a number of polymorphonuclear (PMN) leukocytes. The blood vessels and lacteals were unremarkable.

ELECTRON MICROSCOPY (EM)

Due to the sporadic incidence and slight character of the pathologic changes at day 2 PI, and to the difficulty of recognizing virions when present in very small numbers, EM observations reported



Fig. 13 - Epithelium of ileal villus in non-infected neonatal dog. The enterocytes are uniformly tall and columnar with a regular brush border. The cytoplasm shows apical vacuolation, and fine inclusions. Goblet cells (Gc) are interspersed between enterocytes. Cp, Capillary. Epon section, stained with toluidine blue-pyronin Y; x1,600.



Fig. 14 - Epithelium of infected ileal villus of neonatal dog, 4 days after oral challenge with coronavirus 1-71. The enterocytes are low columnar or cuboidal with a shortened brush border. The cytoplasm contains clear vacuoles and numerous lipid droplets. Epon section stained with toluidine blue-pyronin Y; x1,600.

here were confined to tissues obtained from pups killed on day 4 PI. They confirmed the LM observations described above and revealed further details at the ultrastructural level. The fine structure of the ileum of the noninfected pup was similar to that already described in detail in other neonatal mammals (Stair & Mebus et al. 1973, Staley & Jones et al. 1968). Briefly, the epithelial cells of the villi consisted of absorptive cells (enterocytes) and of goblet cells, while those in the crypts included undifferentiated columnar cells and a small number of goblet and argentaffine cells, but no Paneth cells which are lacking in the dog (Trier, 1966). In the enterocytes of the villi, the microvilli were regular and tall and the glycocalyx poorly developed, while the apical cytoplasm contained an elaborate system of tubules and vacuoles (Fig. 15).

At 4 days PI, the infected ileum, virions were identified only in the enterocytes of mid and apical villus. Virions were not seen in goblet cells or in the crypt epithelium. Virus particles were found in the intestinal lumen, topographically close or attached to altered microvilli of the enterocytes (Fig. 16), or inside the apical pits formed by the invaginated microvillous membranes. Within the cytoplasm of the infected cells, virions were found most frequently singly or in groups, enclosed in vesicles or in smooth membranes vacuoles of various size and shape (Fig. 17). Virions were also observed in cisternae of the Golgi apparatus (Fig. 18); some appeared to be in cisternae of the rough endoplasmic reticulum (ER) and more rarely, in the dilated perinuclear space (Fig. 19). Few virions were free in the cytoplasm (Fig. 18), and none in the nucleoplasm.

Viral particles were mostly circular or, much less frequently, ellipsoidal in profile (Fig. 17). The former averaged 80 nm in diameter and the latter were 75 to 80 nm wide and 180 to 200 nm long. Both forms consisted of an inner core and of an outer envelope formed by two single membranes, each 3-4 nm thick, separated by an electron lucent zone 4-5 nm thick. The inner core consisted of a central vesicular matrix surrounded by a peripheral electron dense zone 10-20 nm thick which was often closely apposed to the inner leaflet of the outer envelope.

Various stages of virus replication were observed in few favorable sections, in association with vesicular membranes (Fig. 20). The earliest event in viral replication appeared to be the formation of crescents from modified portions of the vesicular membrane, which invaginated toward the interior of the vesicles. At the same time a dense layer developed, topographically close to the cytoplasmic aspect of the crescent membrane. This dense layer was approximately 10-20 nm thick and was separated from the outer leaflet of the vesicular membrane by a space 4-8 nm wide. As the crescent of the vesicular membrane, together with underlying dense layer continued to invaginate toward the vesicular space, its edges appeared to pull together. Thus, the underlying dense layer came to be enclosed by the membrane and was transformed into the inner core of the virion. In few instances, it was possible to identify invaginations, already

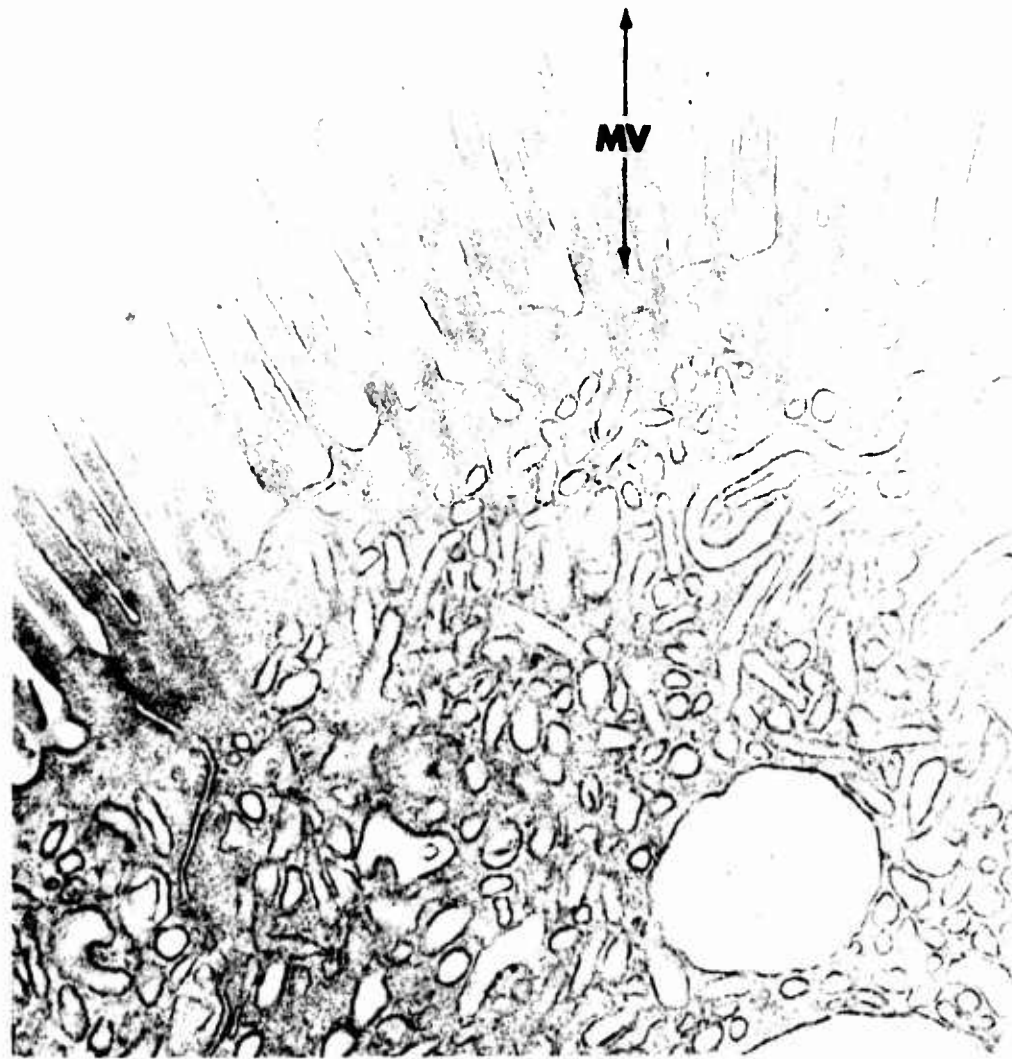


Fig. 15 - Luminal aspect of enterocyte near apex of ileal villus in non-infected neonatal dog. The microvilli are slender, regular and tall. The glycocalyx, attached to the microvillous membrane, is underdeveloped. The plasmalemma between the microvilli invaginates. Note numerous tubules and vacuoles in the apical cytoplasm. x75,000.

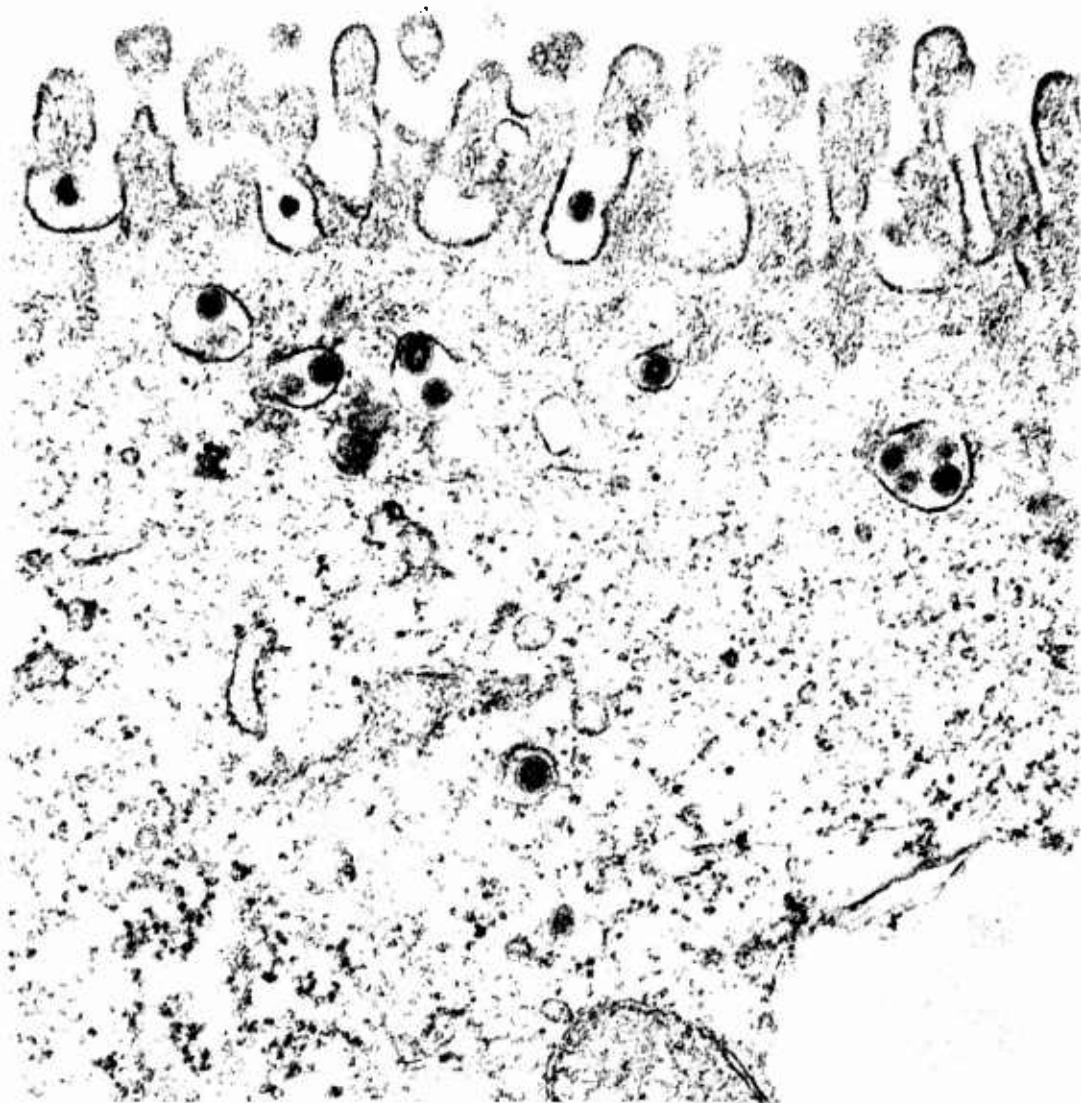


Fig. 16 - Luminal aspect of infected enterocyte near apex of villus of neonatal dog, 4 days after challenge. Microvilli are shorter than in the control, irregular, and blunt. Many of the tubules and vacuoles present in the control have disappeared. In this obliquely cut section, virions are present between microvilli, and enclosed in membrane-bound vesicles in the apical cytoplasm. x52,000.



Fig. 17- Virion containing vacuoles in cytoplasm of infected enterocyte, ileal mid-villus. Note the differences in size and shape of viral particles. M, mitochondria; L, lipid particles. x62,000.



Fig. 18 - Golgi region of infected enterocyte, ileal mid-villus. A virion is enclosed within a Golgi cisterna (thin arrow) and others appear to lie free in the cytoplasm (arrowheads). A vacuole (thick arrow) containing a number of virions is adjacent to a lipid droplet (L). M, mitochondria. x48,000.



Fig. 19- Portion of infected enterocyte, ileal mid-villus.
Virions are present in the dilated perinuclear space.
N, nucleus. x84,000.

rounded, attached to the vesicular membrane only through a short stalk (Fig.20), or in the process of pinching off into the vesicular lumen. From these findings we have concluded that this process represents the budding of the virion. This budding process was never found on the luminal, lateral or basal aspects of the plasmalemma nor in the Golgi and ER cisternae and in the perinuclear spaces of the epithelial cells.

The infected cells showed variety of cytopathic effects (CPE). In general, their cytoplasmic matrix was much paler than that of the noninfected cells. Alterations of the microvilli were common and included shortening and blunting (Fig.16). In some instances they seemed fused together, while in others they were reduced to simple wavelike projections of the plasma membrane. The glycocalyx became indistinct. In the cytoplasm there were numerous, homogeneously electron dense, lipid droplets which ranged from 20 to 1000 nm in diameter and were enveloped by single membranes (Fig. 16-18). Similar, but mostly single, lipid droplets were seen occasionally also in the nucleoplasm of very severely infected cells; otherwise the nuclei were uncringed.

Most of the mitochondria were transformed from their original rod and tubular shapes to rounded or oval shapes. They showed a condensed, electron opaque matrix with deranged cristae and distinct intercrystal spaces. The rough ER exhibited a reduced number of ribosomes and dilated cisternae. The cisternae of the Golgi apparatus and of the ER and especially the perinuclear space (Fig.19) were also dilated. Phagosomes and multilaminar membranes were present in some infected cells.

In addition, two different characteristic structures were identified in the cytoplasm of the infected cells; these were dense filamentous structures and membrane-bound bodies. The first consisted of dense material sequestered by filaments arranged in reticular fashion and forming aggregates which varied in size from cell to cell (Fig.21); they were found in the vicinity of vesicles containing virions, or even in the absence of virions.

The second structures, characterized as membrane-bound bodies, consisted of portions of cytoplasmic matrix enveloped by a system of membrane-enclosed cisternae, which appeared to develop around them, and so isolating them from the surrounding cytoplasm (Fig.17). The membrane-bound bodies ranged from 70 to 200 nm in diameter and were usually seen in the vicinity of viral particles. In certain sections, they appeared to be completely disconnected from the surrounding cytoplasm and looked like round or oval cytoplasmic bodies separated from the cytoplasm by a system of cisternae (Fig. 22). The cisternae often contained finely granular material or else could be obliterated by fusion of their two membranes. Neither filamentous or membrane-bound structures appeared to contain virions.

Some heavily infected cells were apparently in process of detaching from the adjacent epithelial cells and shedding into the lumen (Fig.22). In other cells, were seen ballooning of the micro-

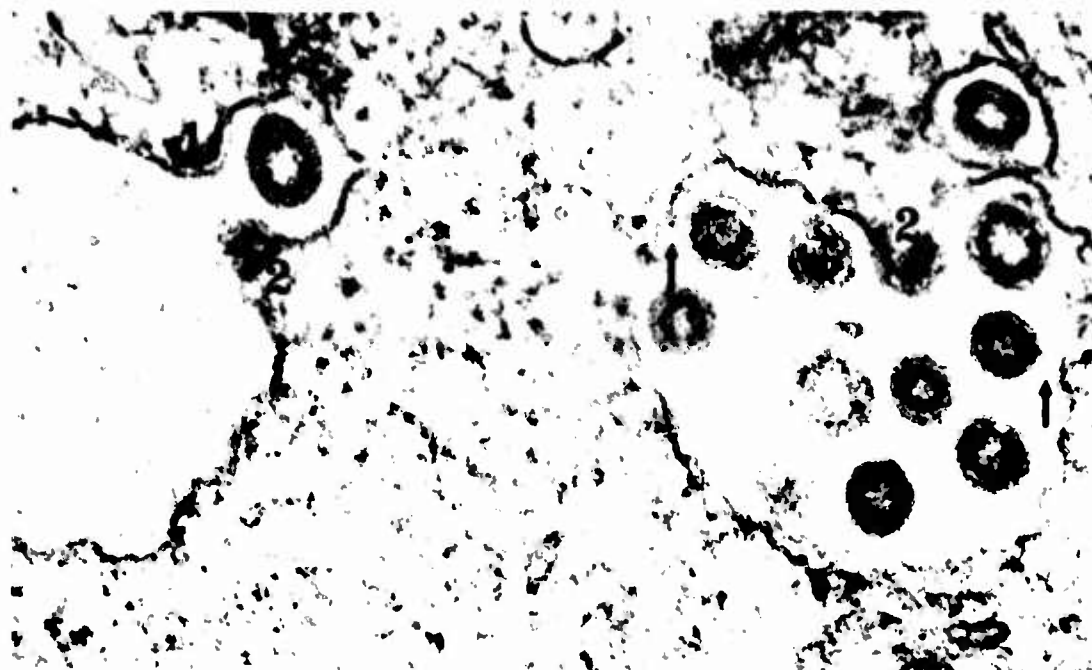


Fig. 20- Viral replication by budding on membranes of dilated vesicles, infected enterocyte; 1, portion of membrane, lined on its outer aspect by a dense layer, begins to invaginate in the form of a crescent; 2, the crescent further invaginates inward and the dense layer closes forming a thick walled ring. Incompletely formed virions seem to be connected to the vesicular membrane with thin stalks (arrows). x98,000.

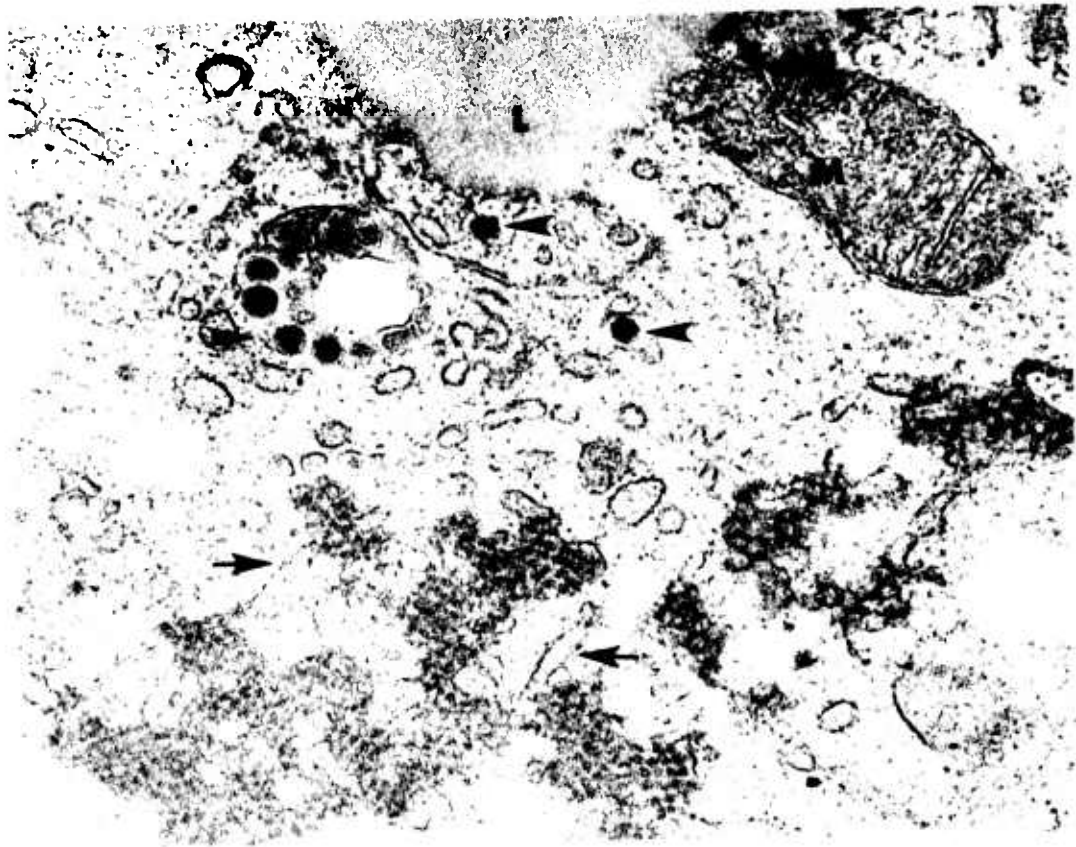


Fig. 21 - Portion of infected enterocyte, ileal mid-villus. A dense filamentous structure is closely associated with cisternae of the smooth ER (arrows). Arrowheads indicate virions. L, lipid droplets; M, mitochondrion. x46,000.

villi, and bleb formation of the apical cytoplasm toward the lumen (Fig. 23). In these cells the plasma membrane might be disrupted resulting in release of the cytoplasmic content, including free virions, virions containing vesicles, and lipids, into the intestinal lumen (Fig. 24).

DISCUSSION

The present EM study has established that the intraepithelial coronavirus 1-71 is localized within the enterocytes of the small intestine of the neonatal dog. This observation correlates well with those based on IFA, reported previously by Keenan *et al.* (Keenan & Jervis *et al.* 1976). IFA findings have indicated that specifically fluorescing coronavirus antigen is present in the epithelium of the upper two thirds of the villi. The EM evidence has ruled out definitely the possibility that the coronavirus might also be found in the goblet cells which are interspersed with the enterocytes in the villus epithelium.

In the enterocytes, the coronavirus 1-71 appears to replicate only by budding of smooth membranes of cytoplasmic vesicles and vacuoles. This observation confirms those made on dog embryo cell cultures infected with the same 1071 coronavirus used in this study (Binn & Lazar *et al.* 1975) and also bears a close resemblance to those described during *in vitro* studies of other coronaviruses (Becker & McIntosh *et al.* 1976, David-Ferreira & Manaker, 1965, Oshiro & Schieble *et al.* 1971). In an EM study of the small bowel loops of swine infected with TGE virus Pensaert *et al.* (Pensaert & Haelterman *et al.* 1970) have suggested that the TGE virus replicates in the Golgi apparatus, smooth membrane-bound vesicles, and also in the microvillus membrane; in the latter case, replication would be accompanied by direct viral release into the intestinal lumen. In the present EM study of a canine 1-71 coronavirus infection, although we have seen virions in close association with the microvilli, the cisternae of Golgi apparatus, and in the perinuclear space, we have not identified budding in any host membranes other than the smooth membranes of cytoplasmic vesicles and vacuoles.

The coronavirus particles appear to penetrate the enterocytes from the luminal surface, at the base of the microvilli, where they seem to be enclosed in the tubular invaginations of the surface membrane. These virion-containing tubules or vacuoles, in the terminal web region, are strikingly similar to the virion-containing tubules seen in the enterocytes of neonatal rats ileal loops infected with an adenovirus (Worthington & Bonnie *et al.* 1973) and of neonatal pig jejunal loops infected with TGE virus (Pensaert & Haelterman *et al.* 1970). In fact Pensaert *et al.* (Pensaert & Haelterman *et al.* 1970) and Wagner *et al.* (Wagner & Beamer *et al.* 1973) have suggested that the TGE virions are taken up by pinocytosis and then transported inward into the epithelial cytoplasm. Unlike the enterocytes of the adult intestine, their neonatal counterparts, are capable of absorbing large amounts of macromolecular substances (Kraehenbuhl & Campiche, 1969); it is conceivable,

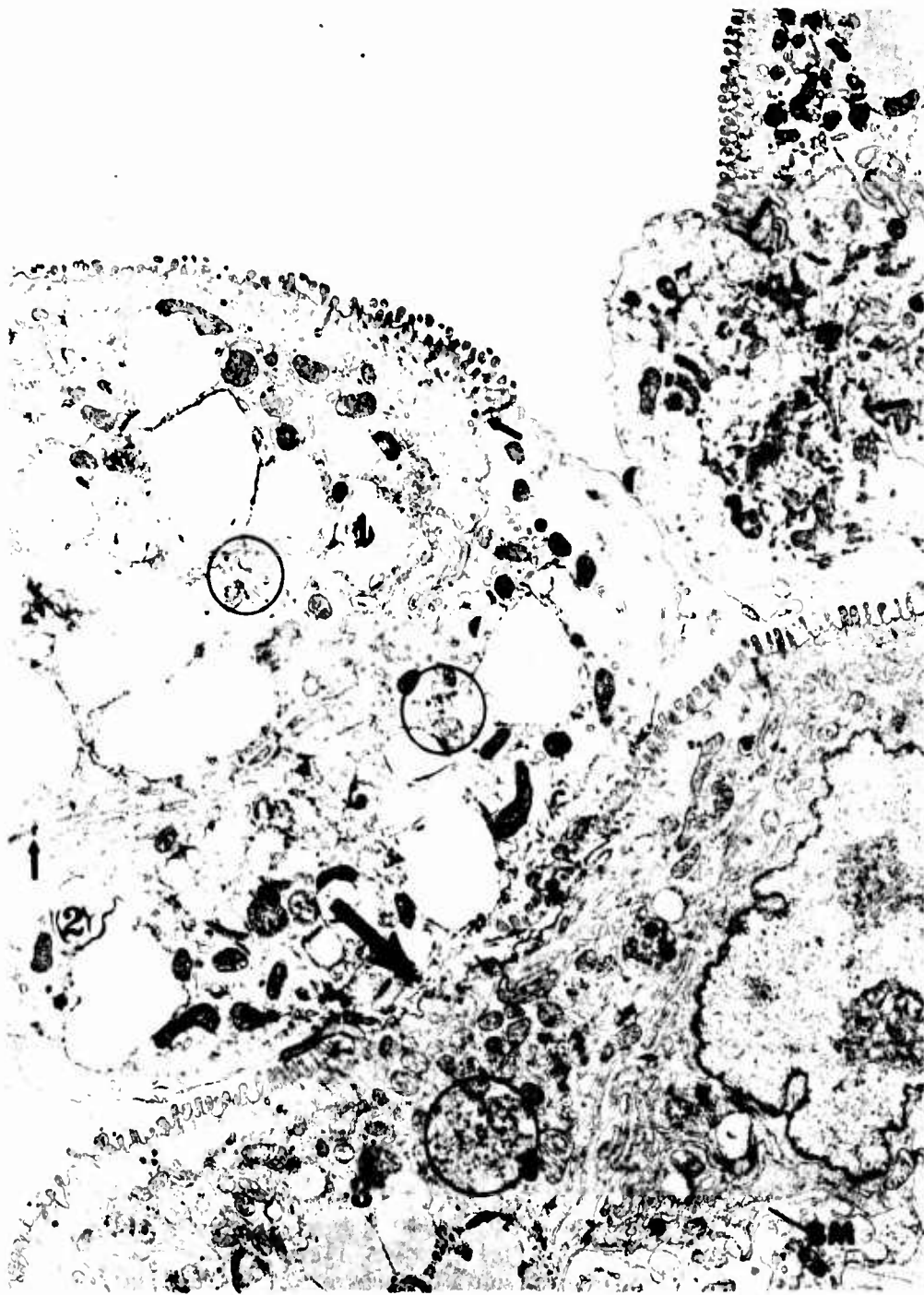


Fig. 22 - Infected enterocytes in process of shedding from ileal epithelium at mid-villus. Two infected cells (1 and 2) are still connected by the interdigitating lateral plasmalemma and the cell junctions (thin arrows). Cell 2 is almost completely detached from cell 3 except at point marked by thick arrow. Cell 4, for the most part, has been extruded in the lumen but is still connected to cell 5 through the lateral plasmalemma and the cell junction (thin arrows). Note the numerous virions (in circles) and the severe CPE in the enterocytes. BM, basal lamina. x81,000.

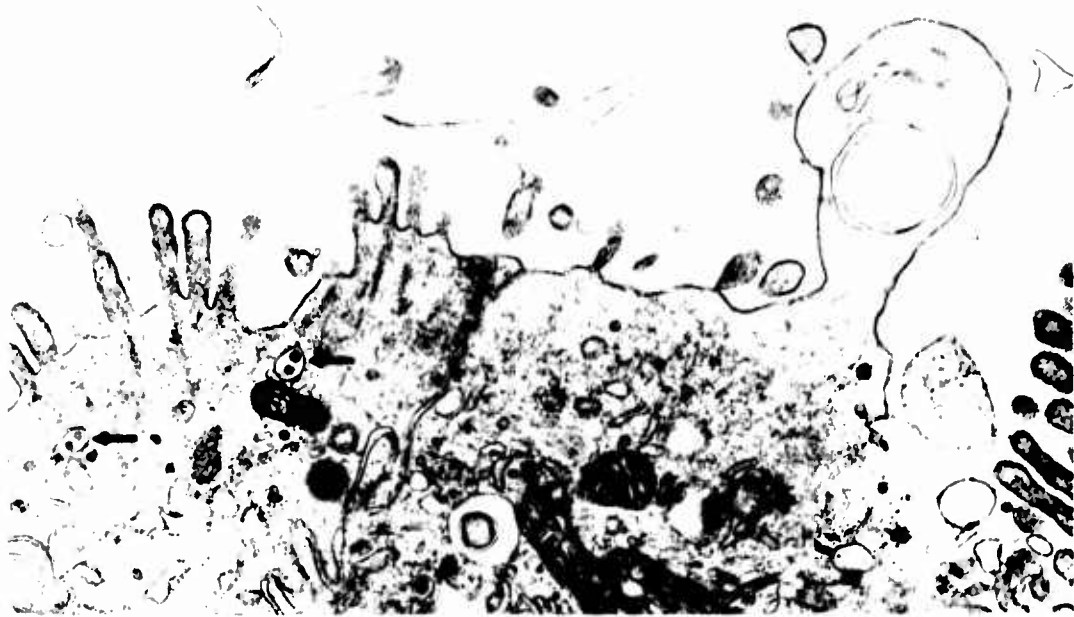


Fig. 23 - Luminal aspect of infected enterocyte, ileal mid-villus. Microvilli are irregular in height and width, some have disappeared and others show ballooning. To the right, the apical cytoplasm shows bleb formation which precedes reapture of the luminal plasmalemma. Arrows indicate virion-containing vesicles. x14,200.



Fig. 24 - Heavily infected enterocyte from ileal mid-villus showing release of cyto-components into gut lumen. Cytoplasmic content, including large lipid droplet (L) and numerous free, as well as membrane enclosed virions are discharging into the lumen. Note difference of cytoplasmic density between the severely infected cell and the adjacent cell on the right, which is less severely affected. Arrow indicates portion of the disrupted luminal plasmalemma. x38,000.

therefore, that penetration of the coronavirus might be facilitated by the physico-chemical character of the absorptive cells in this crucial neonatal period as suggested by Wagner et al. (Wagner & Beamer et al. 1973).

In accord with this surmise, it has been found that adult animals are less severely affected by coronavirus infections. Adult pigs, infected with TGE, develop a milder and more transient form of the disease than the neonatal pigs (Wagner & Beamer et al. 1973). Likewise, the dams of the coronavirus infected pups used in this study remained asymptomatic although they developed humoral antibody to the coronavirus (Keenan & Jervis, et al. 1976). The fact that the coronavirus 1-71 was first isolated from adult German Shepherd dogs during a diarrheal epizootic (Binn & Lazar et al. 1975), may not contradict the above findings, since German Shepherd dogs are known to be more susceptible to certain diseases than other breeds (Hildebrandt & Huxsoll et al. 1973). Resistance in the adult animal may be also closely related to the increased competence of its immune system which may trigger a more rapid and effective production of intraluminal antibodies than in the young.

The CPE revealed by EM, correlate very closely with the histochemical findings reported previously (Keenan & Jervis et al. 1976). The decreased alkaline phosphatase activity in the brush border is but an expression of the disruption of the microvilli, while the loss of acid phosphatase activity in the apical cytoplasm may reflect the disappearance of the network of tubules and vacuoles so prominent in the normal neonatal dog. Similarly the decrease in the activity of marker enzymes for the ER and mitochondria, glucose-6-phosphatase and NAD diaphorase, respectively, reflect the CPE in these organelles.

The formation of lipid droplets in the cytoplasm is one of the CPE seen in the enterocytes. Lipid droplets are easily identified even at the LM level and appear to increase in size and number as the number of intracellular virions increases. Similar intracellular lipid droplets have been reported in the enterocytes of the small and large intestines of various animals, under different pathologic conditions, including acute infections with virus (Adams & Kraft, 1967), bacteria (Takeuchi & Formal et al. 1968), protozoa (Takeuchi & Phillips, 1975) and noninfectious lesions (Jervis & Donati et al. 1969, Jervis & Sheahan et al. 1972). Intraepithelial lipid droplets, therefore, cannot be considered specific for viral infections, but rather a result of non-specific acute response of enterocytes to stimuli. In fact it has been suggested that cytoplasmic lipid droplets may be a morphologic expression of intracellular disturbances in fat metabolism or of abnormal intracellular lipid transport (Takeuchi & Formal et al. 1968). Similarly, other CPE such as alterations of microvilli and mitochondria, and dilatation of ER have been commonly seen in enterocytes of the small intestine during the course of a variety of diseases of the gut (Takeuchi & Formal et al. 1968, Takeuchi & Sprinz, 1967). Thus, these changes again represent non-specific alterations of the enterocytes produced by injurious agents.

In contrast, two other structures in the cytoplasm of the infected cells, identified as filamentous structures and membrane-bound bodies, appear to be CPE characteristic of a viral infection, if not specifically of a coronavirus infection. In fact, the filamentous structures, illustrated in Fig. 9, resemble the "reticular inclusions" described by David-Ferreira and Manaker (David-Ferreira, Manaker 1965) in mouse liver-derived cultured cells infected with a mouse hepatitis virus. These investigators have raised the possibility that the reticular inclusions might be the site of replication of the viral nucleoprotein. On the other hand, the membrane-bound structures illustrated in Fig. 10, are very similar to the bodies enclosed in "membrane-bounded channels" illustrated in Fig. 2 of the study by Dales *et al.* (Dales & Eggers *et al.* 1965) on the development of poliovirus in HeLa cells; poliovirus has been frequently found within these bodies. In this *in vivo* study, however, it has not been possible to find viral particles within either the filamentous or the membrane-bound structures and to relate them to viral replication.

The present EM observations have provided additional information on the mode of viral release from host cells into the intestinal lumen. There seem to be two processes involved; one is accelerated cell desquamation of infected cells from the villus epithelial lining as illustrated in Fig. 11, the other is the disruption of the luminal plasma membrane with discharge of the cytoplasmic contents as illustrated in Fig. 13. In the former, shedding of infected cells into the lumen appears to be followed by the breakage of their cell membrane with subsequent release of virions. Shedding of infected cells is commonly seen in the small intestine infected with adenovirus (Takeuchi & Hashimoto, *in press*), TGE virus (Pensaert & Haelterman *et al.* 1970), and reovirus (Stair & Mebus *et al.* 1973). In the case of direct viral discharge into the lumen, virion-containing vesicles and vacuoles as well as free virions, are expelled through the disrupted luminal plasma membrane of the host cell, as well illustrated by Adams and Kraft (Adams & Kraft, 1967) and also described by Wagner *et al.* (Wagner & Beamer *et al.* 1973). It is, therefore, evident that the coronavirus 1-71 shares with other enteric viruses similar release mechanisms from the epithelial cells into the lumen.

CONCLUSION & RECOMMENDATION

In both adenovirus and coronavirus infections of the gut in experimental animals, the FA, LM and EM studies have provided new information on several important aspects of the interaction between virus and host cell as yet undisclosed *in vitro* observations.

Further studies on the interaction between these enteroviruses and host cells *in vivo* model by FA and EM techniques should provide the precise mechanism of penetration of the gut epithelial cell by viral agents and also information immune response of the gut mucosa to these enteroviruses.

IV. IMMUNE RESPONSE OF THE GUTS

A. Studies on the Effects of Shigella Immunization

Numerous studies, confined principally to immunologic observations, have been made regarding the effects of parenteral or oral immunization against dysentery with a live or killed shigella vaccine. In collaboration with the Department of Applied Immunology, histologic and immunopathologic studies of responses of the gut mucosa to parenteral immunization with shigella bacilli are being carried out. Preliminary observations indicate that parenteral immunization fails to prevent invasion of the mucosa by orally administered shigellae, but appears to prevent clinical dysentery.

In order to clarify these important observations, we continue these studies by electron microscopy and immunofluorescent methods.

B. Studies of Immune Responses by Simple Antigens

In order to obtain fundamental information on the immune process in the gut, there has been established an experimental model in rabbits and guine pigs, based on parenteral immunization with simple antigens such as bovine serum album (BSA) or horseradish peroxidase (HRP). When an immune status is produced in these animals, they are given the same antigen in a gut loop. Preliminary results demonstrate that the acute immune reaction in the gut is morphologically similar with either protein or shigella immunization. These acute immune responses are characterized by severe edema, acute inflammation and hemorrhage of the bowel at the early stages (Fig. 25-29) and at a later stage by severe structural changes of the gut mucosa including necrosis and severe inflammatory exudation into the lumen (Fig. 31-34).

New studies of the gut with oral immunization have begun in order to compare effects of parenteral immunization with those of oral immunization. Further studies by electron microscopy and immunopathology may clarify the basic mechanisms of parenteral and oral immunization and contribute to the development of successful methods of immunization against enteric pathogens.



CONTROL



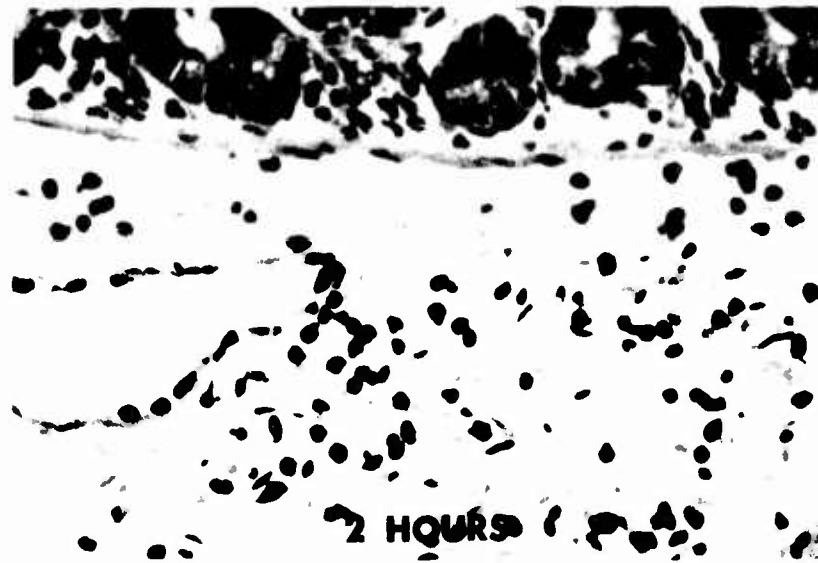
1 HOUR

Fig. 25 - Control ileal loop, rabbit parenterally hyper-immunized by horseradish peroxidase (HRP) as an antigen.

Fig. 26 - Experimental ileal loop, rabbit parenterally immunized by HRP at 1 hour after intraluminal administration of HRP. Edema is obvious at the submucosa, otherwise the bowel is unremarkable.



2 HOURS



2 HOURS

Fig. 27 - Ileal loop, parenterally immunized rabbit at 2 hours post intraluminal administration of HRP. The submucosa shows marked edema and clumps of cellular infiltrates. The lacteals, venules, capillaries and arterioles are dilated. Fine vacuolation is present in the circular muscle coat. Neutrophilic infiltration is obvious at the perivascular area of the submucosa in higher magnification. (Fig. 28)



4 HOURS



4 HOURS

Fig. 29 - Ileal loop, parenterally immunized rabbit at 4 hours post intraluminal inoculation of HRP. Crypt-villus ratio remains unaltered. The number of goblet cells is reduced. Hemorrhage is diffuse.

Fig. 30 - Higher magnification of venule in the submucosa shows attachment of numerous granulocytes to the endothelium.

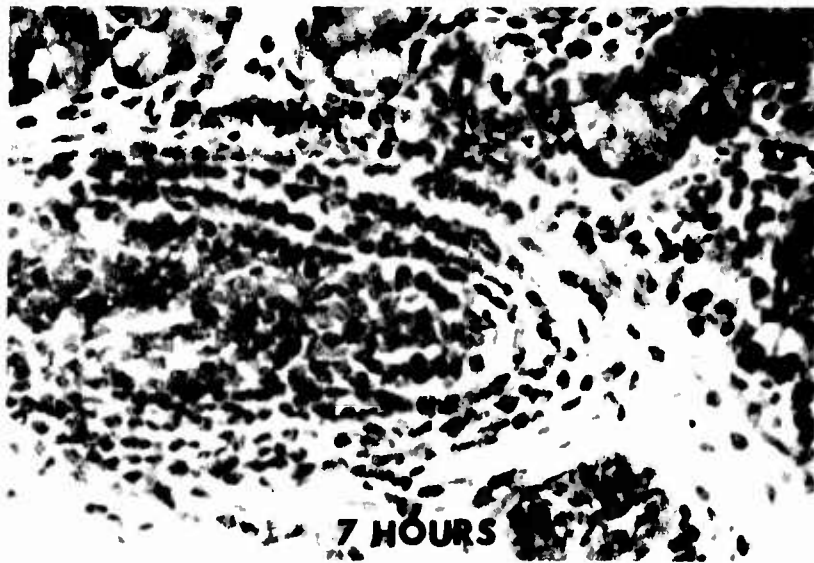


Fig. 31 - Ileal loop, parenterally immunized rabbit at 7 hours post intraluminal inoculation of HRP. Structural changes are evident in villi which are short and often disintegrated. Hemorrhage and cellular infiltrates are extensive involving the entire wall of the ileum. Submucosal vasculatures are heavily infiltrated by numerous granulocytes and RBC (Fig. 32)



17 HOURS

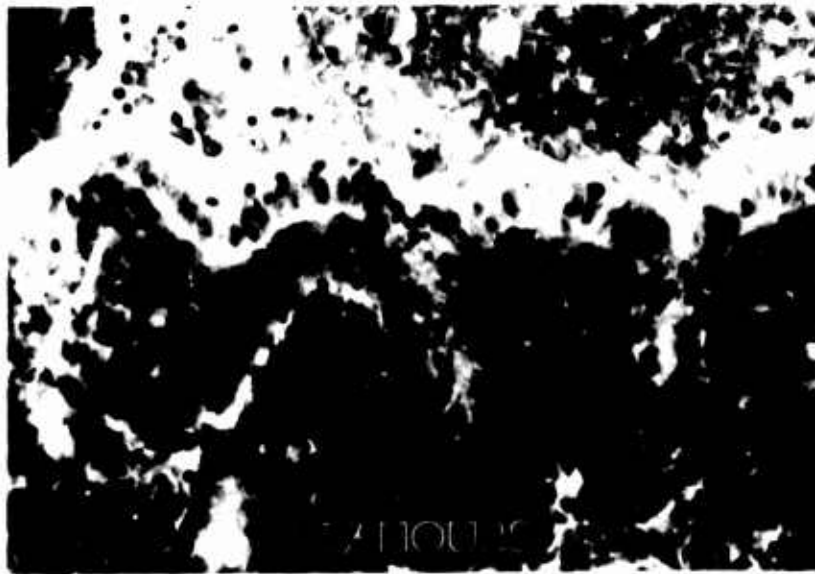


Fig. 33 - Ileal loop, parenterally immunized rabbit by HRP at 17 hours post inoculation by HRP. Structural changes are severe; the villi are flat, fused and show intensive necrosis. Inflammatory exudate containing numerous RBC and granulocytes is present in gut lumen, however, the epithelial lining remains intact (Fig. 34).

V. COLLABORATIVE STUDIES ON TRYPANOSOMA AND RICKETTSIAL INFECTIONS

A. Trypanosoma Infection

1. Ultrastructure of Trypanosoma Rotatorium.

Recent application of the freeze-etching technique to the study of biological specimens has added important information on the intricacies of various cell membranes and cytoplasmic organelles, in particular those of protozoa, not previously disclosed by other electron microscopic techniques (Henley, Lee and Takeuchi, 1975).

In search of new information on the ultrastructure of trypanosoma we have been studying Trypanosoma rotatorium by the freeze-etching technique (FE) of electron microscopy. In this work, we attempt to describe its ultrastructure as revealed by FE and to correlate it with observations made by the thin-sectioning technique (TS). Particularly, attention has been given to the complexity of the flagella attachment to the organism.

Freeze-etched surface replicas of T. rotatorium revealed the organism to be a rectangular profile 25μ to 30μ in length and 15μ to 20μ in diameter, with one of the longer sides appearing smooth and the parallel opposite appearing serrated. Higher magnification of the serrated side showed the continuity of the pellicle with that of the flagella (1μ in diameter) via the undulating membrane (336μ in diameter). The external pellicle is 0.336μ in diameter and is sparsely dotted with ribosomes. The flagella pocket was found to be an invagination of the pellicle with a "collar region" formed at the point where the pellicle invaginated into the cell's cytoplasm. Enclosed by the pellicle is a homogeneously lumpy cytoplasm. Numerous tubular and ovoid inclusions of unspecified origin are found in the cytoplasm. Near the anterior end of the cell are numerous lipid-like globes, 2.5μ to 3.5μ in diameter. The nucleus is sometimes lobed, sometimes elongated, depending on the plane of fracture, and is 6.5μ in diameter.

B. Rickettsial Infection

1. Studies on the Mode of Transmission and the Distribution of Rickettsia Tsutsugamushi in the Tissues of the Vector and the Host

In collaboration with the Department of Hazardous Microorganisms, DCD&I, WRAIR, we intend to clarify the mode of transmission of rickettsial organism between the vector chigger and the host mouse and to follow subsequent spread of the organism in the vector and host tissues by histologic and electron microscopic techniques.

We are currently establishing a satisfactory method of histologic and EM procedures for the vector chigger.

2. Studies on the Effects of Immunization of Mice with Rickettsiae Tsutsugamushi

Utilizing histology, immunofluorescent and electron microscopy, scrub typhus infection is being studied in immunized mice. (Details in Annual Report, 1975-76, Department of Hazardous Microorganisms)

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Disease
and Injuries

Literature Cited.

Publications:

1. Takeuchi, A., Jervis, H. R., Formal, S. B.: Animal Model of Human Disease. Human Shigella Dysentery Animal Model: Monkey Shigellosis. *Am. J. Pathol.* 81: 251-254, 1975.
2. Takeuchi, A.: Electron Microscopy Observations on Penetration of the Gut Epithelial Barrier by Salmonella typhimurium. *Am. Soc. Microbiol.* 44: 174-181, 1975.
3. Takeuchi, A. and Hashimoto, K.: Electron microscopy study of experimental enteric adenovirus infection in mice. *Inf. Imm.* 13: 569-580, 1976.
4. Takeuchi, A. and Phillips, B. P.: Electron microscope studies of experimental Entamoeba histolytica infection in the guinea pig. II. Early cellular and vascular changes accompanying invasion of the lamina propria. *Virch. Arch. B. Cell Path.* 20: 1-13, 1976.
5. Takeuchi, A., Binn, L. N., Jervis, H. R., Keenan, K. P., Hildebrandt, P. K., Valas, R. B., Bland, F. F.: Electron microscope study of experimental enteric infection in neonatal dogs with a canine coronavirus. *Lab. Investigation* 34: 539-549, 1976.
6. Takeuchi, A., Keenan, K. P., Jervis, H. R., Binn, L. N., and Hildebrandt, P. K.: Penetration, replication, and release of coronavirus particles in the infected neonatal small intestinal epithelium. *Fed. Proc.* 35: 394, 1976.
7. Henley, G. L., Lee, C. M., and Takeuchi, A.: Freeze-etching Observations of Trophozoites of Pathogenic Entamoeba histolytica. *Z. Parasitenk.* 48: 181-190, 1976.
8. Colvin, R. and Dvorak, H.: Fibrinogen/Fibrin on the surface of macrophages: Detection, distribution, binding requirements, and possible role in macrophage adherence phenomena. *J. Exp. Med.* 142, 1378-1390, 1975.
9. Colvin, R. B. and Dvorak, H. F.: Cell-mediated hypersensitivity. II. Kinetics of fibrinogen/fibrin accumulation and vascular permeability changes in tuberculin and cutaneous basophil hypersensitivity reactions. *J. Immunology* 114: 377-387, 1975.

10. Kurman, R. J., McIntire, K. R., Colvin, R. B., Scardino, P.T. and Waldmann, T. A.: Cellular localization of alpha fetoprotein and human chorionic gonadotropin in testicular neoplasms using an immunoperoxidase technique. Fed. Proc. 35: 492, 1976.

11. Hyman, L. R., Colvin, R. B., Bernard, E. and Steinberg, A. D.: Major histocompatibility-linked susceptibility to autoimmune tubulointerstitial nephritis (TN) in guinea pigs (GP). Fed Proc. 35: 824, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^b	3 REPORT CONTROL SYMBOL ^c	
				DA OB 6463	76 06 30	DD DR&E(AR)636	
4 DATE PREV SUMMARY	5 KIND OF SUMMARY	6 SUMMARY SCTY ^d	7 WORK SECURITY ^e	8 REGRADING ^f	9A DMB'S INSTN ^g	9B SPECIFIC DATA CONTRACTOR ACCESS ^h	
75 07 01	K. Comp.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10 NO CODES ⁱ		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		62760A	3A762760A822	01	126		
B. CONTRIBUTING							
C. COOPERATION		CARDS 114F					
11 TITLE (Precede with Security Classification Code) ^j							
(U) Infectious Hepatitis							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^k							
002600 Biology 003500 Clinical Medicine							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
69 07		76 06		DA		C. In-House	
17 CONTRACT AMT				18 RESOURCE ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				PRECEDING		B. FUNDS (In thousands)	
B. NUMBER ^l				FISCAL YEAR		75	
C. TYPE				CURRENT		1	
D. KIND OF AWARD				76		1	
E. CUM. AMT.						71	
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME ^m Walter Reed Army Institute of Research				NAME ⁿ Walter Reed Army Institute of Research			
ADDRESS ^o Washington DC 20012				ADDRESS ^p Washington DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Funded DOD or U.S. Academic Institution)			
NAME ^q Joy, COL R. J. T.				NAME ^r Knodell, MAJ R. G.			
TELEPHONE: 202-576-3551				TELEPHONE 202-576-2208			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Conrad, Dr. M. E.			
				NAME: Flannery, LTC E. P. DA			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Liver Disease; (U) Hepatitis; (U) Australia antigen							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) Viral hepatitis has been a major cause of morbidity in military populations and a significant hazard in the military blood program. Investigations were undertaken related to its etiology, epidemiology, and prevention.							
24. (U) A randomized double-blind study was undertaken to determine if gamma globulin with high anti-HBs titer provides protection against transfusion hepatitis and if transfused blood positive for HBsAg only by radioimmune assay (but not by other methods of testing) causes hepatitis.							
25. (U) 75 07 - 76 06. From Aug 72 to Dec 74 all volunteers undergoing cardiac bypass surgery at WRAMC or LAMC received either high titer anti-HBs gamma globulin, conventional gamma globulin, or an albumin placebo solution. Only 6 percent of patients developing either icteric hepatitis or elevated transaminases had HBsAg-related hepatitis as measured by radioimmunoassay (RIA). Fifteen patients (4 percent of group) were transfused with blood HBsAg positive by RIA but negative by counterimmunoelectrophoresis; one of these developed type B hepatitis and 4 had a serologic response. Both conventional and high titer anti-HBs globulin were efficacious in decreasing incidence of icteric hepatitis (p = 0.03) and total hepatitis (p = 0.02) cases. There was no significant difference in protective effect of the two gamma globulin preparations. Publications from this research include R.G. Knodell et al, Etiological spectrum of post-transfusion hepatitis, Gastroent. 69:1278-1285, 1975; M.E. Conrad and R.G. Knodell, Viral Hepatitis, JAMA 233:1277-1279, 1975; R.G. Knodell et al, Efficacy of prophylactic gamma-globulin in preventing non-A, non-B post-transfusion hepatitis, Lancet I:557-561, 1976. This project is being terminated in order to centralize all WRAIR work in hepatitis. After 1 Jul 76 all hepatitis work will be done by DCD&I. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

PII Redacted

DD FORM 1498

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

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 126 Infectious hepatitis

Investigators

Principal: MAJ Robert G. Knodell, M.D., MC

Associate: Marcel E. Conrad, M.D., University of Alabama

LTC E. Patrick Flannery, M.D., MC, Letterman
General Hospital

Allen L. Ginsberg, M.D., George Washington
Medical School

Objectives: To determine if gamma globulin with high anti-HB_s titer provides protection against transfusion hepatitis and if transfused blood positive for HB_sAg only by radioimmune assay (but not by other methods of testing) causes hepatitis.

Technical Approach: During past years there had been a considerable incidence of hepatitis among patients undergoing cardiac bypass surgery at WRAMC. Estimates of the incidence of hepatitis in this group based upon detection of elevated transaminase determinations 3 months after surgery were 10-20% of patients. It was believed that this was caused by the requirement of using many pints of blood and blood products from multiple donors in these patients.

From August 1972 to December 1974, all volunteers who underwent cardiac bypass surgery received a 10 ml injection of either high titer anti-HB_s gamma globulin, conventional gamma globulin, or an albumin placebo solution. These injections were administered double blind under code. Blood was drawn from the volunteers before gamma globulin injection, weekly after surgery while the patient was hospitalized, and 3, 6, and 9 months after surgery. Determinations for HB_sAg, anti-HB_s, SGPT, and serum bilirubin were done on each blood specimen. In addition, a history was obtained from each patient at intervals after surgery. All blood used for transfusion was tested by radioimmune assay for HB_sAg and anti-HB_s. The biologic materials used in this study included a high titer anti-HB_s lot of gamma globulin prepared by the Massachusetts State Laboratories and currently used under NHLI contract in several national studies, a lot of gamma globulin used in 60,000 soldiers in Korea, and a placebo solution used in 40,000 U.S. soldiers in Korea without known complications. All solutions were tested in accordance with U.S.P. regulations.

Progress and Results: This has been a collaborative double blind study conducted at Walter Reed Army Medical Center and at Letterman Army Medical Center. Since the inception of the study, 354 patients undergoing cardiac bypass surgery at either WRAMC or LAMC volunteered as participants in the study. Each patient received under code either 10 ml of high titer anti-HB_s gamma globulin, conventional gamma globulin, or an albumin placebo solution. There were no known adverse reactions to the administration of either the gamma globulin or placebo solutions. Transaminase elevations were observed in 17% of patients 3-6 months after surgery. Only 10 patients had clinical icteric hepatitis. Only 6% of patients with either icteric hepatitis or elevated transaminase determinations had HB_sAg-related hepatitis as measured by radioimmunoassay. Four percent of the patients were transfused with blood that was HB_sAg positive when tested by radioimmunoassay; all blood was HB_sAg negative when tested by counterimmuno-electrophoresis. Of this group of 15 patients, only one patient developed type B hepatitis while 4 others had a serological response only. In the majority of hepatitis cases hepatitis B virus could not be implicated, and different viral agents must be hypothesized. Both conventional and high titer anti-HB_s globulin were efficacious in decreasing the incidence of icteric hepatitis ($p = 0.03$) and total hepatitis ($p = 0.02$) cases. When the gamma globulin groups were combined, p value for the protective effect of gamma globulin versus placebo for icteric hepatitis was 0.003 and 0.007 for total hepatitis. There was no significant difference in the protective effect of the two gamma globulin preparations.

Conclusions: This study has documented that posttransfusion hepatitis B now represents a minority of hepatitis cases following blood transfusion and emphasizes the importance of defining additional hepatitis etiological agents. Demonstration of the efficacy of prophylactic gamma globulin in preventing non-A, non-B posttransfusion hepatitis in cardiac surgery patients provides a means for reducing both the severity and total transfusion of hepatitis by blood transfusions. Additional important information concerning chronic liver disease after non-A, non-B hepatitis will be obtained in long-term followup of study patients.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 126 Infectious hepatitis

Literature Cited.

Publications:

1. Knodell, R. G., Conrad, M. E., Dienstag, J. L., and Bell, C. J.: Etiological spectrum of post-transfusion hepatitis. Gastroenterology 69: 1278-1285, 1975.

2. Knodell, R. G., Conrad, M. E., Ginsberg, A. L., Bell, C. J., and Flannery, E. P.: Efficacy of prophylactic gamma-globulin in preventing non-A, non-B post-transfusion hepatitis. Lancet I: 557-561, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6532	76 06 30	DD-LR&E(AK)836	
3 DATE PREV SUMMARY ^a	4 KIND OF SUMMARY ^a	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A DOW'N INSTR'N	8B SPECIFIC DATA- CONTRACTOR ACCESS	9 LEVEL OF SUM A. WORK UNIT
75 07 01	...	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10 WD CODES ^a		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		62760A	3A762760A822	01	128		
b. CONTRIBUTING							
XXXXXXXXXX		CARDS 114F					
11. TITLE (Precede with Security Classification Code) ^b							
(U) Biochemical Methodology and Laboratory Automation							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^b							
008300 Inorganic Chemistry 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 10		76 06		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. FUNDS (in thousands)	
a. DATES/EFFECTIVE: NA EXPIRATION:				NECESSARY		a. PROFESSIONAL MAN YRS	
b. NUMBER:				75		6	
c. TYPE:				FISCAL YEAR		76	
d. KIND OF AWARD:				76		6	
e. CUM. AMT.						117	
19. RESPONSIBLE ODD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Div of Biochemistry Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursuant to 38 USC 4186)			
NAME: Jov, COL R. J. T.				NAME: Temaree, LTC G.E.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2211			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Bass, B. G., MS; Neeley, MAJ, W.E.			
				NAME: Kazvak, J., BS DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Automation; (U) Toxicology; (U) Drugs of Abuse; (U) Gas Chromatography							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pursuant to individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The technical objective of this work unit is to develop and establish modern automated methods for qualitative and quantitative chemical analyses of military importance in medical laboratories and to adapt these systems for use in support of combat medical operations.							
24. (U) This work unit is terminated with this reporting period.							
25. (U) 75 07 - 76 06 Work was continued on the development and testing of micro-analyzers for hospital and field applications, and on the adaptation of electronic programmable calculators to high speed continuous flow analyzers. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

PII Redacted

DD FORM 1498
1 MAR 68

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

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 128 Biochemical methodology and laboratory automation

Investigators.

Principal: LTC Gale E. Demaree, MSC

Associate: MSG Vaughn G. Ayer; Billy G. Bass, M.S.; Larissa deBaare, M.D.; John I. Davis, B.S.; James E. Doolittle, A.A.; MAJ Thomas P. Gibson, M.C.; SFC Lovelyn Hall; Leo Kazyak, M.A.; SP4 Leighton C. Makanui; Edward J. Matusik, B.S.; Jean E. (Lewis) Matusik, B.S.; SP5 Michael E. McKay; SFC Evelyn Moore; MAJ William E. Neeley, M.C.; Dominic F. O'Donnell; Helen C. Sing, M.S.

The objectives of this work unit are to develop and exploit new laboratory methods for qualitative and quantitative analyses of biochemical substances in support of military medicine and to apply automation to analytical procedures to provide rapid, precise and accurate results. During the reporting period, efforts have been focused on the following areas:

1. Development and evaluation of high speed, miniaturized continuous flow analyzer.
2. Application of electronic programmable desk top calculators to on-line clinical laboratory analyses.
3. Modification of conventional continuous flow analyzers to achieve high speed and high precision analyses.
4. Analysis of TAB nerve agent antidote.

1. Development and evaluation of high speed miniaturized continuous flow analyzer.

A four-channel, high speed, microanalyzer for electrolytes has been operated in the WRAMC clinical laboratory for 6 months. A 2-channel analyzer for BUN-creatinine determinations was operated and evaluated in the laboratory for about two weeks until the flow cell material (Lexan) was determined to be inappropriate for use with picric acid. A quartz flow cell has been designed and tested for this system. A new flame photometer detector system and circuit has been designed for measuring the ratios between sodium and lithium and between potassium and lithium. The advantages of this instrument are high speed, low cost, small size, improved accuracy and precision, reduced sample size and greatly reduced the reagent requirements. Ten units of the high speed microanalyzer were constructed at the US Army Medical Engineering Research

and Development Laboratory. These units have been placed in various local laboratories for operational evaluation. A miniaturized continuous flow analyzer has been coupled to a high performance liquid chromatograph. This effort uses the high resolution separation by the liquid chromatograph to separate the interfering substances from the compounds of interest. Preliminary results on analysis of creatinine and catecholamines show significant improvements in specificity, sensitivity and speed of analysis.

2. Application of electronic programmable desk top calculators to on-line clinical laboratory analyses.

Work has continued on the development of electronic programmable calculators as on-line data processors in the clinical laboratory. Program improvements have been made to reflect changes in analyzer circuitry. Using the H-P 9810A calculator equipped with a cassette magnetic tape memory, point to point values of analog display such as liquid or gas-liquid chromatograms were recorded, and off-line analyses of the data points were made for sample quantification by four different calculation techniques:

- (a) Full area of the peak by point to point integration.
- (b) Full area by product of peak height and sum of half widths at one-half of peak height, [i.e., $P-H \times (1/2 W_{rise} + 1/2 W_{fall})$].
- (c) Half area by product of peak height and half width at half peak height.
- (d) Peak height.

Quantification of high performance liquid chromatograms is usually achieved by peak height measurements. This method underestimated concentrations by as much as 33% (from results in this laboratory). This discrepancy is due to the fact that standards are prepared in aqueous solutions and give gaussian concentration distributions, but serum specimens present broadened distribution curves that may or may not be gaussian in shape. Standard curves generated with aqueous solutions and peak height values may underestimate serum concentrations. Standard curves generated with integration methods are not as dependent on distribution curve shape and lend themselves to a more consistent analysis when applied to serum specimens. Methods (a) and (b) agree. Method (c) underestimates concentration by 1 - 10% compared to methods (a) and (b). The more complex quantification technique is possible with using the electronic programmable calculator as an on-line data processor.

3. Modification of conventional continuous flow analyzers to achieve high speed and high precision analyses.

Work has continued on the modification of conventional continuous flow analyzers to take advantage of some of the developments of the micro-analyzer to improve the speed and precision of analysis of equipment that is readily available in most clinical laboratories. Efforts have been focused primarily on flow cell design and data processing with an on-line calculator. A 1.0 mm quartz flowcell has been built and tested, and a 0.635 mm quartz flowcell has been built. New procedures have been evaluated to eliminate necessity for using known carcinogens as reagents. One example is the use of o-Dianisidine for glucose measurements. A new glucose oxidase (peroxidase method - a modification of Trinder's method - has been evaluated. A two-fold increase in analysis speed with lower reagent consumption was realized.

4. Analysis of TAB nerve agent antidote.

The Division of Biochemistry provided the analytical support for the chemical analysis of TAB nerve agent antidote. Materials from Cartrix Parenteral Systems included Neat compounds used for the production of injectors, and filled cartridges not yet assembled into injectors. Methodology was established and evaluated and new procedures introduced.

The Neat compounds were analyzed by the ultra violet spectroscopy, gas chromatography, high performance liquid chromatography, and thin layer chromatography. The precision of the analytical methods were estimated to be 1.5% for TMB4, 2.3% for atropine sulfate, 4.0% for benactzyine HCl, and 4.4% for the parabens. The Neat compounds met the analytical specifications.

Cartridges (26 Lots) received from Cartrix Parenteral System were analyzed for accuracy of formulation. On the basis of the 26 lots analyzed, if these are representative of all lots of injectors, it was estimated that 95% of all injectors will be between 89% and 115% of the labelled potency for all active ingredients.

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 128 Biochemical methodology and laboratory automation

Literature Cited.

Publications:

1. Neeley, W.E., Sing, H.C., Yates, T.: Novel dual-channel dializer for continuous flow analysis. *Clinical Chemistry*. 21: (8), 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
				DA OB 6503	76 07 01	DD DR&E(AR)656	
3. DATE PREV. SUMM ^c	4. KIND OF SUMMARY	5. SUMMARY ACTY ^d	6. WORK SECURITY ^e	7. REGRADING ^f	8. DRG'S INSTR ^g	9. SPECIFIC DATA CONTRACTOR ACCESS ^h	10. LEVEL OF SUM ⁱ
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. / CODES ^j		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62760A		3A762760A822		01	
b. CONTRIBUTING						129	
c. CONTRACTOR ^k		CARDS 114F					
11. TITLE (Precede with Security Classification Code) ^l							
(U) Epidemiology of Hepatitis in the Military							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^m							
010100 Microbiology							
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 ⁿ				76		2	
c. TYPE				CURRENT		11	
d. KIND OF AWARD:				77		22	
e. AMOUNT:							
f. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Res				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				ADDRESS: Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DRG if U.S. Academic Institution)			
NAME: Joy, COL Robert J.T.				NAME: Segal, Herbert E., LTC, MC			
TELEPHONE: 202-576-3551				TELEPHONE 202-576-2480			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Nowosiwsky, Taras, COL, MC			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Epidemiology; (U) Hepatitis B; (U) Liver; (U) Virus Diseases							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Furnish individual paragraphs identified by number. Precede rest of each with Security Classification Code.)							
<p>23. (U) To define and study the prevalence, incidence, and variables of hepatitis transmission in medical care provider and line military populations. To apply this information to the design of hepatitis prevention and control programs.</p> <p>24. (U) Contemporary epidemiologic methods are employed. Multidisciplinary collaborative approaches are utilized and new methods developed as required.</p> <p>25. (U) 75 07-76 06 Analysis of occupational and assignment data from the two-year followup study of the 1972 cohort of Army health care personnel and association of those data with clinical hepatitis and serologic results were completed. Analysis of data from a one-year prospective study of Hepatitis B antigen and antibody acquisition by personnel newly assigned to Fort Hood is in progress. (These studies are complementary to work described under DA OB 6513, Work Unit 176, entitled "Mechanisms of Transmission of Hepatitis Viruses.") A protocol for the study of chronic hepatitis following Hepatitis B infection was revised and submitted. For technical reports see Walter Reed Army Institute of Research Annual Report 1 Jul 75-30 Jun 76.</p> <p>Support in the amount of \$2,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>							

PII Redacted

DD FORM 1498
1 MAR 68

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

Project 3A762760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 129 Epidemiology of hepatitis in the military

Investigators:

Principal: LTC Herbert E. Segal, MC
COL Taras Nowosiwsky, MC; MAJ Gilbert R.
Irwin, MC; SSG Michael C. Callahan; L.
Charlene Evans

1. Followup Incidence Study of Army Health Care Personnel
for Hepatitis B Antigen and Antibody

Army Medical Department officers who had been enrolled at the Academy of Health Sciences, Fort Sam Houston, Texas between July and October 1972 and had participated in the prevalence study previously reported were eligible for this followup. Newly inducted nurses (ANC), dietitians and physical therapists (AMSC), dentists (DC), physicians (MC), medical administrators and allied health scientists (MSC), and veterinarians (VC) had attended the Basic Officer Courses at the Academy upon their entrance onto active duty. Most of these officers were eligible for release from active duty after two years service. More senior personnel, representing those with career commitments, had been enrolled in the Officer Advanced Course.

Approximately three months before the second anniversary of their enrollment, a mailing was sent to each officer at his or her last known duty station. Included in the mailing were a letter to explain the purpose of the study and request participation, a questionnaire to elicit information on the location, dates, and description of duties performed as well as on selected professional and personal health-related experiences, and blood collection and storage tubes to process and ship a serum specimen for HB_sAg and anti-HB_s testing. Return of the questionnaire and serum specimen was requested before the officer was released from active duty, to the Walter Reed Army Institute of Research by mail.

Sera were tested for Hepatitis B surface antigen (HB_sAg) by radioimmunoassay using the Ausria II kit (Abbott Laboratories), with specificity for HB_sAg confirmed as previously described. Antibody to the surface antigen (anti-HB_s) was determined by the passive hemagglutination

(PHA) test described by Vyas. Serologic evidence of hepatitis B virus infection HB_sAg or anti-HB_s positivity, (HBV positivity) was then correlated with health-related assignment and occupational variables listed in the questionnaire.

RESULTS

Two thousand and seventy-five officers were considered eligible for followup, the remainder having been reportedly released from active duty prior to the scheduled mailing date (Table 1). Slightly fewer than a third of all mailings sent were returned acceptably completed, that is with a usable questionnaire and serum specimen. Completion rates varied by Corps, the overall rate declining from 43.5 percent to 26.1 percent as the mailings proceeded. Sera submitted by officers after they were released from active duty are not considered in this report, and are tabulated as non-responses.

Table 1. Followup Response Rates by Corps

Corps	Total Eligible	Released from Active Duty	Total Mailings	No. (%) Responders
ANC	569	120	449	203 (45.2)
AMSC	48	12	36	16 (44.4)
DC	460	108	352	85 (24.1)
MC	846	63	783	228 (29.1)
MSC	646	236	400	121 (30.3)
VC	61	6	55	26 (47.3)
TOTAL	2630	545	2075	679 (32.7)

Serologic evidence of hepatitis B infection was detected in 20 (2.9%) of those responding (Table 2), being found both in officers with known preexisting seropositivity (group "A") as well as in those seroconverting sometime after their enrollment in the initial prevalence study (group "B"). Twenty-two initially seropositive officers lacked detectable antibody on followup (group "C"). Among

all officers seropositive initially (groups "A" and "C") anti-HB_s positivity persisted in 42.1% of those with antibody detected with titers of \geq 1:32 and in 21.4% of those with titers of \leq 1:16. The majority of officers tested were seronegative on both occasions (group "D").

Table 2. Initial and Followup Serologic Results*

Group	(initial: followup results)	No.(%) officers
"A"	positive: positive	11 (1.6)
"B"	negative: positive	9 (1.3)
"C"	positive: negative	22 (3.2)
"D"	negative: negative	637(93.8)
Total		679(99.9)

* All positive results are for anti-HB excepting one officer in Group B who was HB_sAg positive.

Officers seroconverting during the two-year study period (group "B") are listed in Table 3. None of them reported an illness compatible with a diagnosis of hepatitis. An additional seven seronegative officers (included in group "D") gave a history of clinical hepatitis without diagnosis of metabolic disorders of the liver or infectious mononucleosis during the period under study (Table 4). Two of those reported having been HB_sAg positive during their illness. Combining these two groups, fifteen of the sixteen were nurses (ANC), dentists (DC), or physicians (MC), indicating a relative risk (rr) for personnel serving in these Corps of 5.2, compared with officers in nondirect patient care fields.

Table 3. Professional Characteristics of Officers HBV Seroconverting During the Two-year Followup

Officer	Corps	Specialty	Assignment Area
1	Medical	Pathology	United States

Continued

Table 3. Professional Characteristics of Officers HBV Seroconverting During the Two-year Followup

Officer	Corps	Specialty	Assignment Area
2	Medical	Internal Medicine	United States
3	Medical	Anesthesiology	United States
4	Medical	Otolaryngology	United States
5	Medical	Otolaryngology	United States
6	Medical	General Medicine/ Pediatrics	Germany
7	Nurse	Medical Intensive Care	United States
8	Nurse	General Surgery/ Orthopedics	United States
9	Medical Service	Experimental Psychology	United States

Table 4. Professional Characteristics of Officers Becoming Ill with Hepatitis During the Two-year Followup

Officer	Corps	Specialty	Assignment Area
10	Medical	General Medicine	Germany
11	Medical	General Practice	United States
12	Medical	General Medicine	Germany
13*	Dental	General Dentistry	United States
14*	Nurse	General Medical	United States
15	Nurse	General Medical Emergency Room	United States
16	Nurse	General Medical	United States

* Reported HB_sAg positivity coincident with illness.

Physicians were best represented, 3.9 percent sero-converting or experiencing clinical hepatitis, followed by nurses, 2.5 percent, dentists, 1.2 percent, and allied health scientists, 0.8 percent. Seroconversion or clinical hepatitis among physicians occurred in two of four otolaryngologist-responders, four of 40 general medical officers, one of six pathologists, one of 13 anesthesiologists, one of 56 internists, and none of 26 general surgeons. Of all personnel assigned to Germany, three, 8.5 percent, seroconverted or became clinically ill, compared with 13 or 2.5 percent of all those stationed in the United States ($rr = 3.4$).

Almost all officers assigned to Corps whose officers provide direct patient care responded to the questionnaire that they actually did serve that function (Table 5). Of those, 74 percent "cared for hepatitis patients," 85 percent "drew or handled blood or blood products," 63 percent "contaminated themselves with blood or blood products," and 35 percent "performed laboratory work" on one or more occasions.

Table 5. Questionnaire Responses of Direct Patient Care Providers*

Question	Total	No. (%) Sero- converting or Ill	Relative Risk (rr)	95% Confidence Limits
Provide direct patient care?				
yes	486	14 (2.9)	0.3	-
no	10	1 (10.0)		
Care for hepatitis patients?				
yes	356	9 (2.5)	0.6	-
no	140	6 (4.3)		
Draw or handle blood?				
yes	411	14 (3.4)	2.9	0.44-207
no	85	1 (1.2)		
Contaminated with blood?				
yes	304	8 (2.6)	0.7	-
no	192	7 (3.6)		

Continued

Table 5. Questionnaire Responses of Direct Patient Care Providers*

Question	Total	No. (%) Sero- converting or Ill	Relative Risk (rr)	95% Confidence Limits
Perform laboratory work?				
yes	166	8(4.8)	2.3	0.72-7.4
no	330	7(2.1)		

*Excludes officer #9 not at risk of performing these functions.

There appears to be risk of seroconversion or clinical illness attendant to the drawing or handling of blood or blood products and to the performance of laboratory work. The relative risk for officers who both drew or handled blood or blood products and performed laboratory work was 4.2. Additional questionnaire data suggested risk for officers receiving blood transfusions (rr = 11.4) but not for officers in contact with hepatitis patients outside the medical care setting (rr = 0.2). None of the 15 direct care oriented officers seroconverting or experiencing clinical illness reported receiving gamma globulin during the two-year period covered by the questionnaire, compared with 8 percent of the remaining 481 direct care oriented officers.

2. Epidemiologic Studies of Hepatitis B at Fort Hood, Texas

A one-year prospective study of enlisted personnel newly arrived at Fort Hood during the February-April 1974 period was completed. Each of the 2333 personnel in this cohort was administered a demographic and health experience questionnaire and had serum collected for Hepatitis B surface Antigen (HB_sAg), antibody to the surface antigen (anti-HB_s), and antibody to the core antigen (anti-HBc). The cohort was followed at four month intervals by questionnaire and rebleed; personnel studied at 4, 8, and 12 months after arrival numbered 1915, 1210, and 900 respectively. Correlation of demographic and health histories with serologic data is in progress. Examination of the usefulness of anti-HBc assay in the serologic study of Hepatitis B is reported elsewhere (Project 3A161101A91C, Work Unit 105, Mechanisms of Transmission of Hepatitis Viruses).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUPPLY	4. KIND OF SUMMARY	5. SUMMARY SCTY ³	6. WORK SECURITY ⁴	7. REGRADING ⁵	8. DRGPN INSTR ⁶	9a. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUB A. WORK UNIT
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. CODES ⁷	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62760A	3A762760A822		01	130		
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ⁸							
(U) Gastrointestinal Diseases of Military Importance							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹							
010100 Microbiology 008800 Life Support 002600 Biology							
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		EXPIRATION:		PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER *				FISCAL 76		7	
c. TYPE.		d. AMOUNT:		CURRENT 77		2 75	
e. KIND OF AWARD:		f. CUM. AMT.				335	
20. 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 (Provide SSAN if U.S. Academic Institution)			
NAME: JOY, COL, R.J.T.		TELEPHONE: (202) 576-3551		NAME * BOEDEKER, MAJ, E.C.			
				TELEPHONE (202) 576-2582			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered.				NAME: CHARNEY, MAJ, A.N.			
				NAME: DONOWITZ, MAJ, M. DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Diarrheal Diseases; (U) Salmonellosis; (U) Intestinal Absorption; (U) Shigellosis; (U) Intestinal Cell Surface; (U) Cholera; (U) Immunology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Research efforts in this department will continue to be directed toward gastrointestinal diseases of military importance. In particular, the focus is on the enteropathogenic diarrheal diseases, Salmonellosis and Shigellosis and pathogenic E. coli. These have critical military relevance since they represent a major factor in troop mobility.							
24 (U) Studies will continue to employ several in vivo and in vitro models. These include perfusion models using rhesus monkeys and rabbits, in vivo rat ileal loop models Ussing chamber studies and subcellular membrane fractions. Lymphocyte function (antibody mediated cellular cytotoxicity) is being studied in vitro.							
25 (U) 75 07-76 06 Shigella enterotoxin activates rabbit ileal mucosal adenylate cyclase activity in the presence of sufficient ATP. Methylprednisolone (MP) increases mucosal Na-K-ATPase activity and water and electrolyte transport in rat intestine without affecting adenylate cyclase. MP can prevent and reverse the net secretion of water and electrolytes induced by cholera toxin. Cecal inoculation of Shigella in Rhesus monkeys results in dysentery alone without the changes in jejunal secretion seen after oral inoculation. Antibody-dependent cellular cytotoxicity of Shigella by human peripheral blood lymphocytes has been shown. A model for adherence of pathogenic E. coli to intestinal epithelial membranes has been developed. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 75 - 30 Jun 76.							
Support in the amount of \$98,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							
Available to contractors upon originator's approval							

PII Redacted

DD FORM 1498
1 MAR 66

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

Project 3A762760A822 Military Internal Medicine

Task 01 Military Internal Medicine

Work Unit 130 Gastrointestinal Diseases of Military Importance

Investigators

Principal: Edgar C. Boedeker, MAJ, MC
Associates: Alan N. Charney, MAJ, MC; Dennis P. Collin, CPT, MSC;
Mark Donowitz, MAJ, MC; Ralph A. Giannella, M.D.;
Ester P. Jorolan, Ph.D.; Marshall D. Kinsey, MAJ, MC;
Richard P. MacDermott, MAJ, MC; Alton A. Reeder, LTC, MC;
Yuan-Heng Tai, Ph.D.

Description

The research activities in this Department have continued to focus on the toxigenic and/or invasive enteropathogenic diarrheal diseases caused by Salmonella, Shigella, E. coli and, to a lesser extent, Cholera. Three basic sets of questions are being asked about the pathogenesis of these diseases.

a. Pathophysiology of Intestinal Transport - What enzymatic and hormonal mechanisms mediate bacterial-induced secretion of water and electrolytes by the intestine? What are the normal mechanisms for salt and water absorption? How do absorptive and secretory mechanisms interact? Do they share any pathways? Can pharmacologic agents increase salt and water absorption in the face of infection or decrease secretion induced by bacterial toxins?

b. Role of Host Immune Mechanisms - How do disease causing bacteria interact with the normal cellular (lymphocyte, macrophage, polymorphonuclear leukocyte) and humoral (antibody) immune defense mechanisms in the intestine? What are the most effective means of inducing active cellular and humoral immunity to enteric infection (i.e., how can an effective vaccine for enteric infection be produced)? What factors (antibody, complement) enable lymphocytes to kill enteric bacteria in vitro? Are these factors of equal importance in an in vivo model of enteric infection?

c. Role of Intestinal Surface Characteristics - How do disease causing bacteria interact with the surface of the gastrointestinal tract? What characteristics of this intestinal surface permit disease causing bacteria to adhere to and colonize the small intestine? What

characteristics of the intestinal surface permit specific interactions with toxins produced by bacteria? Can specific interactions of bacteria or their toxins be altered by orally administered agents?

Progress and Results

Sodium potassium-activated adenosine triphosphatase (Na-K-ATPase) is associated with electrolyte transport in many tissues. To help delineate its role in intestinal transport, changes in rat intestinal electrolyte and water transport induced by injecting methylprednisolone acetate (MPA) or deoxycorticosterone acetate (DOCA) were correlated with changes in Na-K-ATPase activity in the jejunum, ileum and colon. MPA increased Na and H₂O absorption and Na-K-ATPase activity in parallel in all segments. DOCA increased Na-K-ATPase and transport in the colon. Permeability, Mg-ATPase and Adenylate Cyclase activities were unchanged by either drug. These data are consistent with a primary role for Na-K-ATPase in intestinal transport (14).

Cholera toxin induced net water and electrolyte secretion and increased adenylate cyclase activity in rat ileum. Utilizing the model of increased intestinal water and electrolyte absorption induced by MPA (14), the relationship of the mucosal enzyme systems Na-K-ATPase and Adenylate Cyclase and their associated transport processes was studied. MPA, administered prior to exposure of intestine to cholera toxin, could promote net absorption of water and electrolytes, without affecting adenylate cyclase. In addition, MPA could reverse ongoing net secretion induced by cholera toxin. Thus, MPA can prevent and reverse the secretory effects of cholera toxin by selectively stimulating a coexisting absorptive process (15,16).

Systematic study of Na-K-ATPase in the above studies led to two technical advances in the measurement of this enzyme. An in vitro inhibitor of Na-K-ATPase was discovered in a commonly used preparation of ATP (17). An automated ATPase assay capable of measuring 60 samples per hour with a high degree of precision and essentially no sample carry-over was developed (1).

Shigella Dysenteriae I is one of several bacteria which produces an enterotoxin capable of stimulating intestinal water and electrolyte secretion. The mechanism whereby *Shigella* enterotoxin induces intestinal secretion has been debated. When studied in vitro in rabbit ileal mucosa in a modified Ussing chamber, the enterotoxin caused net sodium secretion but short circuit current did not change and no change in mucosal cyclic adenosine monophosphate (cyclic AMP) levels could be demonstrated (19).

This suggested that cyclic AMP might not be a mediator of Shigella enterotoxin action. However, in separate studies (18), activation of Adenylate Cyclase in the same time was clearly demonstrated when substrate (ATP) concentrations above the Km of Adenylate Cyclase were employed. These concentrations of ATP are greater than those required to show Adenylate Cyclase activation by cholera toxin. These latter observations strongly suggest that Shigella enterotoxin action may be mediated by the Adenylate Cyclase-cyclic AMP system and provide explanation for the failure of previous investigators to make similar observations.

The differences between cholera enterotoxin and shigella enterotoxin were emphasized in studies of their effect in rat colon. Previously, no effect of cholera toxin on colonic secretion had been shown. A net increase in cecal excretion of water and electrolytes was observed with cholera toxin but not with Shigella toxin (2). E. coli enterotoxin also failed to have an effect.

Most shigella organisms do not produce an identifiable enterotoxin. Shigella flexneri 2a is an invasive enteric pathogen in which enterotoxin has not been identified. Previous studies have shown that oral inoculation of S. flexneri in rhesus monkeys results in dysentery alone or diarrhea plus dysentery. Dysentery alone is associated with invasion of the colon and transport abnormalities only in the colon. The occurrence of diarrhea correlates with transport abnormalities in the jejunum as well, but without jejunal invasion. To determine whether jejunal transport abnormalities seen following oral inoculation resulted from a direct interaction between the organisms and the jejunal mucosa or from an indirect effect of colonic invasion and inflammation, rhesus monkeys were injected by direct cecal inoculation. Sixty-four per cent of animals developed clinical disease and 94% of these developed dysentery alone. Bacterial invasion and colonization was limited to the colon and sodium and water transport remained normal in the small bowel. Thus, the watery diarrhea seen with shigella seems to require an interaction between the jejunal mucosa and shigella organisms (3,20).

The pathogenesis of the increase in ileal secretion caused by Salmonella Typhimurium (Strain TML--an invasive organism), was investigated in rabbit ileal loops. Although this organism does not elaborate a traditional enterotoxin, TML infected mucosa demonstrated a marked increase in Adenylate Cyclase activity and cyclic AMP levels. Indomethacin pretreatment abolished both secretion and Adenylate Cyclase activation (4). The effect of indomethacin suggests that prostaglandins may be intermediates in Salmonella activation of Adenylate Cyclase.

The possible role of changes in intestinal permeability in the pathogenesis of Salmonella Typhimurium (TML) diarrhea was investigated in rhesus monkeys. Normal animals demonstrated a gradient of diminishing permeability from jejunum to ileum to colon. Salmonella injection did not increase permeability for any segments, despite extensive invasion of the ileal and colonic mucosa and increased secretion of water and electrolytes. This study shows that changed mucosal permeability is not a factor in Salmonella diarrhea (21).

The two preceding studies indicate that the diarrhea accompanying enteric infection may be related to specific chemical or hormonal mediators of fluid and electrolyte secretion. This prompted investigation of intestinal fluid and electrolyte movement induced by endocrine syndromes or chemical mediators.

Clinical studies in three patients with carcinoid syndrome and diarrhea using triple lumen jejunal perfusion demonstrated net fluid secretion. Methysergide (an antagonist of 5-hydroxytryptamine (5HT)), was effective in reversing the secretory state in some patients (22). A model for chronic elevation of blood 5HT was developed in the rabbit (23). This was achieved by daily injection of 5HT in oil. The major secretory effect was seen in the ileum where there was increased secretion of H_2O , Na, K and HCO_3 . 5HT-induced secretion is not associated with activation of the Adenylate Cyclase-cyclic AMP system.

A study on the effect of the laxative dioctyl sodium sulfosuccinate in the rat cecum was published (24). In this model net secretion was associated with increased mucosal cyclic AMP levels. An editorial on the mechanisms involved in laxative action was published (25).

Factors responsible for the host cellular immune response to enteric bacteria remain to be clarified. To investigate active destruction of bacteria by host immunocytes, human peripheral blood lymphocytes were isolated. In vitro, in the absence of complement, lymphocytes will kill 80% to 90% of antibody coated shigella. Normal human lymphocytes alone, or lymphocytes from patients recovering from Shigella, did not kill bacteria, indicating that sensitization of lymphocytes does not result in direct cell mediated killing of the bacteria (28). Similar experiments with meningococci demonstrated antibody dependent cellular cytotoxicity as a potential mechanism of destruction of these bacteria (29).

The phenomenon of cellular cytotoxicity was investigated in a series of studies using chromium labeled Red Blood Cells (RBCs) as the target cells. Plant lectins including the mitogenic E-phytohemagglutinin (E-PHA)

and the non-mitogenic Wheat Germ Agglutinin (WGA) produced marked cytotoxicity of human lymphocytes for autologous (human) RBCs. All subpopulations of lymphocytes (T,B or Null) were effective (26,5). Further studies investigating the induction by lectin of cytotoxicity of human lymphocytes for RBCs of several species showed that lectin-induced lymphocytotoxicity is related to a prearmed lymphocyte which seeks out and kills the appropriate target cell (27,6). In a separate system, the ability of a calcium ionophore (A23187) to induce cytotoxicity of lymphocytes for autologous RBCs was investigated. The data indicate a calcium-dependent mechanism in certain cellular cytotoxic processes (7). The analogies between the RBC system and cytotoxicity for enteric bacteria are being explored.

A defect in the ability of lymphocytes from patients with Common Variable Hypogammaglobulinemia (CVH) to synthesize immunoglobulin was demonstrated in vitro. The data indicate the lymphocytes of CVH patients are able to suppress immunoglobulin synthesis in normal lymphocytes, suggesting an abnormality of regulatory T cells which suppress B cell activation (8).

Initial interaction between the intestine and a variety of nutrients (e.g. vitamins) and toxic factors (e.g. bacterial enterotoxins, bacterial cell surfaces) is mediated by receptors at the surface of the intestinal epithelial cell. It is likely that these receptors are glycoproteins or glycolipids. Since plant lectins are known to bind to specific cell surface oligosaccharide structures, the ability of a series of plant lectins to bind to intestinal cell surface membranes (brush borders) was investigated. Wheat Germ Agglutinin, ricinus communis agglutinin and E phytohemagglutinin all bound to proximal and distal guinea pig brush border with about 10^{14} binding sites/mg of brush border protein (9).

In order to investigate the influence of plant lectins on a well characterized intestinal cell surface binding reaction, the binding of intrinsic factor vitamin B₁₂ complex (IF-B₁₂) was chosen. Pre-incubation of E phytohemagglutinin with distal brush border resulted in competitive inhibition of subsequent IFB₁₂ binding (9). This suggests that E phytohemagglutinin interacts specifically with the intestinal cell receptors for IFB₁₂.

Since the proteins and glycoproteins of the intestinal cell surface (brush border) membrane are insoluble when isolated free of phospholipid. Solubilization of these molecules is necessary to investigate their role in the processes of toxin binding and bacterial adherence. A systematic study of the ability of anionic and nonionic detergents

to solubilize Brush Border membrane protein was undertaken. Solubilization of different molecular species was dependent on detergent concentrations. Selective solubilization of membrane proteins and glycoproteins using Triton X-100, and also sodium deoxycholate, was achieved (10,30). The role of these molecules in lectin binding and bacterial adherence is being investigated. The model for bacterial adherence being utilized is that of a South Carolina Strain of *E. coli*, which causes diarrheal disease in rabbits and agglutinates rabbit brush borders in vitro.

A clinical study on the metabolism of deoxycholic acid in a group of patients with alcoholic cirrhosis was reported. In order to explain the low levels of the secondary bile acid deoxycholic acid in bile of patients with cirrhosis, disappearance of ^{14}C labeled deoxycholic acid from stools of patients with cirrhosis, quantitative and qualitative determination of fecal bile acids and in vitro ability of fecal bacteria to metabolize cholic acid were studied. Our in vitro data suggested that unpaired conversion of cholic acid to deoxycholic acid by intestinal bacteria best explains low deoxycholic acid in cirrhotic bile (11).

In additional studies on the same group of patients with cirrhosis, biliary lipid secretion rates were measured to determine whether decreased secretion of bile acids might result in decreased deoxycholic acid levels. These studies demonstrated a decreased secretion of cholic acid and its bacterial product deoxycholic acid in cirrhotics (12).

A sensitive radioimmunoassay for chenodeoxycholic acid has been developed (13) which permits measurement of serum levels of this bile acid. In a clinical study using this technique elevations of serum chenodeoxycholic acid were found to correlate with pruritus in patients with hyperthyroidism (31).

Conclusions and Recommendations

Work in this department has shown that Na-K-ATPase activity is closely correlated with water and electrolyte transport in the intestine. It has further been shown that pharmacological modification of Na-K-ATPase activity can prevent or reverse the net fluid and electrolyte secretion caused by cholera toxin. It appears that two separate and oppositely directed transport processes in the intestine can be modulated independently. These studies offer hope of eventual pharmacological treatment of infectious diarrhea.

Observations that humoral mechanisms rather than simply invasion and inflammation may be responsible for fluid and electrolyte secretion in infectious diarrhea have been extended. Alteration of intestinal permeability has been shown to have no role in Salmonella diarrhea. However, Salmonella infection has been shown to activate the Adenylate Cyclase-cyclic AMP system.

In contrast to observations published previously by other investigators, Shigella toxin has been shown to cause activation of intestinal Adenylate Cyclase.

The molecular composition of the intestinal cell surface has been further defined. Plant lectins have been shown to bind specifically to intestinal surface membranes and many cell surface proteins and glycoproteins have been solubilized and characterized. A system for studying bacterial adherence to intestinal surface membranes has been set up and the mediator of this interaction is being studied.

The ability of human lymphocytes to kill antibody coated enteric bacteria has been identified. The isolation and characterization of subpopulation of human lymphocytes with particular surface characteristics and patterns of response is continuing.

The major aim of the Department continues to be the elucidation of the fundamental mechanism of normal and pathologic intestinal secretion and absorption. It is becoming increasingly clear that infectious diarrhea is related to specific functional alterations in intestinal cells and not to general cell destruction. If the hormonal mediators, enzymatic activities, specific toxins or cell receptors involved in enteropathic diarrhea can be defined, specific pharmacologic reversal or alteration of these mechanisms can be hoped for. In addition, the factors involved in host immunologic resistance to enteric bacteria is being pursued with emphasis on defining the mechanisms of cell killing and the subpopulation of immunocytes involved.

Project 3A762760A822 Military Internal Medicine

Task 01 Military Internal Medicine

Work Unit 130 Gastrointestinal Diseases of Military Importance

Literature Cited

References

1. Collin, D.P., Davis, J.I., and Charney, A.N.: An Improved Automated Determination of Na^+ - K^+ -Activated Adenosine Triphosphatase. Manuscript submitted to *Biochimica, Biophysica Acta*.
2. Donowitz, M. and Binder, H.J.: Effect of Bacterial Enterotoxins on Fluid and Electrolyte Transport in the Colon. *Journal of Infectious Disease*. In Press.
3. Kinsey, M.D., Formal, S.B., Dammin, G.J., and Giannella, R.A.: Fluid and Electrolyte Transport in Rhesus Monkeys Challenged Intracably with *Shigella flexneri* 2a. *Infection and Immunity*. In Press.
4. Kinsey, M.D., Dammin, G.J., Formal, S.B., and Giannella, R.A.: The Role of Altered Intestinal Permeability in the Pathogenesis of Salmonella Diarrhea in the Rhesus Monkey. *Gastroenterology*. In Press.
5. MacDermott, R.P., Nash, G.S., Saint, J.G., Clark, E.A., Zaras, A.G., and Boldt, D.: Autologous Human Cellular Cytotoxicity Induced by Mitogenic and Non-Mitogenic Lectins. *Journal of Immunology*. In Press.
6. MacDermott, R.P., Nash, G.S., and Boldt, D.: Prearmed Effector Cells and the Target Cell Specificity of Lectin Induced Cellular Cytotoxicity. Manuscript submitted to the *Journal of Immunology*.
7. MacDermott, R.P. and Nash, G.S.: Calcium Ionophore A23187 Induced Cellular Cytotoxicity of Autologous Human Red Blood Cells. Abstract submitted to 1976 Leukocyte Culture Conference.

8. Waldmann, T.A., Broder, S., Krakauer, R., MacDermott, R.P., Durm, M., Goldman, C., and Meade, B.: The Role of Suppressor Cells in the Pathogenesis of Common Variable Hypogammaglobulinemia and the Immunodeficiency Associated with Myeloma. Federation Proceedings. In Press.
9. Boedeker, E.C. and Boldt, D.A.: Effect of Plant Lectins on Binding of Human Intrinsic Factor-Vitamin B12 Complex to Guinea Pig Brush Borders. Manuscript in Preparation.
10. Boedeker, E.C., Higgins, Y.K., Donaldson, R.M., Jr., and Small, D.M.: Selective Solubilization of Enzyme Proteins from Microvillus Membranes (MVM) Using Non-Ionic and Anionic Detergents. Manuscript in Preparation.
11. Knodell, R.G., Kinsey, M.D., Boedeker, E.C., and Collin, D.P.: Deoxycholate Metabolism in Alcoholic Cirrhosis. Gastroenterology. In Press.
12. Boedeker, E.C., Kinsey, M.D., and Knodell, R.G.: Decreased Hepatic Synthesis of Cholic Acid May Contribute to Absent Levels of Deoxycholic Acid in Cirrhosis. Presentation at 5th William Beaumont Gastrointestinal Symposium, March 1976-- Manuscript in Preparation.
13. Collin, D.P., Kinsey, M.D., and Donowitz, M.: Determination of Chenodeoxycholic Acid (CDC) in Hyperthyroid (HT) Patients by Gas Chromatography (GLC) and Radioimmunoassay (RIA). Clinical Chemistry. (Abstract) In Press.

Publications

14. Charney, A.N., Kinsey, M.D., Myers, L., Giannella, R.A., and Gots, R.E.: Na^+ - K^+ -Activated Adenosine Triphosphatase and Intestinal Electrolyte Transport. The Journal of Clinical Investigation 56: 653-660, 1975.
15. Charney, A.N. and Donowitz, M.: Prevention and Reversal of Cholera Toxin-Induced Intestinal Secretion by Methylprednisolone (MF). Gastroenterology 70: A11/869, 1976 (Abstract).
16. Charney, A.N. and Donowitz, M.: Prevention and Reversal of Cholera Enterotoxin-Induced Intestinal Secretion by Methylprednisolone Induction of Na^+ - K^+ -ATPase. Journal of Clinical Investigation 57: 1590-1599, 1976.

17. Charney, A.N., Silva, P., and Epstein, F.H.: An in vitro inhibitor of Na-K-ATPase present in an adenosinetriphosphate preparation. *Journal of Applied Physiology* 39: 156-158, 1975.
18. Charney, A.N., Gots, R.E., Formal, S.B., and Giannella, R.A.: Activation of Intestinal Mucosal Adenylate Cyclase by *Shigella Dysenteriae* I Enterotoxin. *Gastroenterology* 70: 1085-1090, 1976.
19. Donowitz, M., Keusch, G.T., and Binder, H.J.: Effect of *Shigella* Enterotoxin on Electrolyte Transport in Rabbit Ileum. *Gastroenterology* 69: 1230-1237, 1975.
20. Kinsey, M.D., Formal, S.B., Dammin, G.J., and Giannella, R.A.: The Importance of Oral Inoculation in the Pathogenesis of *Shigella* Diarrhea. *Gastroenterology* 70: A44/902 1976 (Abstract).
21. Giannella, R.A., Gots, R.E., Charney, A.N., Greenough, W.B., III, and Formal, S.B.: Pathogenesis of Salmonella-Mediated Intestinal Fluid Secretion. *Gastroenterology* 69: 1238-1245, 1975.
22. Donowitz, M., and Binder, H.J.: Jejunal Fluid and Electrolyte Secretion in Carcinoid Syndrome. *Digestive Diseases* 20:1115-1122, No. 12, 1975.
23. Donowitz, M., and Charney, A.N.: Effect of Chronic Elevation of Blood Serotonin on Intestinal Transport. *Gastroenterology* 70: A22/880, 1976 (Abstract).
24. Donowitz, M., and Binder, H.J.: Effect of Dioctyl Sodium Sulfosuccinate on Colonic Fluid and Electrolyte Movement. *Gastroenterology* 69: 941-950, 1975.
25. Binder, H.J., and Donowitz, M.: A New Look at Laxative Action. *Gastroenterology* 69: 1001-1005, 1975.
26. MacDermott, R.P., Nash, G.S., Saint, J.G., Clark, E.A., Zaras, A.G., and Boldt, D.: Autologous Lectin Induced Lymphocytotoxicity of Human Red Cells. *Federation Proceedings* 35: 233, 1976 (Abstract).
27. MacDermott, R.P., Nash, G.S., Clark, E.A., Saint, J.G., and Boldt, D.: Target Cell Specificity in Lectin Induced Lymphocytotoxicity. *Clinical Research XXIV*: 332A, 1976 (Abstract).

28. MacDermott, R.P., Summers, P.L., Reeder, A.A., Lowell, G.H., and Formal, S.F.: Antibody-Dependent, Cell-Mediated Cytotoxicity of Shigella. *Gastroenterology* 70: A52/910, 1976 (Abstract).
29. Lowell, G.H., Smith, L.F., MacDermott, R.P., and Artenstein, M.S.: Lymphocyte-Mediated Antibody-Dependent Killing of Meningococci (mgc). *Federation Proceedings* 35: 738, 1976 (Abstract).
30. Boedeker, E.C., Higgins, Y.K., Donaldson, R.M., Jr., and Small, D.M.: Selective Solubilization of Enzyme Proteins from Microvillus Membranes (MVM) Using Non-Ionic & Anionic Detergents. *Gastroenterology* 70: A7/865, 1976 (Abstract).
31. Kinsey, M.D., Donowitz, M., Wartofsky, L., Boehm, T.M., and Collin, D.P.: The Correlation Between Pruritus and Elevated Serum Chenodeoxycholic Acid (CDC) Levels in Hyperthyroidism. *Gastroenterology* 70: A44/902, 1976 (Abstract).

Project 3A762758A823
MILITARY PSYCHIATRY

Task 00
Military Psychiatry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OA 6470	760630	DD DR&E(AR)636	
3 DATE PREV SUMRY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING ⁷	8A DIS'N INSTR'M	8B SPECIFIC DATA FOR TRACTOR ACCESS	9 LEVEL OF SUM
75 07 01	K. Comp.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
10 NO CODES ¹⁰	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	A. PRIMARY	62758A	3A762758A823	00	030		
	B. CONTRIBUTING						
	C. CONTRIBUTING	Cards 114F					
11 TITLE (Precede with Security Classification Code) ¹¹							
(U) Military Psychiatry							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ¹²							
003500 Military Psychiatry							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
54 09		76 06		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE N/A				PREVIOUS		B. FUNDS (In thousands)	
D. NUMBER *				FISCAL		75	
E. TYPE				CURRENT		4	
F. CUM. AMT.				76		4	
20 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME * Walter Reed Army Institute of Research Washington, D.C. 20012				NAME * Walter Reed Army Institute of Research Division of Neuropsychiatry Washington, D.C. 20012			
ADDRESS *				ADDRESS *			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME. Joy, COL R.J.T.				NAME * Marlowe, D.H., Ph.D.			
TELEPHONE (202)576-3551				TELEPHONE (301)427-5210			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Pearson, MAJ D.W.			
				NAME: Rothberg, J.M., Ph.D.			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Drug Abuse Prevention; (U) Epidemiology; (U) Military Psychiatry; (U) Demography							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number precede text of each with Security Classification Code)							
23. (U) The mission of this unit is to identify psychiatric, psychologic, sociologic and organizational factors which predispose the soldier to perform ineffectively or develop psychiatric illness and to develop more effective preventive treatment techniques.							
24. (U) The research methods of psychology, sociology, clinical psychiatry, anthropology, and social work are used to identify and modify factors that contribute to ineffective military performance.							
25. (U) 75 07 - 76 06 This work unit has been terminated at the end of FY 76. The military family and adolescent dysfunctioning study which describes the families of problem adolescents who seek help at an Army out-patient psychiatric service, is in the final stages of data analysis and write up. The mental health care utilization pilot study examined patterns of mental health care and the utilization of mental health facilities by active duty personnel and their dependents. The Career Outcome Study is a study of the military and medical careers of urine positive and negative soldiers. A cohort selected from three basic training posts has been followed for 24 months. Techniques of personnel and IPDS record analysis have been developed. The Social Adjustment and Multiple Interactive Determinants of Stress Study will study the way in which social factors and social support systems affect responses to chronic stress using hemodialysis and kidney transplant patients as well as control groups as participants in the study. For technical report see WRAIR Annual Progress Report 1 July 75 - 31 June 76. Non-drug related aspects of the research are continuing under new work units 032 and 079.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Investigators.

Principal: COL Harry C. Holloway, MC

Associate: David H. Marlowe, Ph.D.; CPT George D. Bishop, MSC;
MAJ T. Peter Bridge, MC; Linda J. Cunningham, BA;
Rosemary A. Diliberto, MSW; MAJ Juan M. Garcia, MC;
MAJ Steven P. Gilbert, MC; CPT Eugene E. Grossman,
MSC; Glenn T. Gurley, BA; E6 Nelson S. Henry; MAJ
William Hollinshead, MC; MAJ Larry H. Ingraham,
MSC; Ellen N. Levine, MS; E4 James M. Maedke; MAJ
John H. Newby, Jr., MSC; William G. Palm, MA; MAJ
David W. Pearson, MC; E5 Stephen A. Perkins, MAJ
Daniel Redmond, MC; E5 Alberto J. Rivas; Joseph
M. Rothberg, Ph.D.; CPT Robert J. Schneider, MSC;
Boyd Stephenson; LTC Albert Tahmoush, MC; SSG
Charles I. Taylor

Description

This work unit has been terminated due to the reorganization of the Division of Neuropsychiatry. Much of the research will be carried forward in the new Psychiatric Epidemiology, Preventive Psychiatry and Follow-up Medical Evaluation work units for FY 77. The Military Psychiatry work unit included the following investigations: Military Family and Adolescent Dysfunctioning Study; Studies of Individual and Group Responses to Chronic and Acute Stress, the Career Outcome Study, Studies of Psychiatric Epidemiology in Active Duty Populations, Demographic Analysis of the WRGH Psychiatric Patient Study, Utilization of Operationalized Anchor Points with the Brief Psychiatric Rating Scale Study, and Follow-up Studies of Human Volunteers Who Ingested Psychoactive Substances.

1. The Military Family and Adolescent Dysfunctioning Study

Description

a. The Military Family and Dysfunctioning Study describes the families of problem adolescents who seek help at an Army outpatient psychiatric service. The purpose of this study is to differentiate one family from another in terms of the internal family relationships, the external social relationships, and to classify the problems described behaviorally.

b. The military family has been described as being subjected to considerably more family¹²⁴ dislocation and separation from the father than the civilian family. The possibility that separation, frequent moves, and changing interfamilial roles may contribute to the disorganization and dissolution of the military family is of clinical interest. Data collected from this project may contribute to the development of new primary and secondary preventive programs within Army psychiatry.

c. In order to accomplish the study objectives a number of data-gathering techniques have been employed. These techniques include questionnaires, structured and semi-structured individual and group interviews, individual diaries and structured and unstructured video taped family transactions. A self administered questionnaire pertaining to aspects of the study contained in the three major sections has been developed and currently is being tested, for feasibility and validity in further studies.

d. The study is divided into three main sections: the problem section, the social networks section, and the family activity section. Sub-sections of the study direct their attention to: patterns of interaction and communication among family members during structured and unstructured time periods, non-compatible role relationships within families and the effects of recent life events on the behavior of family members. In relation to the latter, all research subjects were administered an instrument which measured the occurrence of recent events within the family milieu.

Progress

The data collection portion of the study has been completed and detailed data analysis has begun. Provisionally, a number of impressions have emerged which seem worthy of reporting at this time.

The data appear to indicate that the primary problems involved in adolescent dysfunction and its stressful effects on the military family are more readily referable to the social milieu of the family than to intrinsic pathology within the family unit. Characteristically the families have indicated stress within the family unit as generated by (1) vague and unclear patterns of communications and (2) failure of families to negotiate or re-negotiate and abide by behavioral norms consistent with psycho-social and maturational changes occurring during adolescence. In an effort to resolve problems, family members tend to move toward, away from or against individuals within the family demonstrating the problem behaviors. The data further indicate (1) a general lack of confluence between the social networks of parents, (2) high levels of "perceived isolation" in families with female identified patients and (3) fragmented patterns of interaction among family members i.e., parents tend to go their separate ways in relation to leisure-time pursuits outside of the home and there is a tendency for family members to isolate themselves in different parts of the home

even though other family members may be present. Analysis of the recent life events data on 9 identified patients and the 15 siblings shows that the SRE distinguishes symptomatic from non-symptomatic subjects at a significant level ($p=0.01$) and in the predicted direction, i.e., the identified patients accumulated higher scores during the 6 months prior to intake at the Child Guidance Clinic than their siblings. It was also noted that parents showed a tendency to under-report significant events reported by their children (both patient and siblings). We have yet to trace out the relationship, if any, between the patterns of family interaction, the structure of their social network relationships, family activities, and variations in role behaviors within family units.

Description

Neuropsychiatric casualties have represented a major source of manpower loss and related costs in military operations in every armed conflict in which the U.S. has been involved in this country. These disturbances appear to be more closely related to sustained performance and intense pulsed combat than to any clear defect in the affected soldier. Therefore Military Psychiatry has maintained a longstanding interest in and commitment to stress research. The symptomatic responses to stressful events are in part determined by the "coping styles" of the individuals undergoing stressful events; that is, one man's stress is another man's challenge, and in part to the social milieu in which an individual experiences a stressful event, the most dramatic example being panic and flight by a group of soldiers vs. continued advance in the face of seemingly insurmountable odds. At present we have only the barest appreciation of how the social milieu organizes, shapes, and reinforces responses to stressful events and processes interact with the coping styles of the individuals who respond to stress. Two studies, one analogue and the other a simulation, have been initiated to explore these issues. The first concentrates on chronic, life-threatening stress experienced by renal transplant and hemodialysis patients. The objective of this research is to describe the events experienced by these patients, their coping styles, and the responses of such social support systems as their families and the care-giving staff.⁵⁻⁶ Assessment of these parameters will be made in terms of outcome of treatment and social adjustment. The second study is being conducted in collaboration with the Department of Medical Psychophysiology at WRAIR and the U.S. Army Research Institute of Environmental Medicine. Subjects for this study will be artillery fire direction control teams from the 82nd Airborne Division who will be brought to the laboratory in Natick to perform their military mission in a simulated battlefield scenario that requires continuous performance over several days under conditions approaching and exceeding work capability for successful performance. Measures will be taken of group performance, physiological functioning, and group/individual responses to the performance demands.

Progress

Contacts with hemodialysis and renal transplant patients as well as suitable controls have been made and follow-through interviewing and observation has begun. Attaining the full complement of 20 research participants has been hindered by administrative changes that have reduced the number of qualified patients at WRAMC. Thus far 5 subjects and their spouses have been seen and evaluated. With respect to the Fire Direction Control project, two teams will play out the scenario at Natick, MA., in February and April 1977. Members of the department will observe both runs, collect relevant questionnaire data on the participants, and describe the ecological context of the experiment as well as the sequence of events and coping strategies of the groups. These descriptive studies will provide an empirical basis for deciding on the suitability of such laboratory simulations for future studies of work groups under stress.

3. The Career Outcome Study

Description

A cohort of drug users who entered the Army during 1972-1973 was defined in order to prospectively study their individual military and medical careers. Since the Army draws from civilian age group at highest risk for drug abuse, urinalysis screening for illicit drugs was routinely done at the reception stations and all positives were to have been medically evaluated. This study was designed to assess the long range behavioral implications of urine positivity at the reception station and, indirectly, to evaluate the medical evaluation procedure. After matching against the Army personnel files, the cohorts consist of 1967 individuals with positive urines and 2432 negative urine controls. The rate of matching was slightly over 80% for each cohort.

Progress

a. Retention on active duty: The time course of the attrition rates for the two groups was similar with 44% of the (U-pos) and 53% of the (U-neg) group remaining on active duty after their first 24 months. The groups were initially 18.6% and 15.0% draftee at the time of entry into the service.

b. Medical impact: Provisional analysis of the combined hospitalization and personnel data (episodes per mean strength) shows that:

- (1) The total hospitalization rates during the 1st 6 months of service were over 4 times greater than the total hospitalization rates during the 4th 6 months of service, for each group.

- (2) The respiratory disease hospitalization rates was almost 30 times greater during the 1st 6 months of service than the respiratory disease hospitalization rate during the 4th 6 month period, for each group.
- (3) The (U-pos) group had 1/3 more hospitalizations than the (U-neg) group.
- (4) The (U-pos) group had 1/7 more hospitalizations than the (U-neg) group after discounting all drug caused hospitalizations.
- (5) The (U-pos) group had 1/2 more hospitalizations for mental disease than the (U-neg) group.
- (6) The (U-pos) group had 1/4 fewer hospitalizations for mental disease than the (U-neg) group after discounting all drug caused hospitalizations.

4. Studies of Psychiatric Epidemiology in Active Duty Populations

Description

Utilizing material available in the Individual Patient Data System a series of pilot analysis have been completed. These provisional studies concerned with the distribution and affects upon The Army Medical Departments resources of psychiatric and psychosomatic disease represent the initial phase of the Department's program in Psychiatric Epidemiology.⁷⁻⁸

Progress

The following provisional summaries have thus far been completed. The data are considered provisional until IPDS tabulations are available for confirmation. Further summaries are in process.

- a. Alcohol, Drug and Psychiatric Data Summary
- b. Hospital and Total Days Lost Data Summary
- c. RVN Intentionally Self Inflicted Wound Data Summary
- d. NP Case Registry; counts of individuals, episodes, records, hospital days and non effective days for selected diagnostic categories by sex by region by year for selected active duty Army hospital disposition records.
- e. Sexual Deviation Summary

- Medical Data by Sex Data Summary
- g. Drug/Alcohol Disease/Symptom Data Summary

5. Demographic Analysis of WRGH Patient Populations 1973-5

Description

Data on all admissions to the Psychiatric Service at WRAMC for the period 1973-5 originally gathered by COMPSY (Computer Support in Military Psychiatry) has been turned over by MISO, WRAMC, to members of the Department of Psychiatry. The analyses planned are designed to determine whether demographic factors affect patterns of diagnosis and disposition of psychiatric patients.

Progress

The data are presently being arrayed and analyzed. It is anticipated that the project will be completed by the end of 7T.

6. Utilization of Operationalized Anchor Points with the Brief Psychiatric Rating Scale

Description

This study is being carried out in collaboration with the National Institute of Mental Health. It involves an attempt to evaluate the function of operationalized anchor points in relation to inter-judge reliability with a frequently used patient rating scale. The hypothesis is that presence of such anchor points on the scale will lead to greater diagnostic consensus among physicians.

Progress

Videotaped interviews have been conducted, under an NIH Protocol and two of these selected for showing to a panel of 40 psychiatrists, both military and civilian. 30 psychiatrists have thus far participated in the study. Completion of the study is anticipated before the end of 7T.

7. Follow-up Studies of Human Volunteers Who Ingested Psychoactive Substances (LSD)

Description

At the Mandate of the Surgeon General, the Division of Neuropsychiatry, WRAIR, was tasked with the development of a research plan and protocol to determine whether or not there have been long-term medical or psychological sequelae following exposure of human volunteers to LSD.

Progress

As per the tasking letter assigning the WRAIR this project, an operations plan was devised, a pilot group of 28 former subjects, specially evaluated by HSC at WRGH, and a second pilot plan formulated. Contact letters have been sent to 488 former participants in Army Psychoactive drug experiments and, at present, 218 individuals are awaiting examinations while attempts are being made to contact others. Special psychological and psychiatric examination modules have been designed as has a comprehensive physical examination system. Control groups have been chosen. The follow-up evaluation study will enter its second pilot phase in 7T and should commence as a full scale operation, with the cooperation of Hospitals in Health Services Command, during FY 77.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Literature Cited.

References:

1. Baker, S.L. et.al: Impact of father absence on personality factors of boys: I. an evaluation of the military family's adjustment. Paper presented at American Orthopsychiatric Association Meeting, Washington, DC, March 1967.

2. Baker, S.L. et.al.: Impact of father absence: III. problems of family reintegration following prolonged father absence. Paper presented at American Orthopsychiatric Association Meeting, Chicago, IL, 1968.

3. Pederson, F.A.: Relationships between father absence and emotional disturbance in male military dependents. Merrill - Palmer Quarterly, 12:321-322, 1966.

4. Pederson, F.A.; Sullivan, E.J.: Relationships among geographical mobility, parental attitudes, and emotional disturbances in children. American Journal of Orthopsychiatry, 34:575-580, 1964.

5. Bridge, T.P.; "Suicide Prevention Centers: an ecological evaluation." Accepted for publication in Journal of Nervous and Mental Disease.

6. Bridge, T.P.; "Distributed adolescent's recall of recent events" Paper presented at A.O.A., Atlanta, GA.

7. Datel, W.E.; A summary of source data in military psychiatric epidemiology. Alexandria, Va.: Defense Documentation Center, AD #A021265, 1976. Prepared while author was on assignment to Division of Neuropsychiatry, Walter Reed Army Institute of Research.

8. Wells, J.D.; Need assessment in human sources consolidation efforts. Alcohol and Drug Abuse Conference, U.S.A. TRADOC, Norfolk, VA.

Publications

1. Rothberg, J.M. A small sample application of discriminant analysis. Abstracts of the Advanced Seminar on Classification and Clustering, 1976, University of Wisconsin Mathematics Research Center.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
				DA OA 6456	76 06 30	DD DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^c	6. WORK SECURITY ^d	7. REGRADING ^e	8A. DSN ^f INSTR ^g	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
75 07 01		U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES ^h	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62758A	3A762758A823	00	031			
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ⁱ (U) Military Performance and Stress: Factors Leading to Decrements of Performance & Disease							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^j 016200 Stress Physiology 013400 Psychology 009400 Man-Machine Relat.							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
61 07		76 06		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
NA				PRECEDING		F. FUNDS (In thousands)	
20. DATES/EFFECTIVE				FISCAL YEAR		CURRENCY	
EXPIRATION				75		3.5	
21. NUMBER ^k				76		3	
22. TYPE				23. AMOUNT		24. FUNDS (In thousands)	
25. KIND OF AWARD				26. CUM. AMT.		27. FUNDS (In thousands)	
				76		215	
28. RESPONSIBLE DOD ORGANIZATION				29. PERFORMING ORGANIZATION			
NAME ^l Walter Reed Army Institute of Research				NAME ^l Walter Reed Army Institute of Research			
ADDRESS ^m Washington, DC 20012				ADDRESS ^m Division of Neuropsychiatry Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME ⁿ Joy, COL R. J. T.				NAME ⁿ Hegge, F. W. Ph.D.			
TELEPHONE ^o 202-576-3551				TELEPHONE ^o 202-427-5521			
30. GENERAL USE				31. SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				[REDACTED]			
32. KEYWORDS (Precede EACH with Security Classification Code) ^p (U) Electrophysiology; (U) Biorhythms; (U) Psychophysiology; (U) Operant Conditioning; (U) Stress; (U) Performance; (U) Human Volunteer				33. ASSOCIATE INVESTIGATORS			
				NAME: Jennings, CPT J. R.			
				NAME: Wood, CPT C. C.			
34. TECHNICAL OBJECTIVE, 35. APPROACH, 36. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Stressful environments, physiological conditions and performance demands likely to produce significant deterioration in the accomplishment of a soldier's mission are studied. The behavioral and physiological functions that contribute to deteriorated performance are identified and therapeutic and prophylactic strategies are developed.							
24. (U) Using psychophysiological and operant methodology, time series analysis, and computer-based control and analysis techniques, behavioral and physiological events are isolated, analyzed, and controlled. Endogenous and exogenous factors contributing to behavioral and physiological rhythmicity and performance levels are studied under specified normal and stressful conditions.							
25. (U) 75 07 - 76 06 This work unit has been terminated as a result of the re-organization of the Division of Neuropsychiatry. Work was accomplished in the areas of stress effects on speech recognition, and the role of cardiac cycle changes in a performance task involving a trade-off between speed and accuracy. The technology needed for the monitoring of psychophysiological functions under field conditions was advanced. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 75 - 30 JUN 76.							
*Available in contractor upon originator's approval.							

PII Redacted

DD FORM 1493

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1493A 1 NOV 65 AND 1493 1 MAR 65 FOR ARMY USE ARE OBSOLETE.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 031 Military performance and stress: Factors leading to decrements of performance and disease

Investigators.

Principal: Frederick W. Hegge, Ph.D.
Associate: CPT John R. Jennings, MSC; CPT Charles C. Wood, MSC; David R. Thorne, Ph.D.; Alison L. Lee; Stanley Hall, B.A.; SP6 Theodore M. DeWitt

Description

An understanding of the biological substrates of stress and performance decrement is important both to military psychiatry and to the performance of normal military missions. The basic research strategy of this work unit is psychophysiological in nature, involving concurrent measures of behavioral processes and physiological activity. Special attention is paid to stressors having their origin in continuous performance requirements, sleep deprivation, temporal disorientation, and drug use. Due recognition is given to the fact that performance is not a unitary construct, but a continuum of human activity ranging from simple motor behavior to the most complex cognitive activity. Research is directed to the experimental delineation of interactions between stressors and complex performance that are functional analogs of militarily relevant activities. These include vigilance, the integration of multiple sources of information, and decision processes. When necessary for scientific clarity, complex performances are analyzed in terms of more basic processes involving sensorimotor, attentional and mnemonic components. A major attempt is made to distinguish between variations in performance which reflect: a) changes in an individual's basic sensitivity, efficiency, or capacity to perform a given task; and b) changes in the response biases, response criteria, or strategies employed in task performance. Whether stress-induced performance decrements involve one or both of these components can have important implications for an understanding of stress-performance relationships and for the design and implementation of counteractive measures. This work unit is terminated, however, non-drug related aspects of the research are being continued under work unit 039, Military Stress: Health, Performance, and Injury Factors.

Progress

1. Development of Speed-Accuracy Tradeoff Functions as Performance Measures

An important example of the distinction between efficiency and response bias components of performance is the development of speed-accuracy tradeoff functions for use in choice reaction time (RT) and other experiments which measure response latency. Choice RT techniques

have played an important role in our human performance research for a number of reasons. First, the requirement for rapid, accurate execution of specified behaviors is critical to successful performance of many military tasks. Second, by varying the form and complexity of the decisions required in choice RT tasks, sensitive measures of a wide variety of human information-processing capabilities can be obtained. Third, choice RT procedures permit a decomposition of task performance into a series of conceptual stages or processes which may be influenced to varying degrees by stress and other variables (34, 37).

Despite these advantages, choice RT procedures as traditionally employed do not allow efficiency and response bias components of performance to be distinguished. For example, the time required for an individual to detect the occurrence of a target signal might be increased under stress for either of the following reasons: a) because stress decreases basic performance efficiency; or b) because a more conservative criterion for responding has been adopted under the stressful conditions. These two alternative explanations would require very different means for counteracting the stress-induced decrement in performance, yet conventional experimental procedures are powerless to distinguish between them.

Over the past two years we have contributed to the development of speed-accuracy tradeoff functions as performance measures which are capable of distinguishing between efficiency and response bias effects. These measures are based on the observation that individuals are capable of trading increases in speed for decreases in accuracy and vice versa over the entire range from chance to near-perfect accuracy (e.g., 23, 31, 35). By inducing subjects to systematically vary their relative emphasis on speed versus accuracy (i.e., their speed-accuracy criterion), a complete speed-accuracy tradeoff function may be derived which expresses the functional relationship between RT and accuracy over a wide range of possible criteria. Such functions may be thought of as operating-characteristics for response latency in the same sense that the functional relationship between hit rate and false alarm rate constitutes an operating-characteristic for signal detection performance (12, 36). Changes in an individual's speed-accuracy criterion are represented by shifts in performance along a single speed-accuracy tradeoff function. In contrast, changes in performance efficiency are represented by a shift in performance from one speed-accuracy tradeoff function to another.

Two papers describing our initial work on speed-accuracy tradeoff functions have been published in the past year. The first (Pub. 8) presents the results of empirical and computer-simulated comparisons of alternative procedures for computing empirical speed-accuracy tradeoff functions. This paper also discusses the importance of the relationship between speed and accuracy within and between different speed-accuracy criteria. Some theories (e.g., 33) predict that the speed-accuracy relationship should be invariant across changes in

criteria, while others predict different functions for different criteria. Our data which are summarized in Figure 1 demonstrate that the speed-accuracy relationship is not invariant across changes in criteria. This figure displays five different speed-accuracy tradeoff functions, each obtained under different speed-accuracy criteria. As these functions clearly show, the relationship between accuracy and RT varied systematically across criteria. That is, accuracy appeared to be more closely related to the RTs that subjects were attempting to achieve than to the actual RTs attained.

The second paper (Pub. 2) describes the application of our methodological developments to the problem of assessing the effects of low-to-moderate doses of alcohol on choice RT performance. Although previous experiments using conventional RT methodology have produced conflicting conclusions regarding alcohol effects (5), the use of the speed-accuracy tradeoff approach revealed a clear decrease in performance efficiency under alcohol which was monotonically related to alcohol dose. The results of this experiment are described more fully in last year's Annual Report.

We have continued to investigate both conceptual and methodological aspects of the speed-accuracy tradeoff procedure with the goal of maximizing the stability of the data obtained while minimizing the time and experimental effort required. These characteristics are highly desirable for the application of speed-accuracy tradeoff procedures to the study of continuous performance and other stress-related variables. A significant improvement has been achieved by changing the reward contingencies from the "RT deadline" procedure employed previously to a "RT target" procedure. The continuous and symmetric reward function of the latter procedure appears to generate more stable and systematic variations in performance than the discontinuous reward function used previously. In the RT target procedure subjects receive monetary reward for correct responses in proportion to the similarity between obtained RTs and the specified RT target. Rewards are maximal for RTs exactly equal to the target value and decrease linearly and symmetrically for both shorter and longer RTs. By systematic variation of the RT target values in different blocks of trials, performance over the full range of speed-accuracy criteria may be assessed.

We have also continued to investigate the question of invariance of the speed-accuracy relationship over changes in speed-accuracy criteria. The conclusion of Wood & Jennings (Pub. 8) that the speed-accuracy relationship is not invariant over changes in criteria has been confirmed in two subsequent experiments. Together, these data provide additional support for our previous conclusion that accuracy is more closely related to the RTs that subjects were attempting to achieve than to the actual RTs attained. In collaboration with Dr. Joseph S. Lappin of Vanderbilt University, we will reanalyze data from

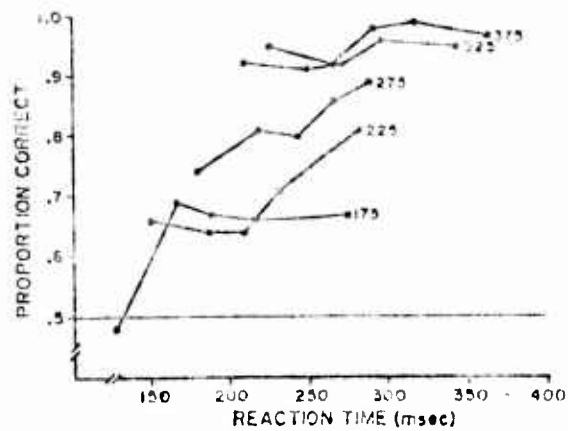


Figure 1: Empirical speed-accuracy tradeoff functions derived from different RT deadline conditions. Subjects were rewarded for correct responses occurring before the specified deadline values (175, 225, 275, 325, and 375 msec, respectively).

previous experiments and collect new data concerning the relative effects of variability in performance efficiency and response criteria on speed-accuracy tradeoff functions. These results should increase our understanding of the properties of speed-accuracy tradeoff functions and make possible their direct application in continuous performance and related experiments. A number of the experiments in the remainder of this report will illustrate the use of the speed-accuracy tradeoff and related signal detection techniques in the study of stress-performance relationships. Future work will apply these procedures to military task environments such as the team performance of a Fire Direction Center as described in Section 4 below.

2. Cardiac Cycle Effects and Speed-Accuracy Trade-Off

The speed-accuracy trade-off design was applied to a potentially important problem in stress research which might be clarified by the improved performance measurement inherent in the design. Certain neurophysiological as well as performance studies have reported a phasic variation in an organism's arousal or capability to perform that is synchronized with the cardiac cycle (2, 21). Autonomic feedback from cardiovascular events is viewed as inhibiting central nervous system activation. Under relatively normal conditions, evidence for such a central-autonomic interaction can be provided by changes in performance associated with changing autonomic tone within the cardiac cycle. Under highly stressful conditions producing continuous sympathetic hyperactivity, a chronic performance decrement might be observed. In the simplest model of such regulation, sympathetic nervous system hyperactivity would lead to heightened autonomic afferent input to the brain. This would decrease central nervous system activity and result in a performance decrement.

Our initial work on this problem was focused on attempting to confirm the existence of changes in performance or cardiac responsivity as a function of the timing of task-related events relative to the cardiac cycle. The term "cardiac cycle time" is used to refer to the time interval between the R wave of the electrocardiogram and the task-related event of interest. The cardiac cycle time of stimulus onset was manipulated to investigate three specific relationships:

- a) cardiac cycle effects on choice RT performance;
- b) cardiac cycle effects on the correlation between anticipatory cardiac deceleration and RT;
- c) cardiac cycle effects on cardiac inter beat intervals (IBIs) during and immediately following the RT stimulus.

The latter two relations refer to the well-established cardiac deceleration which occurs immediately prior to a temporally predictable RT stimulus (16, 21). The Lacey's (21, 22) have previously reported that this cardiac deceleration is correlated to RT and that the magnitude

of this correlation is a function of the timing of the RT stimulus relative to the R wave. In addition they have reported that the IBI concurrent with the RT stimulus is lengthened if the RT stimulus occurs early as opposed to late in the IBI.

In the current experiment, five target RT conditions were employed with RT stimuli presented either simultaneous with the R wave or 350 msec later. Performance was assessed using RT, accuracy, and speed-accuracy tradeoff measures. The results failed to support any effect of cardiac cycle time on any performance measure or on the correlation of IBI and performance. In other words, none of the performance-related measures differed significantly between the 0 and 350 msec delay conditions. This experiment cannot itself disprove the functional significance of autonomic feedback for central nervous excitability. In combination with previous reports, however, it seems unprofitable to continue study of this effect in humans until further physiological details of the phenomenon can be garnered from animal preparations.

Strong support was found for the effect of cardiac cycle time on IBIs concurrent and subsequent to the RT signal. Figure 2 presents the effect cardiac cycle time on cardiac IBIs during the task. The arrows on the abscissa indicate the occurrence of the RT stimulus. When the RT stimulus was delayed by 350 msec., the shift from deceleration to acceleration was delayed into the beat following the stimulus. The influence of this delay continues to be seen throughout the next three beats.

The use of the speed-accuracy trade-off design allowed a detailed assessment of the above cardiac cycle effect and also produced confirming evidence of the effects of attention and motor demands on cardiac IBI. The speed-accuracy design required Ss to pace their RT to various targets producing five sets of RT data differing in mean RT for each subject. An examination of these data showed that the cardiac cycle effect differed as a function of RT. Figure 3 illustrates this effect by plotting for each RT grouping beats 3, 4, and 5 of the cardiac response. Looking particularly at the point when cardiac deceleration shifts to acceleration, one sees that this point successively shifts to later times throughout the initial RT blocks of the 0 delay condition. This shift is largely completed prior to the RT blocks of the 350 msec delay condition. This data suggested that the appropriate unit of time for the expression of cardiac cycle time effects is the time interval between the R wave and response completion.

In addition to the cardiac cycle effects just described, the amount of anticipatory cardiac deceleration and accelerative recovery also varied systematically with RT. These variations suggested attentional and motor influences on the cardiac response. The requirements of the fast targets for attention might be presumed to be higher than that for relatively slower targets. Cardiac deceleration, under certain conditions, has been associated with attention (6, 16). In the

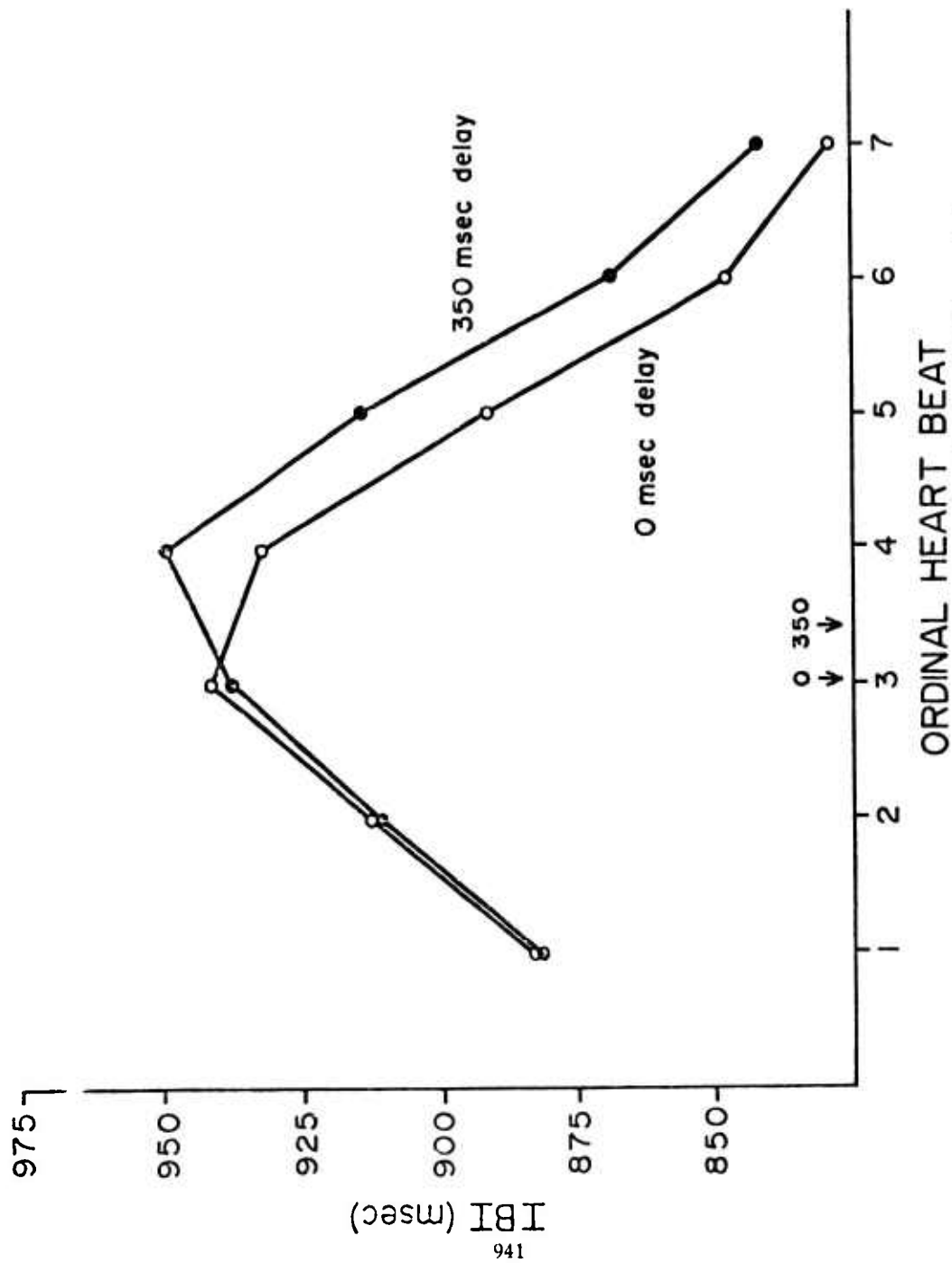


Figure 2. Cardiac IBI plotted over ordinal heart beats for the 0 and 350 msec delay cardiac cycle time conditions. Stimuli were presented at the arrows.

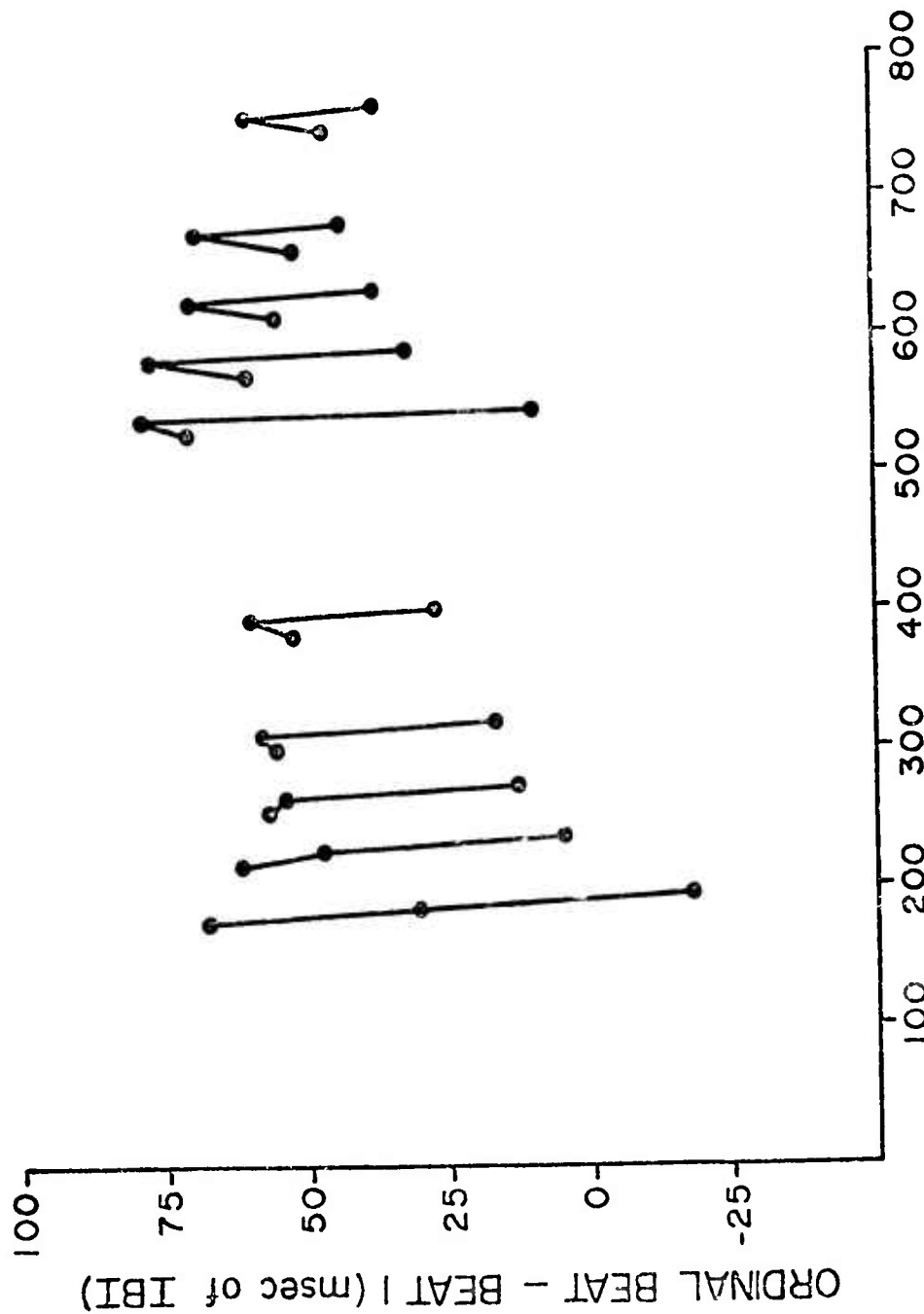


Figure 3. IBI response proximal to stimulus and response as a function of time from the R Wave to response. The difference between the IBIs of Beats 3, 4, and 5 and Beat 1 is presented for each quintile of the RT distribution.

current data the magnitude of deceleration prior to the stimulus decreased as the RT's slowed progressively. A similar trend in magnitude of cardiac acceleration after the RT was observed: acceleration was maximal at short RT and minimal at long. This appears to reflect a coupling between IBI and motor requirements (30).

An interpretative summary relating the findings to neurophysiological studies of vagal effects on IBI may be useful. Two background findings must be mentioned: a) cardiac deceleration in similar RT situations is due almost entirely to vagal activation (29) and b) vagal input to the pacemaker is known to occur predominantly in the initial one-third of the cardiac cycle (7, 17, 19, 24). With these facts in mind, it appears that the RT task sets up a clear temporal-attentional anticipation of the stimuli. This state induces a cardiac deceleration proportional to the strength of the anticipatory state. The deceleration is terminated by the conjunction of task completion (RT) and the timing of completion relative to the R wave. Deceleration is terminated immediately at task completion only if completion occurs prior to the major vagal input for the beat. The strength of accelerative recovery following the RT is then viewed as a function of the motor requirements of the RT performed.

The results of the experiment provide a detailed, consistent picture of the psychophysiological control of cardiac IBI during an RT task. They fail, however, to show significant effects of IBI or cardiac cycle on RT performance. The seemingly direct relationship between the current cardiac IBI results and neurophysiological evidence of the timing of vagal influence on the pacemaker may prove to have significant applications. These would center around use of the RT technique to noninvasively assess vagal tone and responsivity. Timing anomalies in the cardiac IBI response to RT tasks might reasonably be seen as indicative of abnormalities in the vagal control of the heart. It is conceivable that such abnormalities may occur under conditions of stress as well as under conditions of physical disease.

3. Efficiency and Response Bias Effects in Performance Under Speed Stress

Optimal performance in a wide variety of military tasks requires the use of two fundamentally distinct classes of information: a) the information immediately available in stimuli which are present concurrently during task performance; and b) information concerning the probabilistic structure of task-related events derived from an individual's history with the task. The relative contributions of these two classes of information were investigated in a speed-accuracy trade-off design which imposed varying degrees of speed stress on performance in a two-alternative RT task. The RT target procedure described in Section 1 was used to induce different amounts of speed stress in different blocks of trials. Five different RT targets (150, 200, 250, 300, and 350 msec, respectively) were employed. The probabilistic

structure of the task was manipulated by varying the a priori probability of the two stimulus alternatives. In one condition the stimuli were equiprobable ($P(S_A) = P(S_B) = .50$), while in the other the probabilities were asymmetric ($P(S_A) = .30$; $P(S_B) = .70$).

In agreement with a large number of previous experiments (1), error rate decreased significantly in the asymmetric probability condition. However, speed-accuracy tradeoff functions for the two conditions were not significantly different. These results support the conclusion of Lappin & Disch (23) that the improvement in RT and/or accuracy under asymmetric probability conditions reflects not changes in performance efficiency but adjustments in subjects' response criteria.

A comparison of accuracy and response bias measures revealed a systematic decrease in accuracy and an increase in response bias with increasing amounts of speed stress. Under relatively mild speed stress, subjects relied primarily upon concurrently available stimulus information to control performance. Consequently, there was negligible response bias induced by the probabilistic structure of the stimuli under these conditions. However, as speed stress increased in the faster RT targets, subjects relied less upon concurrently available stimulus information and more upon the probabilistic structure of the task. This shift in strategy was reflected in an increase in response bias and a decrease in accuracy under high speed stress conditions.

The results of this experiment were used further as an empirical test of the relative judgment theory (RJT) of psychophysical discrimination recently proposed by Link (26, 27). The concepts embodied in this theory provide a means of conceptualizing the present results which is unavailable in alternative theories. The RJT approach conceives of discrimination as a process of accumulating differences between a given stimulus and an internal reference or standard. The accumulation of such differences is represented mathematically as a random walk process which begins at a starting point under the subject's control and terminates at one of n boundaries representing the n response alternatives in a given task. The distance between boundaries is also postulated to be under the subject's control.

The most important feature of RJT in the context of the present experiment is that it postulates the existence of two distinct types of response bias or response criteria. The first is the degree of bias toward one response alternative or another, represented by the location of the starting point of the random walk process. The second is the total accumulated difference required before a response is generated, represented by the distance between the terminating boundaries. The present results appear to require both of these types of response bias: subjects demonstrated the capability of varying both the amount of information required before a response was generated as well as their bias toward or against each response alternative. Although other mathematical approaches may ultimately prove more appropriate, the dual criterion concept inherent in RJT represents a

significant departure from theories such as classical signal detection theory (12) and choice theory (28) which postulate only a single criterion.

Empirical tests of key predictions of RJT based on the present data support the descriptive validity of the approach. RJT predicts linear relationships between RT and parameters representing the location of the starting point and terminating boundaries. The latter can be directly estimated from empirical data. Within acceptable limits of statistical error, these linear predictions were verified in the present data.

In summary, this experiment provides a clear demonstration of the interaction between concurrently available stimulus information and information concerning the probabilistic structure of task-related events in the control of performance. Subjects used these two types of information differentially under different conditions of speed stress. These results suggest that an important source of error in military tasks involving heavy time pressure is an excessive reliance on expectancies to the exclusion of available stimulus information.

4. Performance decrement and physiological functions in members of a Fire Direction Center (FDC) engaged in continuous, simulated combat

Data analysis of an initial Fire Direction Center exercise is in progress. An FDC team was observed in collaboration with USARIEM, Natick, Mass. over multiple sessions at sea level and altitude. The FDC team was engaged in continuous, simulated combat problems during the sessions. Heart rate data collected continuously throughout each session were assessed for changes in biological rhythms and for changes in brief, phasic responses occurring during fire missions.

Although technical difficulties prevent definitive interpretation, two results are suggested by the available data. First, circadian rhythmicity seemed to be suppressed during performance at altitude. This effect, which was probably a joint effect of fatigue and altitude sickness, represents a significant change in the physiological functioning of the team members. The decrease in amplitude of the rhythmicity occurred despite the fact that the team engaged in shift-work allowing sleep time on a rotating basis. Second, cardiac rate was shown to increase during fire missions although clear, time-locked phasic response to specific events could not be identified. Data analysis has not progressed sufficiently to indicate whether the cardiac responsivity of the team to fire missions changed with continued performance and altitude stress.

At present the primary value of this data lies in its use to develop measurement techniques for the FDC exercise planned in February. Improvements in electronic techniques and research design should allow a clear assessment of the role of cardiac changes within

performance decrements due to continuous performance and altitude stress.

5. Sensitivity and Response Bias in Speech Discrimination

Because of the obvious importance of verbal communication in military performance, a series of experiments has been designed to investigate the relative contribution of sensitivity and response bias to speech recognition performance. In the first experiment in this series, the ability to classify speech stimuli into phonetic categories and to discriminate within and between categories has been investigated. The stimuli were computer-generated synthetic speech stimuli which constituted a continuum from one phoneme category to another. Listeners were presented pairs of stimuli which were either identical or adjacent stimuli along this continuum and were required to discriminate between them. An important finding from previous experiments was verified; namely, that sensitivity was higher at the boundary between phoneme categories than within either category (25). However, the present experiment demonstrated further that the superiority of discrimination at the phoneme boundary was associated with large changes in response bias as well as the increase in sensitivity. Similar results were obtained when the acoustic cues for the phonetic distinction were removed from syllable context and were not categorized linguistically. These later results suggest that the parallel variations in sensitivity and response bias are not limited to linguistic stimuli but may be characteristic of a broader class of information-processing tasks. A paper describing these results is now in press (40). Subsequent experiments have extended these questions to other linguistic stimulus continua and have investigated variables such as extended practice and linguistic expectancy on the sensitivity and response bias effects. These experiments provide additional support for the important role of response bias in the control of military performance. Together with the response bias effects discussed in other sections of this report, these results imply that strong consideration must be given to both the beneficial and harmful effects of response bias in the design and implementation of military tasks.

6. Performance and Autonomic Activity in a Multiple Task Environment

The requirement to perform two or more tasks at the same time is a common stressor which frequently produces decrements in performance. Situational factors and the ability to allocate our attention determine whether all tasks show a decrement, whether performance on one is maintained at the expense of others, or whether some other strategy of resource allocation is employed. The situation in which one task is primary and one secondary provides a means of studying the allocation of performance resources when faced with concurrent tasks. Ongoing research is examining the temporal course of cognitive resource allocation in a serial memory task with a secondary simple RT task. Initial

work demonstrated that RT changed consistently with both learning and recall trials. RTs were longest when the reaction signal occurred simultaneously with item onset and were longer on recall trials than on learning trials. These results were interpreted in terms of selective attention within a limited capacity information processing organism (4, 10, 20). In these terms, retrieval of information from memory and initial perceptual registration of an item seemed to require greater selectivity of attention than other aspects of the task. Looking at the cost rather than the benefit side of this selective attention, one could expect these processes to be relative more prone to performance decrement in the presence of strong competing tasks.

The effect of stress on the allocation and intensity of attention is also relevant to the autonomic components of stress and attention. Stress may have multiple effects on the processes involved in selective attention. It may decrease the intensity of attention available, it may reduce the capability to distribute attention, it may change the relative priority of different tasks, or it may change the criterion for deciding when a task is done and thus change the criterion for shifting attention. Autonomic nervous system functioning has been related to selective attention in two ways: first, arousal-like changes have been said to narrow the focus of attention and induce shifts in the criterion for responding to a task (4); second, specific patterns of autonomic responses have been observed in responses to tasks requiring attention (6, 16, 22).

In order to explore the relationship of autonomic activity and selective attention as indexed by the secondary simple RT task, an experiment was designed to collect both types of data in a multi-trial serial memory task. Degree of attention required was studied by examining changes in RT, cardiac IBI, and respiration over learning trials. As the subject learns an item over trials, the resources (attention) devoted to that item should decrease. Initial results suggest a) that consistent RT and HR changes can be seen during learning and recall trials, and b) that RT and HR are also responsive to degree of learning. Final and specific conclusions must await completion of the study.

7. Effects of Speed Stress on the Processing of Multiple Sources of Information

A related set of experiments concerns tasks which require the processing and integration of multiple sources of information. Such tasks are typical of military performance requirements and may be particularly sensitive to speed stress and continuous performance requirements. An individual's ability to process multiple sources of information has been shown to depend on the stimulus properties of the information sources as well as demands imposed by the specific processing tasks involved (10). Previous experiments have demonstrated that when task-related information from multiple sources is redundant (i.e., statistically correlated), an improvement in performance is often obtained

relative to identical tasks without redundant information sources. Such "redundancy gains" in performance have been obtained in a variety of tasks using both simple unidimensional stimulus continua as well as multidimensional stimulus patterns including written and spoken language (e.g., 9, 10, 39).

We have previously employed a normative mathematical model to study the process of combining redundant sources of information. This model is normative in the sense that it predicts optimal performance levels achievable under a given set of assumptions; departures from optimal performance can then be used as measures of performance impairment. The basic assumptions of the model involve statistically independent and temporally parallel processing of multiple sources of information, with the subsequent response based on whichever component process is completed first. These assumptions are represented mathematically as the problem of determining the minimum of n independent probability density functions. A paper describing the model and its empirical application has been published (Pub. 5).

Subsequent experiments have used the normative model to investigate the processing of redundant sources of information under speed stress induced by the speed-accuracy tradeoff design. The effects of speed-stress clearly disrupt the total amount of redundancy gain in performance relative to conditions of minimal speed stress. However, such decreases in redundancy gains are also predicted by the normative model due to the properties of the RT distributions under speed stress. Analysis of the data now in progress will determine whether the speed stress effects disrupted performance beyond that predicted by the model.

8. Autonomic Correlates of Memory

An additional project is directly concerned with the role of autonomic changes, particularly changes in cardiac inter beat interval, in the amount of attention required by different cognitive tasks or processes. This work proceeds from previous experiments suggesting: a) that memory requirements produce relatively more cognitive load than cognitive manipulation requirements; and b) that such memory requirements also produce a consistent inhibition of cardiac deceleration and a maintenance of acceleration (6, 16, Pub. 1). These results may be set in two general contexts differing in emphasis but not necessarily mutually exclusive. First, the apparent cognitive load induced by memory can be viewed as a stressor and the cardiac responses are then seen as an acute response to this stress. Second, the autonomic nervous system can be viewed as primarily concerned with the regulation of the organism's effort. When greater effort is required, such as by the memory requirements, heart rate responds accordingly reflecting the organism's adjustment to its momentary processing load. The first view would suggest a correlation between cardiac IBI and performance errors and might also predict changes in response bias as a result of the induced arousal. The second view would suggest that cardiac IBI

would be a function of task load even under conditions of error-free performance, and might predict a positive correlation between cardiac acceleration and memory performance. A complex, but perhaps realistic, alternative is to suggest that the cardiovascular control system is responsive to both types of factors and responds in an interactive fashion.

An experiment is in progress which manipulates memory load (number of items to be retained) and measures performance, heart rate, and respiration. This experiment attempts to verify the memory-cardiac IBI relationship using improved performance measures and also attempts to gather data relevant to the above issues. A recognition memory technique is used which allows the measurement of signal detection parameters reflecting strength of recognition memory and biases toward certain judgments. Memory load is varied from 5 to 10 items, thus spanning a region from near perfect performance to error-prone performance.

Initial data verified the presence of a consistent cardiac IBI response to the memory task. Comparisons between memory load levels and performance levels cannot be made at this time.

9. Measurement Technology for Assessing the Effects of State Variables.

The term "state variable" refers to a broad class of variables that produce changes in the physiological state, which in turn modify behavior (drugs, stress, disease, etc.). State variables are sometimes distinguished from "learning variables" that also modify behavior (training procedures, contingencies of reinforcement, practice effects, reinforcement parameters, etc.).

The behavioral assessment techniques currently employed by most laboratories were originally devised for basic research on learning variables, and later adopted for applied research on state variables. These procedures have proved quite valuable but in general they have not been retailored or optimized for their current use, and in some instances have not capitalized on the information, instrumentation and analytical techniques that have emerged since their creation. The department has been testing techniques designed to improve traditional procedures for measuring the psychophysiological effects of state variables.

Our studies have been comparing alternative methods for measuring changes in timing behavior (time estimation, time perception, temporal discrimination). Temporal discrimination is one of the most frequently chosen dependent variables since it is an important component of most behaviors, is sensitive to many environmental and physiological factors, and yields orderly results that generalize across species.

The most widely used procedure for establishing and monitoring a temporal discrimination is the Differential Reinforcement of Low rates

(DRL), a procedure in which an animal or human is trained to make responses at some nominally constant rate. Responses spaced more than t seconds apart are reinforced while shorter inter-response times (IRTs) merely restart the task. This procedure generates a distribution of IRTs clustered about the criterion value. The mean of this distribution is generally decreased by excitatory agents (e.g., stimulants, anxiety) and increased by inhibitory ones (e.g., depressants, fatigue).

The DRL procedure is effective but has two undesirable features. First it confounds timing responses with the time required for reinforcement delivery and receipt. This contamination is asymmetric since it occurs only for the "correct" responses, the proportion of which is controlled by the subject not the experimenter. This artifact leads to several analytical problems and reduces both the sensitivity and face-validity of the procedure. The second undesirable feature of DRL is that it frequently generates a number of extralong IRTs that are off the distribution. The arithmetic mean weights these large values disproportionately and their effect is to constrain or conceal small changes in the mean while distorting its absolute value.

Both of these faults can be corrected by differentially reinforcing response duration instead of inter-response times. This alternative procedure (DRD) has an added advantage in that it still allows IRTs to be measured, independently of duration, yielding a bonus datum for distinguishing between rate and accuracy.

The pilot results reported in last year's Annual Report have now been empirically substantiated for both long and intermediate timing intervals: DRD not only avoids the two problems noted above but also generates significantly sharper response distributions than an equivalent valued DRL, allowing smaller effects to be detected with greater reliability. The study will be extended to the short timing intervals possible with DRD but inappropriate with DRL, where the use of shorter intervals would provide the additional cost advantage of yielding more data points per unit time.

Future plans are to develop and evaluate: (a) a simplified reaction-time procedure that can be trained and stabilized more rapidly than the current techniques; and (b) an interactive temporal baseline that separates the normally confounded contribution of time estimation from other reinforcement and response variables in temporal discrimination tasks.

10. Statistical Problems in the Measurement and Analysis of Psychophysiological Data

a) The ϵ -Adjustment Procedure for Repeated-Measures Analyses of Variance

Psychophysiological data arising from experiments in stress and other areas almost always involve multiple measures on the same

subject. For example, successive heart beats after a stress-inducing event, EEG, and skin conductance waveforms all involve repeated measures on the same individual. Such measures are statistically dependent and the degree of this dependency changes as a function of both time and the effects of stress and other independent variables. In terms of the frequently applied analysis of variance model, these data can be characterized as a repeated measures design with nonhomogeneity in the variance-covariance matrix. Conventional analyses performed on such data have been shown to result in an excessive probability of incorrectly rejecting the null hypothesis (Type 1 error). Two univariate adjustments for the nonhomogeneity problem based on adjustments of degrees of freedom (df) have been developed by Box (3) and Greenhouse & Geisser (11, 13). A conservative, but easily computed solution simply uses $df=1$ in the numerator and the number of independently observed events (e.g., subjects) minus 1 in the denominator. A procedure which more accurately reflects the true Type 1 error probability involves adjustment of df by a factor ϵ based on the degree of homogeneity in the variance-covariance matrix.

A brief paper was recently published from this laboratory (Pub. 3) pointing out the failure of psychophysicists to adequately correct for the nonhomogeneity problem. A survey of the 1975 volume of Psychophysiology showed that 84% of the articles failed to take the repeated measures problem into account. In this light, the paper summarized both the homogeneity problem and the ϵ -adjustment procedure. A Fortran program for computing the ϵ correction was made available.

b) Application of Principal Components Analysis to Psychophysiological Data

The statistical dependencies in psychophysiological data discussed above also raise the question of the intrinsic dimensionality of the data involved. For example, consider the seven sequential cardiac inter-beat intervals (IBIs) measured during each trial in the cardiac cycle time experiment described in Section 2 above. Each seven-IBI response may be thought of as a multivariate observation in seven dimensions, each dimension corresponding to a given IBI. If the sequential IBIs are statistically independent (i.e., uncorrelated), then all seven dimensions are required to represent the data structure completely. However, if the seven dimensions are statistically interdependent (i.e., correlated) as is often the case in psychophysiological data, then all seven dimensions are not necessary and the dimensionality of the data structure may be reduced accordingly.

Principal components analysis is a procedure which empirically assesses the number of statistically independent dimensions necessary to account for an n-dimensional body of multivariate data. This procedure is based on the use of a classical linear model to describe the n original correlated variables in terms of n uncorrelated variables in the following manner:

$$z_j = a_{j1}F_1 + a_{j2}F_2 + \dots + a_{jn}F_n \quad (j = 1, 2, \dots, n)$$

where z_j is the standardized form of the original variable j ; F_1, F_2, \dots, F_n are the derived uncorrelated components; and $a_{j1}, a_{j2}, \dots, a_{jn}$ are weights representing the contribution of each component to the original variable j . The principal components may be ordered in terms of the proportion of the total variance of the original variables accounted for by each component, permitting a direct estimate of the minimum number of components required to approximate the original variables to a desired degree of accuracy.

The basic principle of reducing the dimensionality of multivariate data in this manner was first suggested by Pearson (32) and later developed by Hotelling (14). Our application of this approach is most closely related to that of Tucker (38) who employed the procedures of Eckart and Young (8, 15) for the approximation of a matrix of rank n by a matrix of lesser rank m . This procedure differs somewhat from the approach of Hotelling and from classical factor analytic approaches in that the variance-covariance matrix is the basis of the component decomposition instead of the correlation matrix commonly employed which standardizes each variable (i.e., zero mean and unit variance) prior to component decomposition. Following the principal components solution, the resulting vectors are rotated using the VARIMAX criterion (18).

The decomposition of psychophysiological data into principal components has both methodological and conceptual advantages. Methodologically, it provides a means of approximating a large multidimensional body of data using only a few dimensions. An added advantage is that such dimensions are, by definition, statistically independent. As an example of the utility of the procedure in reducing dimensionality, we have found that the sensory evoked potential represented by 120 sequential time points can be adequately approximated by 5 or 6 principal components. In one example the first two components accounted for 38% and 28% of the variance, respectively, while the first six components together accounted for over 89% of the total variance of the original variables.

The conceptual advantage of principal components analysis of psychophysiological data is that a particular observed variable may reflect the contribution of multiple underlying processes. For example, in the cardiac IBI data discussed above the IBIs occurring in time near the stimulus and response may reflect both the amount of pre-stimulus anticipation and the amount of effort devoted to generating a response. Principal components analysis permits an empirical assessment of the number and form of statistically independent variables which contribute to the observed data.

We are continuing to investigate the most useful and statistically appropriate ways of applying principal components analysis to psychophysiological data. The cardiac IBI data from the speed-accuracy

tradeoff experiment described in Section 2 are being analyzed extensively to determine whether principal components related to attentive and motor processes can be isolated. Such analyses are also being applied to the problem of extracting evoked potential activity related to human performance from single trial EEG waveforms. Preliminary results indicate that a greater proportion of the EEG variance may be related to stimulus processing than has been suggested by conventional signal averaging procedures.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 031 Military performance and stress: Factors leading to decrements of performance and disease

Literature Cited.

References:

1. Audley, R. J.: Some observations on theories of choice reaction time: Tutorial review. In S. Kornblum (Ed.), Attention and performance IV, New York: Academic Press, 1973.
2. Birren, J. E., Cardon, D. V., & Phillips, S. L.: Reaction time as a function of the cardiac cycle in young adults. Science 140: 195-196, 1963.
3. Box, G. E. D.: Some theorems on quadratic forms applied in the study of analysis of variance problems. I and II. Ann. Math. Stat. 25: 290-302 and 485-498, 1954.
4. Broadbent, D. E.: Decision and Stress. New York: Academic Press, 1971.
5. Carpenter, J. A.: Effects of alcohol on some psychological capacities: A critical review with special reference to automobile driving skill. Quart. J. Stud. Alcoh. 23: 274-314, 1962.
6. Coles, M. G. H., & Duncan-Johnson, C. C.: Cardiac activity and information processing: The effects of stimulus significance and detection and response requirements. J. Exp. Psychol: Human Percep. Perf. 1: 418-428, 1975.
7. Dong, E., Jr., & Reitz, B. A.: Effect of timing of vagal stimulation on heart rate in the dog. Cir. Res. 27: 635-646, 1970.
8. Eckart, C., & Young, G.: The approximation of one matrix by another of lower rank. Psychometrika 1: 211-218, 1936.
9. Garner, W. R.: Information integration and form of encoding. In A. W. Melton & E. Martin (Eds.), Coding Processes in Human Memory. Washington, D. C. Winston, 1972.
10. Garner, W. R.: The processing of information and structure. Potomac, Md., Erlbaum Associates, 1974.
11. Geisser, S., & Greenhouse, S. W.: An extension of Box's results on the use of the F distribution in multivariate analysis. Ann. Math. Stat. 29: 885-891, 1958.

12. Green, D. M., & Swets, J. A.: Signal Detection and Psychophysics. New York: Wiley, 1966.
13. Greenhouse, S. W., & Geisser, S.: On methods in the analysis of profile data. Psychometrika 24: 95-112, 1959.
14. Hotelling, H.: Analysis of a complex of statistical variables into principal components. J. Ed. Psychol. 24: 417-441 and 498-520, 1933.
15. Householder, A. S., & Young, G.: Matrix approximation and latent roots. Amer. Math. Monthly 45: 165-171, 1938.
16. Jennings, J. R., Averill, J. R., Opton, E. M., & Lazarus, R. S.: Some parameters of heart rate change: perceptual versus motor task requirements, noxiousness, and uncertainty. Psychophysiol. 7: 194-212, 1971.
17. Jewett, D. L.: Activity of single efferent fibres in the cervical vagus nerve of the dog, with special reference to possible cardioinhibitory fibres. J. Physiol. (London) 175: 31-357, 1964.
18. Kaiser, H. F.: The varimax criterion for rotation in factor analysis. Psychometrika 23: 187-200, 1958.
19. Katona, P. G., Poitras, J. S., Barnett, G. O., & Terry, B. S.: Cardiac vagal efferent activity and heart period in the carotid sinus reflex. Amer. J. Physiol. 218: 1030-1037, 1970.
20. Kerr, B.: Processing demands during mental operations. Mem. & Cog. 1: 401-413, 1973.
21. Lacey, J. I.: Some cardiovascular correlates of sensorimotor behavior: Example of visceral afferent feedback? In C. H. Hockman (Ed.), Limbic System Mechanisms and Autonomic Function. Thomas: Springfield, Illinois, 175-201, 1972.
22. Lacey, B. C., & Lacey, J. I.: Studies of heart rate and other bodily processes in sensorimotor behavior. In P. A. Obrist, A. Black, J. Brener, & L. DiCara (Eds.), Cardiovascular psychophysiology -- Current issues in response mechanisms, biofeedback, and methodology. Chicago, Ill: Aldine-Atherton, 1974.
23. Lappin, J. S., & Disch, K.: The latency operating characteristic: I. Effects of stimulus probability on choice reaction time. J. Exp. Psychol. 92: 419-427, 1972.
24. Levy, M. N., Martin, P. J., Iano, T., & Zieske, H.: Effects of single vagal stimuli on heart rate and atrioventricular conduction. Amer. J. Physiol. 218: 1256-1261, 1970.

25. Liberman, A. M., Cooper, F. S., Shankweiler, D., & Studdert-Kennedy, M.: Perception of the speech code. *Psychol. Rev.* 74: 431-461, 1967.
26. Link, S. W.: The relative judgment theory of two-choice response time. *J. Math. Psychol.* 12: 114-135, 1975.
27. Link, S. W., & Heath, R. A.: A sequential theory of psychological discrimination. *Psychometrika* 40: 77-105, 1975.
28. Luce, R. D.: A threshold theory for simple detection experiments. *Psychol. Rev.* 70: 61-79, 1963.
29. Obrist, P. A., Webb, R. A., Sutterer, J. R., & Howard, J. L.: Cardiac deceleration and reaction time: An evaluation of two hypotheses. *Psychophysiol.* 6: 695-706, 1970.
30. Obrist, P. A., Howard, J. L., Lawler, J. E., Galosy, R. A., Meyers, K. A., & Gaebelein, C. J.: The cardiac-somatic interaction. In P. A. Obrist, A. H. Black, J. Brener, and L. V. DiCara, Cardiovascular Psychophysiology: Current issues in response mechanisms, biofeedback and methodology. Chicago: Aldine, 1974.
31. Pachella, R. G.: The interpretation of reaction time in information processing research. In B. Kantowitz (Ed.), Human information processing: Tutorials in performance and cognition. New York: Lawrence Erlbaum, 1974.
32. Pearson, K.: On lines and planes of closest fit to systems of points in space. *Phil. Mag.* 6: 559-572, 1901.
33. Schouten, J. R., & Bekker, J. A. M.: Reaction time and accuracy. In A. F. Saunders (Ed.), Attention and performance. Amsterdam: North Holland, 1967.
34. Sternberg, S.: The discovery of processing stages: Extensions of Donders' method. *Acta. Psychol.* 30: 276-315, 1969.
35. Swensson, R. G.: The elusive tradeoff: Speed vs. accuracy in visual discrimination tasks. *Percept. & Psychophys.* 12: 16-32, 1972.
36. Swets, J. A.: The relative operating characteristic in psychology. *Science* 182: 990-1000, 1973.
37. Taylor, D. A.: Stage analysis in reaction time. *Psychol. Bull.* 83: 161-191, 1976.
38. Tucker, L. R.: Learning theory and multivariate experiment: Illustration by determination of generalized learning curves. In R. B. Catell (Ed.) Handbook of Multivariate Experimental Psychology, Chicago: Rand McNally, 1966.

39. Wood, C. C.: Parallel processing of auditory and phonetic information in speech discrimination. *Percept. Psychophys.* 15: 501-508, 1974.

40. Wood, C. C.: Discriminability, response bias, and phoneme categories in discrimination of voice onset time. *J. Acous. Soc. Amer.*, in press.

Publications:

1. Jennings, J. R.: Information processing and concomitant heart rate changes in the obese and underweight. *Physiol. Psychol.* 3: 290-296, 1975.

2. Jennings, J. R., Wood, C. C., & Lawrence, B.: Effects of graded doses of alcohol upon speed-accuracy tradeoff in choice reaction time. *Percept. Psychophys.* 19: 85-91, 1976.

3. Jennings, J. R., and Wood, C. C.: The ϵ -adjustment procedure for repeated-measures analyses of variance. (Letter). *Psychophysiol.* 13: 277-278, 1976.

4. Wood, C. C.: Average evoked potentials and phonetic processing in speech perception. In J. E. Desmedt (Ed.), Recent Developments in the Psychobiology of Language. Oxford University Press, 1976.

5. Wood, C. C.: A normative model for redundancy gains in speech discrimination: In F. Restle, R. M. Shiffrin, N. J. Castellan, H. Lindman, and D. Pisoni (Eds.), Cognitive Theory: Volume I. Potomac, Maryland: Erlbaum Associates, 1975.

6. Wood, C. C.: Discriminability, response bias, and phoneme categories in voice onset time discrimination. (Abstract). *J. Acoust. Soc. Amer.* 58: S58, 1975.

7. Wood, C. C.: Discriminability, response bias, and phoneme categories in speech discrimination: II. Place of articulation. (Abstract). *J. Acoust. Soc. Amer.* 59: S26, 1976.

8. Wood, C. C., & Jennings, J. R.: Speed-accuracy tradeoff functions in choice reaction time: Experimental designs and computational procedures. *Percept. Psychophys.* 19: 92-101, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6438	76 06 30	DD-DR&E(AH)636	
3 DATE PREV. SUMMRY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^b	6 WORK SECURITY ^b	7 REGRADING ^c	8A DDM'S INSTR ^d	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
75 07 01	H. Termin.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
10 NO. CODES ^e	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	62758A	3A62758A823	00	C33			
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11 TITLE (Precede with Security Classification Code) ^f							
(U) Anatomical and Physiological Correlates of Brain Function in Stress and Disease							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^g							
002400 Bioengineering				016200 Stress Physiology			
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
63 08		TERMINATED 76 06		DA		C, In-House	
17 CONTRACT GRANT							
A. DATES/EFFECTIVE NA				B. RESOURCES ESTIMATE			
EXPIRATION				C. PROFESSIONAL MAN YRS			
C. NUMBER ^h				D. FUNDS (In thousands)			
D. TYPE				FISCAL YEAR			
E. KIND OF AWARD				CURRENT			
I. CUM. AMT				76			
75				6			
76				5			
178				587			
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME ⁱ Walter Reed Army Institute of Research				NAME ⁱ Walter Reed Army Institute of Research			
ADDRESS ^j Washington, D. C. 20012				ADDRESS ^j Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DDAN if U.S. Academic Institution)			
NAME ^k JOY, COL R.J.T.				NAME ^k Spector, N.H., Ph.D.			
TELEPHONE ^l 202-576-3551				TELEPHONE 202-576-3457			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Petras, J.M., Ph.D.			
				NAME: Wylie, R.M., Ph.D.			
22 KEYWORDS (Precede EACH with Security Classification Code) ^m							
(U) Neurophysiology; (U) Neuropsychiatry; (U) Neuroanatomy							
(U) Combat Shock; (U) Autonomic Nervous System; (U) Biomedical Engineering; (U) Stress							
23 TECHNICAL OBJECTIVE, ⁿ 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The principal research objectives are to identify the anatomical and physiological mechanisms by which the central nervous system maintains vital functions during or following physiological stress, disease, or trauma in the military environment.							
24. (U) The disciplines and techniques of neuroanatomy, neurophysiology, physiological psychology, and neurochemistry are used.							
25. (U) 75 07 - 76 06 Research continued on brain mechanisms mediating control of skeletal muscle and on those mediating equilibrium of internal organs. Experiments directed toward the neural control of posture and movement included study of the activity of vestibular neurons during purposeful movement, morphology of vestibular nerve synapses, anatomic identification of spinal cord systems providing input to the cerebellum, purposeful movement and fatigue in primates deprived of sensory input from the limbs, and organization of neuron populations in sensory-motor cortex. Experiments directed toward neural control of homeostasis included identification of spinal cord centers controlling blood pressure, hypothalamic regulation of body temperature, anatomic identification of spinal cells of origin of specific visceral nerves, and central pathways for analysis of visceral sensory information. Work unit terminated due to Division re-organization; elements of this work to be continued under work units 071 and 072 of task area 01, project 3A161102B71P. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 01 July 75 - 30 June 76.							

PII Redacted

DD FORM 1498

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 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 033 Anatomical and physiological correlates of brain function in stress and disease

Investigators.

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

Associate: J.F. Cummings, Ph.D. DVM; George F. Koob, CPT, MSC; Gregory E. Martin, Ph.D; Epp A. Miller, Ph.D.; J.M. Petras, Ph.D.; Andrew T. Pryzbylik; Maurice E.T. Swinnen; C.F. Tyner, MAJ, MC; R. M. Wylie, Ph.D.

DESCRIPTION

Experiments in this work unit are designed to bring the techniques of physiology and anatomy to bear on those aspects of brain function which are essential to sustained performance in the military environment or to the body's response to stress and injury in that environment. Current experiments include study of: a) hypothalamic neurons critical to body temperature control; b) spinal cord nuclei on control of the cardiovascular system; c) neural regions which control posture and movement and the recovery of useful movement after brain injury.

PROGRESS

Hypothalamic Neurons Concerned with Temperature Regulation

A population of neurons in the medial preoptic and anterior areas of the hypothalamus have been found to have unusual Q10s when defined in terms of spontaneous firing rates in vivo. These cells have been called internal temperature sensors and are believed to play a major role in temperature regulation of endotherms and perhaps exotherms. No data are available, however, on which to postulate the basis of this temperature sensitivity; in particular, it is unclear whether the property is intrinsic to the neurons or is a function of the circuits to which the cells belong. This issue can be resolved if these neurons are available for individual study in tissue culture. Techniques have been developed here for growing these cells in culture and for recording their spontaneous and evoked electrical activity under a wide variety of controlled temperature conditions. During the past year, the media composition and culture methods have been standardized, pre-optic neurons have been grown for up to five months and have shown development of substantial morphological diversity during that time, an accurate means of controlling

media temperature has been developed, and preparations have been made to record the neuronal electrical activity.

Spinal Autonomic Nuclei: Pressor Centers

Investigations have been underway on the influence of spinal autonomic nuclei on cardiovascular function. Initial experiments in spinal cats involved exploring the cord with stimulating micro-electrodes to locate cell groups able to cause increases in blood pressure. Results indicate that these cells are well localized in the intermediolateral nucleus (ILN) of the thoracic spinal cord. Contrary to earlier reports, these pressor neurons are distributed over the entire longitudinal extent of the thoracic region. Furthermore, there is a substantial lateralization of pressor influence: ILN activation on the right side of the cord produces substantially greater increases in systemic blood pressure than does comparable activation on the cord's left side. This lateralization parallels that described by others for the neurons controlling heart rate although the longitudinal distribution of the two neuron sets appears different. Future work will concern spinal effects on the specific components of a pressor response: heart rate, cardiac contractility, and peripheral vasoconstriction.

Spinal Autonomic Nuclei: Input Systems

The preganglionic neurons are the final common pathway for the autonomic nervous system's output from the spinal cord: all neural control of the viscera by the spinal cord involves participation of these neurons. The pathways by which other parts of the nervous system control these final output elements are poorly known, however. Recently begun experiments in the Department are designed to: a) describe the distribution and cellular appearance of spinal visceral motor neurons; b) describe the input to these motor neurons from elsewhere in the spinal cord; c) describe the inputs which descend to these motor neurons from sites above the spinal cord, with particular emphasis on nuclei in the brainstem. The principal techniques in use are anatomic: retrograde degeneration, transport of horseradish peroxidase, and transport of radioactive amino acids. Initial results indicate that retrograde transport of horseradish peroxidase from peripheral sympathetic ganglia to the spinal cord is an effective means of locating visceral motor neurons in the cord with much accuracy.

Spinal Autonomic Nuclei: Routes for Visceral Sensory Information

Three major ascending pathways in the spinal cord transmit somatic sensory information to higher centers; dorsal columns, spinothalamic tract, and spinocervical tract. The first two have been previously shown to transmit sensory information from the viscera as well. Recent experiments in this Department, using the single-unit recording method, have demonstrated that the lateral cervical nucleus -- the principal relay in

the spino-cervical system, located in the upper cervical spinal cord -- also transmits information from the viscera. This input is carried over the greater splanchnic nerve, and possibly other visceral nerves as well, and may signal information about the heart and abdominal blood vessels. Current work is directed toward precise delineation of the interactions between visceral and somatic sensory inputs in the lateral cervical nucleus. An abstract on the work has been accepted for presentation at a forthcoming symposium on the abdominal nervous system (Visceral Afferent Projections to the Lateral Cervical Nucleus, D.D. Rigamonti and D. DeMichelle, 1976).

Posture and Movement: Neuron Populations in Sensory-Motor Cortex

Experiments designed to describe the input-output relations and interactions among neuron populations in the sensory-motor cerebral cortex have been continued, using single-unit recording methods. The most recent experiments have been directed at the wide-field population in this tissue as these neurons may be exemplary of motor cells throughout mammalian nervous systems. Work during the past year has extended the earlier ideas that these cells are receptive to a great variety of inputs by showing them to be activated from two separate spinal cord pathways, the dorsal columns and the spino-cervical tract, and by showing them to respond to activation of the splanchnic nerve in the abdomen. In addition, analysis of the increasing data base for these cells indicates that the wide-field population may have an internal organization in the form of a serial ordering of its members and that this ordering may be a central factor in the population's behavior.

Posture and Movement: The Cervical Spino-cerebellar System

Although anatomists have traditionally recognized two pathways providing input to the cerebellum from the spinal cord, the spinal nuclei of origin of only one of these -- the dorsal spino-cerebellar tract -- is known. Both anatomists and physiologists have disputed the origin of the ventral tract and the situation has been complicated by the claim that the tract arises partly in the lumbar and partly in the cervical spinal regions. Recent experiments in the Department have established conclusively that cells in the cervical spinal cord do project to the cerebellum, and have allowed identification of the nuclear groups involved. Identical results from work with horseradish peroxidase and retrograde degeneration techniques indicate that the nucleus cervicalis centralis and the nucleus centrobasis in the cervical cord project to the cerebellum, the former contralaterally and the latter ipsilaterally. In addition, an electron microscope examination of these nuclei has made possible a description of the fine structure of the cells involved and of the several synaptic types responsible for input to these cells. Two manuscripts have been prepared for submission to the Journal of Comparative Neurology.

Posture and Movement: Activity of Vestibular and Fastigial
Neurons in Waking Cats

Prior work by others has implicated the vestibular nuclei of the brainstem and the fastigial nuclei of the cerebellum in control of posture and movement although the details of their participation are unclear. In an effort to detect the predicted changes in neuron firing which should accompany movement, experiments in the Department have been directed at recording neuron activity in these nuclei in awake cats trained to perform limb movements on command. Although the animals are still under study so that conclusive information on the location of all cells is not yet available, results to date indicate fewer than ten percent of neurons studied show changes in firing pattern clearly related to limb movement; this result is in striking contrast with those of similar studies conducted on a wide variety of other central nervous system motor nuclei. Study of these cells is continuing, as are efforts to suggest alternate theories concerning the roles of these nuclei in the control of skeletal muscle.

Posture and Movement: Recovery of Limb Use After Dorsal Rhizotomy

Severance of dorsal roots innervating a monkey's limb produces profound sensory and motor deficits, although limited recovery of the motor loss is possible. Experiments in the Department have been directed at producing quantitative descriptions of the recovery process and the nature of recovered functions after such surgery. Normal monkeys have readily learned to lift weights to a given height when suitably rewarded and to perform the task with great accuracy over a wide range of imposed weights. As the weight is increased, the total work performed in a session changes as does the muscle fatigue and the monkey's behavioral compensation for it. In contrast, a de-afferented monkey trained to perform the same task, initially with no imposed load and later with a moderate load, showed no ability at first to compensate for these factors, and the animal's present performance remains substantially poorer than that of normals.

Biomedical Instrumentation

Instrumentation development included design and fabrication of a fast-tracking digital voltmeter able to capture and hold peaks, a digital ECG calibrator, a photocell amplifier to pinpoint an animal's exact position in a test apparatus, and several signal simulation systems for testing data processing equipment. In addition, instrumentation personnel offered a course in digital logic circuitry to Department investigators and technicians.

Work in this unit was terminated on 30 June 1976; elements of the program will be continued in work units 072, Biological Modulation of Military Performance, and work unit 040, Behavioral Variables in Autonomic Function and Disease in Military Personnel.

Project 3A762758A823 MILITARY PSYCHIATRY

Tast 00 Military Psychiatry

Work Unit 033 Anatomical and physiological correlates of brain
function in stress and disease

Literature Cited.

Publications:

1. Aminoff, M. J., Layzer, R. L., Satymurti, S. and Faden, A.I.: The declining muscle response to repetitive nerve stimulation in myotonia. *Neurology* 26: 337, 1976.
2. Faden, A. I. and Townsend, J. J.: Myoclonus in Alzheimer Disease. *Arch. Neurol.* 33: 278, 1976.
3. Faden, A. I.: Encephalopathy following treatment of chronic pulmonary failure. *Neurology* 26: 337, 1976.
4. Faden, A. I., Collins, M., and Spire, J. P.: Electroencephalographic changes following intra-amniotic prostoglandin F2 administration for therapeutic abortion. *Obstetrics and Gynecology* 42: 607, 1976.
5. Neeley, W. E., Wardlaw, S. S., and Swinnen, M.E.T.: Design of a novel electronics debubbler for continuous flow analyzers. 9th International Congress on Clinical Chemistry 1975.
6. Neeley, W. E., Wardlaw, S. S., Yates, T., Hollingsworth, W. G., and Swinnen, M.E.T.: A high performance colorimeter with an electronic bubble gate for use in miniaturized continuous flow analyzers. *Clin. Chem.* 22: 227, 1976.
7. Pryzbylik, A. T. and Howe, R. C.: A low-noise cable and slip-ring assembly for recording of small amplitude physiological signals in chronic animals. *Med. Biol. Eng.* in press, 1976.
8. Tyner, C. F.: The naming of neurons: applications of taxonomic theory to the study of cellular populations. *Brain, Behav., Evol.* 12: 75-96, 1975.
9. Towe, A. L., Tyner, C. F., and Nyquist, J. K.: Facilitatory and inhibitory modulation of wide-field neuron activity in the post-cruciate cortex of the cat. *Exper. Neurol.* 50: 734-756, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD DR&E(AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A ORG'N INST'N	8B SPECIFIC DATA - CONTRACTOR ACCESS	
75 07 01	H.Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
9 NO CODES ^a		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		672758A	3A762758A823	00	034		
B. CONTRIBUTING							
C. CONTRIBUTING		CARDS 114F					
11 TITLE (Precede with Security Classification Code) ^a (U) Influence of Stress on Hormone Response, Performance and Emotional Breakdown in Military Personnel							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 013400 Psychology 016200 Stress Physiology 005900 Environmental Bio							
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 ^a				FISCAL		75	
C. TYPE				CURRENT		4	
D. KIND OF AWARD				76		302	
E. CUM. AMT.							
20 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Institute of Research				NAME: ^a Walter Reed Army Institute of Research			
ADDRESS: ^a Washington, DC 20012				ADDRESS: ^a Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursue MAN if U.S. Academic Institution)			
NAME: JOY, COL RJT				NAME: ^a Meyerhoff, JL, MD			
TELEPHONE: (202) 576-3551				TELEPHONE (202) 576-3559			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Mason, JW, MD			
				NAME: Mougey, EH			
22 KEYWORDS (Precede EACH with Security Classification Code) (U) Stress; (U) Emotions; (U) Homeostasis; (U) Psycho-physiology; (U) Neuroendocrinology; (U) Psychoendocrinology; (U) Human Volunteer							
23. (U) Principal objective is to study the integrating influences of the central nervous system in controlling and coordinating the organs of the body and their metabolic functions under environmental and emotional stresses which are likely to produce casualties due to psychiatric or psychosomatic disease in military personnel.							
24. (U) This involves measurement of plasma and urinary hormone levels in humans and monkeys in a variety of acute and chronic stress situations. One important conceptual approach is that the organization of endocrine regulation can only be understood by viewing "overall" hormonal balance, or multihormonal patterns.							
25. (U) 75 07 - 76 06 Collaborative studies of physical stress with ARIEM have continued. Studies of acute heat exposure (49 degrees C for 90 min) with and without superimposed muscular exertion in normal young men have shown that heat, even in the presence of some discomfort and psychological disturbance, produces no increase or a mild decrease in plasma cortisol levels and that the further superimposition of muscular exertion does not result in increased pituitary-adrenal cortical activity. A recent pilot experiment indicated a striking 12 fold increase in plasma renin levels in response to capture and restraint in the rhesus monkey. This is the largest renin response to any type of stress yet recorded and indicates that renin secretion is sensitively responsive to psychological stress and should be considered more fully in future psychoendocrine studies. Elements of this work will be continued under Work Unit 073 Neuroendocrine Response to Military Stress, Program Element 6.27.71A. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

PII Redacted

DD FORM 1498 1 MAR 68

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

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 034 Influence of stress on hormone response, performance and emotional breakdown in military personnel

Investigators.

Principal: James L. Meyerhoff, M.D.

Associate: John W. Mason, M.D.; Robert H. Lenox, M.D., MAJ, MC;
Gregory L. Belenky, M.D., MAJ, MC; G. J. Kant, Ph.D.;
E. H. Mougey, M.S.; F. E. Wherry, A.B.; J. W. Holaday,
M.S.; D. R. Collins, B.S.; L. L. Pennington, B.S.

I. Endocrine Profile as Indicator of Stress Response

Description.

This program is concerned primarily with the role of the central nervous system in the control and especially the coordination of endocrine regulation. As this program has gradually developed, some general concepts have emerged which appear to have major and far-reaching implications for the field of stress research. Included among such concepts which have been particularly important in opening up productive new avenues of stress research are:

- 1.) The neuroendocrine apparatus represents a third effector system of the brain (along with the autonomic and skeletal-muscular systems) providing sensitive and objective reflections of central integrative processes, such as emotions and psychological defenses, which are of key importance in human performance under stress, but which have so far been extraordinarily difficult to bring under rigorous experimental investigation.
- 2.) There is now serious doubt concerning the validity of Selye's "non-specificity" concept in stress theory, because of considerable recent research indicating that psychoendocrine reactions are frequently and inadvertently elicited during experiments designed for the study of physical stimuli. There is a pressing need, therefore, for a thoroughgoing reevaluation of past research on neuroendocrine responses to the physical stresses, with closer scrutiny of independent variables and special attention to the possible contamination of physical stress experiments by psychoendocrine reactions reflecting attendant discomfort, pain, or emotional reactions. Our recent studies of muscular exertion, fasting, and heat, for example, indicate that erroneous conclusions concerning the effects of these stimuli upon neuroendocrine systems are likely to be drawn unless rigorous and systematic efforts are made to evaluate and to minimize interfering psychoendocrine reactions.
- 3.) While it has long been the prevailing practice in stress research to study isolated endocrine systems, usually one at a time, it is increasingly clear that the

many neuroendocrine systems are closely interdependent in their functions and that a key to the understanding of the principles underlying the integration of these systems lies in the study of relative changes between interacting endocrine systems, as manifested in the organization of patterns or profiles of multiple hormonal responses to various stressful stimuli. In recent years, we have learned that relatively distinctive, broadly organized patterns of hormonal changes, involving many hormones in addition to those of the adrenal systems, occur in response to various types of psychological and physical stress. A major immediate goal, therefore, is to define as conclusively as possible the characteristic hormone response profiles for various stressful stimuli, with principal emphasis on psychological stimuli, but also including exercise, heat, cold, fasting, hypoxia, infection, and other physical stimuli encountered in military stress situations. Such basic knowledge of the organization of integrative machinery is an essential foundation for more complex neuroendocrine approaches to the study of stress-related clinical and field problems concerned with such parameters as endurance, fatigue, host resistance, performance, and the pathogenesis of some psychosomatic disorders. It is clear that this approach logically must move eventually through a series of successive stages, beginning with 1.) basic definition of response profiles for the various stressful stimuli, 2.) determination of the degree of response profile specificity for diverse, discrete stimuli, 3.) evaluation of factors which determine response profile priorities when there are various natural admixtures of multiple, concurrent stimuli, 4.) evaluation of the physiological significance of differing neuroendocrine response profiles by extending the approach to the concurrent study of their metabolic or physiological concomitants or consequences, and 5.) evaluation of the degree to which both acute and chronic hormone response profiles may be adaptive or maladaptive. It is evident that each of the above projected stages in the development of this conceptual approach is in large measure dependent upon establishment of prerequisite knowledge in the preceding stage, so that the stages must generally best be pursued in logical sequence. Our efforts at present, therefore, are still largely limited to the sizeable task involved in just the first two stages of this approach. The amount of stress response profile data accumulating, however, is already quite substantial and is providing an increasingly useful basis for the clarification and revision of stress concepts, as discussed in some detail in a recent overview of our approach (Toronto Neuroscience Symposium). During the past year, a major portion of our effort has been devoted to continued collaboration in physical stress with the Army Research Institute for Environmental Medicine (ARIEM) at Natick, MA.

Developmental work has also continued on new or refined hormone assay procedures in order to provide the necessary, up-dated methodological foundation for this stress research program.

Progress

A. Organization of Neuroendocrine Responses to Psychological Stress. Profile of Acute Hormonal Responses to Capture and Chair Restraint in Monkeys: The development of highly sensitive and reliable new methods for the measurement of plasma levels of certain hormones has now made possible, for the first time, in our laboratory, the study of a relatively detailed profile of neuroendocrine responses during acute emotional reactions. The selection of hormonal indices in our research program is based primarily on the rationale that it is logical in the study of neuroendocrine organization to begin with endocrine systems that have well-established neuroendocrine linkages, that is, systems in which endocrine cells articulate with nerve cells, either via neural or neurohumoral connections. The battery of methods used in this study includes those for the measurement of plasma cortisol, epinephrine, norepinephrine, total thyroxine, thyrotropin, testosterone, growth hormone, insulin, prolactin, and glucagon. Chair restraint and capture was selected as a particularly suitable situation for response profile study, since previous research with the adrenal systems had shown it to be an especially potent, reliable, and convenient psychoendocrine stimulus. Following a period of at least one week of cage housing in a quiet, stable environment, monkeys were captured as rapidly as possible and blood samples obtained by saphenous venipuncture at intervals of 1, 3 and 5 minutes. The monkey was then installed in the restraining chair and additional samples obtained at 20, 40 and 60 minutes, 2, 4, 6, 24 and 48 hours. A primary series of 8 capture and restraint experiments and 12 catheter control experiments have now been completed and all hormonal analyses are now completed. A family of hormonal response curves has been defined, with different hormones having remarkably different dynamic characteristics. Prolactin levels show about a four-fold increase, peaking at 20 minutes after onset of restraint. Growth hormone shows greater than a fifteen-fold elevation peaking at 40 minutes. Cortisol shows about a three-fold elevation peaking at 4 hours. Thyrotropin levels show nearly a two-fold increase during the first hour, but total thyroxine rises only very slowly to about a two-fold elevation at 48 hours. Testosterone levels, after a mild and transient rise, show a slow but marked decline over a 24 hour period and remain very low for at least a week. Plasma insulin levels generally tend to be suppressed after 4 to 6 hours. Preliminary evidence indicates that a four-fold increase in plasma glucagon levels may occur within 5 minutes after capture.

During the past year a new series of these capture-restraint experiments has been completed which will permit the addition of plasma epinephrine, norepinephrine and renin measurements to enlarge the profile. In the first experiment with plasma renin measurement, a marked, approximately eight-fold elevation was observed, peaking at 2 hours and remaining high for at least 6 hours after restraint onset. This is the most striking evidence yet available for the responsiveness of renin levels to psychological stress.

In general the overall pattern of acute neuroendocrine responses observed following capture and chair restraint is closely similar to that observed earlier in conditioned avoidance experiments and chair restraint experiments which were of longer duration and dependent largely upon 24-hour urinary hormonal excretion measurements. In these earlier studies it was difficult to evaluate in some instances the extent to which such factors as altered food intake, altered sleep patterns, prolonged postural changes, or altered muscular activity might have been determinants of the neuroendocrine response pattern along with psychological factors. The present study, however, using plasma hormone measurements during the initial minutes and hours of restraint, before the above non-psychological variables become significant factors, provides a much stronger basis for interpreting these data as representing primarily a psychoendocrine response pattern. The data on prolactin, glucagon, and renin provide some of the most striking data available so far, particularly with regard to control of non-psychological variables, which indicate that these three hormones should be included among the growing assemblage of hormones which are sensitively responsive to psychological stimuli.

Finally, control experiments incorporated in this study include experiments in which blood samples were taken by saphenous venipuncture on exactly the same schedule after one month of restraint and three subsequent control experiments performed in each monkey at weekly intervals in which blood samples on the same schedule were withdrawn remotely through chronic indwelling venous catheters in order to minimize psychological disturbance of the monkeys. Only very minor hormonal fluctuations have been observed in the catheter control experiments. In the control saphenous venipuncture experiments, a pattern of response very similar to that seen with acute capture and restraint has been observed, but generally hormonal changes are of substantially smaller magnitude.

B. Organization of Neuroendocrine Responses to Physical Stress.

1. Neuroendocrine Studies of Heat Acclimatization.

In continuation of our studies of heat exposure in collaboration with Dr. John Maher of the ARIEM laboratories at Natick, we have studied hormonal profiles in two groups, each composed of four normal young men; one group during a "control" first week with no treatment and a second week with daily 90 minute exposure to 43°C (30% R.H.); the second group had mild daily 90 minute physical training sessions at 25°C the first week and 43°C (30% R.H.) the second week.

Cortisol. Plasma cortisol levels declined rather sharply during heat exposure alone, but showed less of a decline when muscular exertion was superimposed. It is especially noteworthy that cortisol levels showed no significant increase in any of the heat exposure experiments although exercise and presumably emotional reaction from the discomfort was present during exposure to such intense heat.

Growth Hormone. Plasma growth hormone levels did not change significantly during heat exposure alone, but did show moderate elevations when muscular work was superimposed. However, levels were also elevated in the exercise group on the control day with catheterization alone.

Testosterone. Plasma testosterone levels showed a slight trend upwards with both heat and exercise-heat conditions but the changes are not significant.

Insulin. Plasma insulin levels did not change significantly with heat alone but decreased sharply when exercise was superimposed.

Total Thyroxine. Plasma thyroxine levels did not change significantly during the brief heat exposure periods nor with exercise although there was a mild upward trend in levels.

In general, these findings confirm those of a previous study of acute heat exposure, particularly with regard to the failure of heat "stress" to stimulate the pituitary-adrenal cortical system, thus providing still further data militating against the "non-specificity" concept of Selye in stress theory. In addition, this study indicates that heat may exert a suppressive influence which overrides the concurrent excitatory influence of psychological stress and exercise. Growth hormone and insulin responses to exercise do apparently occur, however, during heat exposure.

It is questionable whether any conclusions concerning heat acclimatization effects can be drawn from these experiments, since physiological measures such as rectal temperature and heart rate indicated only a mild degree of heat acclimatization at the end of the second week in the exercise-heat exposure group. It appears likely that there was appreciable residual summer acclimatization in this group of young men from Ft. Jackson, S.C., studied in Boston in October. It appears advisable to view these present data, then, largely in terms of acute and repeated short term heat exposure with and without concurrent muscular exertion, as discussed above. Further studies would be needed of subjects showing more marked physiological evidence of heat acclimatization before firmer conclusions could be drawn regarding possible hormonal correlates of such acclimatization.

II. Mechanism of Neuroendocrine Response to Stress

In order to study neurochemical mechanisms concomitantly with hormonal response to environmental stress, it has become necessary to develop new models. It is well established that stress causes elevations in plasma corticosterone (1-5) and prolactin (6) while lowering plasma growth hormone (3,4). Corticosterone levels following stress vary with time of day, reflecting the circadian rhythm in testing levels (2). It has been demonstrated that habituation occurs to the corticosterone response induced by handling (3,4). Stress is reported to activate central noradrenergic (NE) dopaminergic (DA) and serotonergic (5HT) neurons (6-12). It has been recently reported that cold stress elevates cerebellar cyclic GMP in rats (13) and mice (14). Cyclic nucleotides are known to activate protein kinases. Rodnight (15) notes a possible stress component in brain protein kinase activity. Increases in cAMP levels in brain tissues have been demonstrated following exposure to NE, DA or 5HT in vitro (16,17) and to NE (but only minimally to DA) in vivo (18).

The foregoing has led us to predict that psychological stressors will elevate cGMP and/or cAMP. An ACTH fragment reportedly lowers brain GABA (19). As ACTH is released in stress and GABA administration lowers cGMP (20), it would seem highly desirable to study the effects of stress (as well as ACTH and other peptides) on brain cGMP and GABA, as well as norepinephrine and cAMP. A method has been established in our laboratory which permits assay of gamma-aminobutyric acid (GABA), glutamic acid (GLU), cyclic adenosine 3'5', monophosphate (cAMP), cyclic guanosine 3'5' monophosphate (cGMP) and norepinephrine in the same sample of brain tissue after microwave inactivation of enzymes, thereby increasing the amount of information obtainable from a single experiment. The assays employed are the radioimmunoassay of Steiner for cyclic nucleotides and the enzymatic method of Graham and Aprison for GABA and GLU. It is thought that cGMP is responsive to cholinergic transmission and under various

conditions, brain tissue cAMP is stimulated by norepinephrine, dopamine, serotonin and histamine. Emphasis in the field is shifting to cAMP/cGMP ratios, and the capacity to study both is essential. Further studies in our laboratory have demonstrated that the technique of using high-intensity microwave irradiation for enzyme inactivation as indispensable for determining levels of cAMP, cGMP and GABA in brain regions. The elimination of artifact has permitted the establishment of new levels of these substances in the regions studied previously. In addition, for many of the regions, the work is unique in that levels have never previously been reported.

We have recently completed a preliminary study of the effect of footshock on levels of cyclic nucleotides in brain. A significant increase in cGMP in cerebral cortex, as well as cerebellum was found in animals exposed to that stressor. No significant changes in cAMP were found. Several additional studies are planned which will evaluate the effects of a range of stressors on brain GABA and cGMP as well as NE and cAMP. Future work will include examination of sex differences in neurochemical and hormonal responses to stress, as well as neurochemical mechanisms involved in habituation to chronic exposure to stress.

A new area of critical importance to understanding the effects of stress on emotional breakdown is being opened up by studies indicating direct effects of steroid and peptide hormones on brain. Corticosteroids bind preferentially to specific regions in brain, notably brainstem and hippocampus (21,22). The binding is both to cytosol and to nuclei and is influenced by level of circulating corticoids (21). Corticosterone administration causes a marked increase in the activity of tryptophan hydroxylase (the enzyme that synthesizes serotonin) in midbrain (24). These observations have led to speculation that the serotonin neurons which project from the raphe cells to the hippocampus (16,25) might mediate the feedback inhibitory effect of glucocorticoids on the pituitary-adrenal axis. There is controversy over whether or not glucocorticoids depress the single unit firing from raphe cells (26,27). An increase in hippocampal theta is reported to occur 1 hr following glucocorticoid administration (29). Raphe stimulation, which releases 5HT (34), blocks habituation to auditory startle response (35). In one study of glial tumor cells in culture, glucocorticoids doubled the cAMP response to NE but at least a 20 hr exposure to the glucocorticoids was required for minimal effect (23). From the above description, it appears likely that the direct effect of glucocorticoids on brain is more related to chronic, rather than acute effects of stress.

As an initial approach to study of effects of hormones on brain, in collaboration with Dr. Barry Hoffer at St. Elizabeth's Hospital, Washington, D.C., we have carried out several experiments involving the measurement of cyclic nucleotides in a chronic preparation of

hippocampal explant. Dr. Hoffer and his colleagues have carried out electrophysiological studies demonstrating cholinergic innervation of the explant via the superior cervical ganglion. Our efforts are directed toward understanding the receptor response in this system as manifested by possible cyclic nucleotide level changes in the explant. Effects of superfused steroids and peptide hormones will be evaluated in this system.

In another approach to study the effects of hormones on the brain, we will study the effects of administered hormones on the cyclic nucleotide response to stimulation of specific monoamine tracts, particularly those innervating the hippocampus. In preparation for these studies, we have studied the effect of urethane on cyclic nucleotide levels. A marked reduction of cGMP levels was found in the cerebellum.

To ensure optimal support of the stress studies, we have modified assay procedures to reflect the most current technological advances. To increase the sensitivity of our assay system we are presently working with modifications of techniques for acetylating our samples as described by Harper and Brooker (J. of Cyclic Nucleotide Research 1:207-218, 1975). This appears to be a promising method to increase our sensitivity into the femtomole range. In collaboration with Dr. David Rodbard at NIH, we have completed a study evaluating the 4 parameter logistic model for statistical analysis of cyclic AMP and cyclic GMP radioimmunoassays. In an effort to maintain the quality and stability of the assay procedure, efforts this past year have been directed toward producing and characterizing our own antibodies for cyclic AMP and cyclic GMP. The protein-conjugates of the ScAMP and ScGMP derivatives were prepared in our laboratory and used for immunization in our rabbit colony. At this time we have several potentially good cyclic GMP antibodies which are being titrated and evaluated for cross-reactivity.

Because many of our neurochemical studies require the use of high-intensity microwave irradiation to accomplish enzyme inactivation, we have continued to initiate improvements in, and document limitations of, the microwave technology. Modifications of our present microwave power source, Varian PPS-2.5, introducing electronic circuitry to precisely control power delivered to the animal as well as exposure duration, have been completed by Peter Brown in the Department of Microwave Research and are presently in operation. Studies have continued in conjunction with Dr. Om Gandhi and Department of Microwave Research to develop an appropriate waveguide applicator to achieve more uniform enzyme inactivation in the brain. As noted in last years report, we have a waveguide applicator suitable for high energy exposures at 985 MHZ. We have proceeded with testing of this applicator for uniformity of inactivation using histochemical techniques in collaboration with AFIP. By evaluating the pattern of presence or absence of succinic dehydrogenase activity, we hope to demonstrate the degree of uniformity of inactivation not only at 985 MHZ but also at 2450.

High power microwave irradiation at 2450 MHZ has been used in the measurement of a number of metabolites (Cyclic AMP, Cyclic GMP, ATP, Creatine-P, etc.) and putative neurotransmitters (GABA, Glutamate, and Acetylcholine) in the brains of both rats and mice. Whole brain levels of these compounds have compared favorably to rapid freezing techniques. Although determination of many of these substances in brain regions has been performed after microwave inactivation, the possibility of cell membrane disruption secondary to rapid heating with subsequent diffusion has been considered but never demonstrated. Studies in our laboratory and one other have demonstrated the use of microwave irradiation at 2450 MHZ in the measurement of norepinephrine (NE) and dopamine (DA) in whole brain. Regional brain studies in our laboratory, however, indicate highly significant increases in DA concentration in specific areas including regions of the cortex, amygdala and septal area following irradiation at 2450 MHZ. Concentrations of NE in all 17 regions studied were comparable to the decapitated control values. Since the precise pattern of microwave energy deposition into the brain is not homogeneous and is affected by frequency as well as applicator characteristics, studies of regional levels of NE and DA in rat brain were carried out at 985 MHZ. Once again levels of DA were significantly elevated over decapitated controls in the same regions, while NE values remained unaffected. These data suggest that a problem of diffusion with microwave inactivation may occur independent of the frequency used, and may be significant in the case of compounds with high regional concentration gradients in brain.

Histological examination accomplished in the Department of Neurophysiology and in the AFIP using Woelke's or Luxol-Fast Blue staining indicated a marked breakdown of myelinated bundles in corpus callosum and corona radiata which might have facilitated diffusion from the corpus striatum to the adjacent frontal and parietal cortex as well as to the septal nuclei and amygdala.

Elements of this work will be continued under Project No. 3A762758A823, Work Unit 037 and Project No. 3A161102371P, Work Unit 070.

Project 3A762758A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 034 Influence of stress on hormone response, performance and emotional breakdown in military personnel.

Literature Cited.

References:

1. Henkin, R.I., and Knigge, K.: Effects of sound on the hypothalamic-pituitary-adrenal axis. *Am. J. Physiol.* 204:710, 1963.
2. Dunn, J., Scheving, L., and Millet, P.: Circadian variation in stress-evoked increases in plasma corticosterone. *Am. J. Physiol.* 222(2):402-406, 1972.
3. Kokka, G.J.F., George, R., and Elliott, H.W.: Growth hormone and ACTH secretion: evidence for an inverse relationship in rats. *Endocrinology* 90(3):735-743, 1972.
4. Takahashi, K., Daughaday, W.M., and Kipnis, D.M.: Regulation of immunoreactive growth hormone secretion in male rats. *Endocrinology* 88:909-917, 1971.
5. Brown, G.M., Martin, J.B.: Corticosterone, prolactin, and growth hormone responses to handling and new environment in the rat. *Psychosom. Med.* 36(3):241-247, 1974.
6. Corrodi, M., Fuxe, K., and Hokfelt, T.: The effect of immobilization stress on the activity of central monoamine neurons. *Life Sci* 7:107-112, 1968.
7. Korf, J., Aghajanian, G.J., and Roth, R.M.: Increased turnover of norepinephrine in the rat cerebral cortex during stress: role of the locus coeruleus. *Neuropharmacology* 12:933-938, 1973.
8. Thierry, A.M., Blanc, G., and Glowinski, J.: Effect of stress on intraneuronal storage forms in the brain stem of the rat. *J. Neurochem.* 18:449-461, 1971.
9. Thierry, A.M., Javoy, F., Glowinski, J., and Kety, S.S.: Effects of stress on the metabolism of norepinephrine, dopamine and serotonin in the central nervous system of the rat. I. Modifications of norepinephrine turnover. *J. Pharm. Exp. Ther.* 163:163-171, 1968.
10. Bliss, E.: Relationship of stress to brain serotonin and 5-hydroxyindoleacetic acid. *J. Psychiat. Res.* 9:71-80, 1972.

11. Morgan, W.: Effect of immobilization stress on serotonin content and turnover in regions of the rat brain. *Life Sci.* 17: 143-150, 1975.
12. Bliss, E.: Relationship of stress and activity to brain dopamine and homovanillic acid. *Life Sci.* 10:1161-1169, 1971.
13. Mao, C.C., Guidotti, A., and Costa, E.: Interactions between γ -aminobutyric acid and guanosine 3',5' cyclic monophosphate in rat cerebellum. *Mol. Pharmacol.* 10:736-745, 1974.
14. Dinnendahl, V.: Effects of stress on mouse brain cyclic nucleotide levels in vivo. *Brain Res.* 100:716-719, 1975.
15. Rodknight. Personal communication.
16. Huang, M.J.: Documentation of cyclic adenosine monophosphate in incubated slices of brain tissue. I. Structure activity relationships of agonists and antagonists of biogenic amines and of tricyclic tranquilizers and antidepressants. *Medicinal Chem.* 15:458, 1972.
17. Forn, J.: Adenosine 3',5'-monophosphate content in rat caudate nucleus: demonstration of dopaminergic and adrenergic receptors. *Science* 186:1118-1120, 1974.
18. Burkard, W.P.: Catecholamine induced increase of cyclic adenosine 3'5'-monophosphate in rat brain in vivo. *J. Neurochem.* 19:2615-2619, 1972.
19. Leonard, B.E.: The effect of two synthetic ACTH analogues on the metabolism of biogenic amines in the rat brain. *Arch. Int. de Pharmacodynamie* 207:242-253, 1974.
20. Mao, C.C., Guidotti, A., and Costa, E.: The regulation of cyclic guanosine monophosphate in rat cerebellum: possible involvement of putative amino acid neurotransmitters. *Brain Res.* 79:510-514, 1974.
21. Steiner, A.L., Kipnis, D.M., Utiger, R., and Parker, C.W.: Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. *Proc. Nat. Acad. Sci.* 64:367, 1969.
22. Steiner, A.L., Wehmann, R.E., Parker, C.W., and Kipnis, D.M.: In *Advances in Cyclic Nucleotide Research*, Vol. 12, Ed. by P. Greengard, G. Alan Robison, and R. Paoletti. Raven Press, New York, 1972.
23. Graham, L.T., and Aprison, M.H.: Fluorometric determination of aspartate, glutamate, gamma-aminobutyrate in nerve tissue using enzymic methods. *Analytical Biochem.* 15:487-497, 1966.

24. Ferrendelli, J.A., Steiner, A.L., and McDougal, D.B.: The effect of oxotremorine and atropine on cGMP and cAMP levels in mouse cerebral cortex and cerebellum. *Biochem. and Biophys. Res. Comm.* 41:1061-1067, 1970.
25. Brown, J.H., and Makman, M.H.: Stimulation by dopamine of adenylate cyclase in retinal homogenates and of adenosine 3'5'-cyclic monophosphate formation in intact retina. *Proc. Nat. Acad. Sci.* 69(3):539-543, 1971.
26. Keibarian, J.W., Petzold, G.L., and Greengard, P.: Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain, and its similarity to the "dopamine receptor". *Proc. Nat. Acad. Sci.* 69: 2145-2149, 1972.
27. Lenox, R.H., Meyerhoff, J.L., and Wray, H.L.: Regional distribution of cyclic nucleotides in rat brain as determined after microwave fixation technique. *Proc. Soc. for Neurosciences Fourth Annual Meeting*, 1974.
28. Balcom, G.J., Lenox, R.H., and Meyerhoff, J.L.: Regional gamma-aminobutyric acid levels in rat brain determined after microwave fixation. *J. Neurochem.* 24(4):609-613, 1975.
29. McEwen, B.S., Wallach, G., and Magnus, C.: Corticosterone binding to hippocampus: immediate and delayed influences of the absence of adrenal secretion. *Brain Res.* 70(2):321-334, 1974.
30. Kraulis, I., Foldes, G., Traikov, H., Dubrovsky, B.: Distribution, metabolism and biological activity of deoxycorticosterone in the central nervous system. *Brain Res.* 88(1):1-14, 1975.
31. Azmitia, E.C.: Corticosterone regulation of tryptophan hydroxylase in midbrain of the rat. *Brain Res.* 166:1274-1276, 1969.
32. Lorens, S.A., and Guldborg, H.C.: Regional 5-hydroxytryptamine following selective midbrain raphe lesions in the rat. *Brain Res.* 78(1):45-56, 1974.
33. Dahlstrom, A.: A method for the demonstration of monoamine-containing nerve fibers in the central nervous system. *Acta Physiol. Scand.* 62, Suppl. 232:1-55, 1964.
34. Mosko, S.S., and Jacobs, B.L.: Midbrain raphe neurons: sensitivity to glucocorticoids and ACTH in the rat. *Brain Res.* 89 (2):368-375, 1975.
35. Foote, W.E.: Effect of hydrocortisone in single unit activity in midbrain raphe. *Brain Res.* 41:242-244, 1972.

36. Martin, S.M., Moberg, G.P., and Horowitz, J.M.: Glucocorticoids and the hippocampal theta rhythm in loosely restrained, un-anesthetized rabbits. *Brain Res.* 93(3):535-542, 1975.

37. Aghajanian, G.K.: Serotonin: release in the forebrain by stimulation of midbrain raphe. *Science* 156:402, 1967.

38. Sheard, M.H.: Stimulation of midbrain raphe neurons: behavioral effects of serotonin release. *Life Sci.* 7:19-25, 1968.

Publications:

1. Mason, J.W. Emotion as Reflected in Patterns of Endocrine Integration. Karolinska Symposium on Parameters of Emotion. *Emotions--Their Parameters and Measurement*, Ed. by L. Levi, pp. 143-181, Raven Press, New York, 1975.

2. Mason, J.W.: A historical view of the stress field, Part I. *J. Human Stress* 1(1):6-12, 1975.

3. Mason, J.W.: A historical view of the stress field, Part II. *J. Human Stress* 2(2):22-36, 1975.

4. Mason, J.W.: Psychological Stress and Endocrine Function. *Topics in Endocrinology*, Ed. by E. Sachar, Grune & Stratton, New York, pp. 1-18, 1975.

5. Mason, J.W.: Clinical Psychophysiology: Psychoendocrine Mechanisms. *Amer. Handbook of Psychiatry*, Vol. IV, Ed. by M. Reiser, Basic Books, New York, pp. 553-582, 1975.

6. Natelson, B.H., Holaday, J., Meyerhoff, J.L., and Stokes, P.E.: Temporal changes in growth hormone, cortisol, and glucose: relation to light onset and behavior. *Am. J. Physiol.* 229(2):409-415, 1975.

7. Lenox, R.H., Gandhi, O.P., Meyerhoff, J.L., and Grove, H.M.: A microwave applicator for in-vivo rapid inactivation of enzymes in the central nervous system. *IEEE Transactions on Microwave Theory and Techniques*, January 1976, pp. 58-61.

8. Rodbard, D., Lenox, R.H., Wray, H.L., and Ramseth, D.: Statistical characterization of the random errors in the radioimmunoassay dose-response variable. *Clin. Chem.* 22(3):350-358, 1976.

Project 3A762758A824
RADIATION INJURY AND PROTECTION

Task 00
Microwave Radiation

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
				DA OB 6484	76 07 01	DD DR&E (AR) 16 30	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^c	6 WORK SECURITY ^d	7 REGRADING ^e	8A ORG'N INSTR'N	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 ^f		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		62758A	3A762758A824	02	057		
b. CONTRIBUTING							
c. CONTRIBUTING							
11 TITLE (Precede with Security Classification Code) ^g							
(U) Biological Effects and Hazards of Microwave Radiation							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^h							
013400 Psychology 016208 Stress Physiology 005700 Electronic & Electrical Engineering							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
71 07		CONT		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE				PRECEDING		b. FUNDS (In thousands)	
b. NUMBER ⁱ NA				76		6	
c. TYPE				CURRENT		7	
d. KIND OF AWARD				7		694	
20 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME ^j Walter Reed Army Institute of Research Washington, DC 20012				NAME ^k Walter Reed Army Institute of Research Div of Neuropsychiatry Washington, DC 20012			
ADDRESS ^l				ADDRESS ^m			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME if U.S. Academic Institution)			
NAME ⁿ Joy, COL R. J. T.				NAME ^o Hunt, F. L.			
TELEPHONE ^p (202) 576-3551				TELEPHONE ^q (202) 427-5262			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				[REDACTED]			
ASSOCIATE INVESTIGATORS				NAME ^r Larsen, MAJ. L.E.			
				NAME ^s Jacobi, J.H.			
22 KEYWORDS (Precede EACH with Security Classification Code) (U) Microwave hazards; (U) Nonionizing Radiation; (U) Dosimetry; (U) Behavioral Effects; (U) Physiological Effects; (U) Military Medicine							
23 TECHNICAL OBJECTIVE ^t 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number Precede text of each with Security Classification Code)							
<p>22. (U) To establish meaningful criteria for delimiting human occupancy in an electromagnetic (EM) environment in support of maximum operational effectiveness of Army and other military personnel with minimum medical and health risk. Delineate the interaction of radio frequency and microwave radiation (10 MHz to 100 GHz) with biological systems, Survey and evaluate known methods and techniques of EM dosimetry and develop same for control and measurement of incident and absorbed energy where appropriate and necessary.</p> <p>24. (U) Investigate each major organ system and biological process where EM effects might occur at reasonably low power intensities. Where indicated, determine the military significance of the effects and the measures necessary to obviate them. Scientific methods of engineering, biophysics, physiology, neurophysiology and experimental psychology will be used. Exposure parameters will be chosen to provide information relevant to Army radiating equipment and operational requirements.</p> <p>25. (U) 75 07-76 06. Research is conducted on: energy absorption and distribution in experimental animals and figurine models of humans using temperature rise measurements and absolute calorimetry; modeling of resonant and non-resonant absorption conditions as determined by frequency, field orientation and presence of reflecting surfaces; development of implantable, radio transparent temperature probe system; characterization of blood-brain barrier permeability changes with brief exposure to low-level pulsed fields; modeling of microwave transmission in anisotropic tissues; investigation of microwave field escape and behavioral activation with low-level exposures. (For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 75 - 30 Jun 76). Support in the amount of \$30,000 from FY 77 funds is program for the period 1 July 76 through 30 Sept 76.</p>							

PII Redacted

DD FORM 1498
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 55 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

074

Task 02 Microwave Radiation

Work Unit 057 Biological effects and hazards of microwave radiation

Investigators.

Principal: Edward L. Hunt, B.A.

Associate: MAJ Lawrence E. Larsen, M.D., MC; John H. Jacobi, M.Sc.; T. Daryl Hawkins, M.A.; Peter V.K. Brown, B.S.; John F. Schrot, Ph.D.; Sandra H. Githens, B.S.

Description

This research task is to develop information on the potential biological hazards of electromagnetic radiations (EMR), primarily microwaves, to Army and other military personnel. Of particular interest are the biological effects of acute and chronic exposure to radiation fields produced by military sources, such as the high peak power, pulsed fields produced by radars. A wide variety of biological effects data needed for hazards analysis are being developed in experimental animal investigations to characterize (1) microwave energy absorption and distribution in biological systems, (2) effects on nervous and sensory systems, (3) effects on behavior and performance, (4) stress reactions to microwave irradiation and (5) physiological and biophysical effects at the cellular and tissue level of function. This research depends on an extensive in-house engineering capability to establish, operate and maintain specialized exposure systems (anechoic chambers), high power generators and other specialized sources, and complex control, instrumentation, data acquisition and analysis systems. This engineering resource is utilized also in (6) a collaborative, programmatic development of tissue enzyme inactivation systems with the Department of Medical Neurosciences (see FY77 Work Unit 070) and in (7) providing direct research support to extramural research investigators using the WRAIR's Microwave Research facilities.

Progress

1. Energy Absorption and Distribution

Investigations have continued on the development of measurement devices and techniques, on the physical modeling of field energy coupling to biological objects and on biodosimetry studies of absorption in experimental animals.

A. Measurement developments include work on improving the RF-transparent, implantable temperature electrode, construction and testing of an absolute calorimetric device for whole-animal (and physical model) dosimetry and, recently started, development of methods for microwave interrogation of biological targets in situ.

Temperature measurements made locally in tissues of interest during microwave exposure of the animal are often required to make interpretable the results of bioeffects experiments. The microwave integrated circuit electrode, initially developed by Larsen, Moore and Acevedo (Larsen et al, 1974), has been further developed to improve its performance. The resistance of the chip thermistor transducer at the tip end of the sapphire needle is now measured by the four terminal method, eliminating RF sensitive bridge circuitry; a hyperthin film transmission line now is used to reduce RF coupling; the transducer is now glass encapsulated to improve long term stability and physiological inertness; materials and structural design have been selected to enhance the electrode's electro-thermal matching with tissues. The electrode is fabricated using standard quantity production methods. Thermography was used to verify RF properties of the electrode and transmission line in simulated tissue and in air. RF transparency has been improved significantly, the electrode operating successfully in a 250 mW/cm^2 field (CW, 2.45 GHz) as compared with the previous 50 mW/cm^2 field.

Whole-animal calorimetry (Hunt and Phillips, 1972) provides an integrated dose measurement of absorbed energy that is of primary interest biologically and is of particular value for microwave bioeffects research since the measurement is independent of incident field measurements and can be used to characterize various exposure systems (Phillips et al, 1975). Twin-well calorimeters were designed and constructed in two sizes, suitable for small rodents (< 100 g weight) and for larger animals and physical models (< 30 cm length), respectively. When tested, both models exhibited excessive sensitivity to ambient temperature variations and excessive balance voltage drift. Some improvements have been made that have increased thermal and electronic stabilities. The larger unit has been used in a limited manner in measuring absorption of microwave energy in physical modeling investigations (see below). Further improvements in performance of these units will be made before standardization of the design is attempted.

Investigations have been started on the potential use of microwaves to interrogate complex dielectric objects to obtain information on the physical shape and structure and the RF electrical characteristics of discontinuities therein, a first approximation to characterizing organs and tissues in situ. In one series of studies, mechanical scanning methods were used to investigate spatial resolutions of single and multiple dielectric discontinuities for

objects immersed in deionized water by measuring scattering parameters. Amplitude and phase of S_{11} and S_{21} for a phase-locked 3243 MHz interrogation source were obtained using dielectrically loaded, matched antennas incorporated in a digitally controlled scanner which was interfaced to an automatic network analyzer (HP 8542A). The complex functions of space obtained in this manner were processed further to create a real valued function of space for intensity representation of the data. Detection of single and multiple discontinuities with dimensions much less than one wavelength in water were obtained. This method can be further generalized to collect multiple projections of S_{21} and from such data the three dimensional pattern of microwave energy absorption can be obtained.

In a second, parallel series of studies with the same scanning system, the velocity of propagation, which is a function of the dielectric constant of the transmission medium, was used to determine time delays in transmission for mapping dielectric inhomogeneities represented by the immersed object. Time and frequency of the transmitted interrogation signal are related with a linear chirp. The received signal is mixed with a reference signal that does not pass through the target object and time delays appear as frequency shifts in the mixer output is processed by generalized harmonic analysis. Path lengths equivalent to 40 picoseconds of propagation time were resolved using this method and it was possible to detect targets with dimensions of less than one wavelength in water for the highest frequency transmitted.

To resolve phase ambiguities and multipath effects in the measured projections of S_{21} , it will be necessary to use path length information. The results of both studies demonstrate the feasibility of obtaining three dimensional dosimetric plots of dielectrically heterogeneous objects in a non-invasive manner.

B. Physical modeling of EMR energy absorption in an isotropic, sizescaled model of man provides a means for testing general concepts that relate absorption to frequency and field geometry or structure and to the size of man and his location and orientation in the field. Once tested, these concepts can serve to determine maximally hazardous exposure conditions suitable for regulation of occupancy. This same information can be used also to establish reliable exposure parameters for experimental animal investigations of bioeffects.

In this report period, Prof. Om P. Gandhi, University of Utah, has continued collaborative work at the WRAIR, employing the free-space exposure facilities to test and extend his "dipole" theory of absorption. According to this theory, man exposed to a free space, plane wave field absorbs field energy similarly to a dipole antenna, with maximum absorption occurring at a resonant frequency at which the model length is about $0.36-0.4\lambda$ and the long axis of the body is parallel to the E-field vector. For 1.75 m tall man, this frequency

is about 65 MHz. This work (Gandhi, 1975) has been extended to investigate a wider range of model lengths to wavelength ratios ($L/\lambda = 0.5-2.5$) using models ranging from 3 to 16 in length, rendered by a professional sculptor to preserve exact proportionalities. At resonance, the absorption cross section is approximately 4 times the shadow cross section and as the frequency increases the absorption cross section approaches 0.5 of the shadow cross section. Fig. 1 shows the absorption characteristics for these and other models for a broad region of the EM spectrum. When electrically grounded, the model acts electrically as a monopole; the resonant frequency becomes approximately half the frequency when ungrounded and absorption is nearly quadrupled.

To test the dipole theory of absorption further, studies were conducted with far-field, plane-wave exposures with reflectors located immediately behind the model. Dipoles located at appropriate distances from such reflectors would form receiving antennas with an enhancement of absorption corresponding to the antenna gain. When placed at certain distances, such as 0.25λ in front of a flat (180°) reflector, absorption by the model was enhanced by a factor of 4, an amount that would be expected also by standing wave theory. At a still closer distance, still greater enhancement would occur with a "receiving antenna" and would be reduced according to standing wave theory. The model exhibited an enhancement factor of 5.5 at the distance of 0.125λ consistent with the antenna theory. With 90° corner reflectors, appreciably greater antenna gains can be achieved, and enhancements in rates of energy absorbed in models exposed at their resonant frequency were increased in a manner consistent with the antenna theory. At a distance of 0.4λ , for example, a 100 g rat that without the reflector would be absorbing energy at the rate of 0.8 W from a 10 mW/cm^2 field at its resonance frequency of 985 MHz would be absorbing energy at the rate of 8.8 W, an enhancement factor of 11. The comparable gain in rate of absorption for the 1.75 m tall "standard" man similarly oriented in the 10 mW/cm^2 , 65 MHz field would be from 151 W to 1,660 W. In parallel with these investigations with size-scaled models of "antenna" enhancements with reflectors, rats of appropriate size were employed in microwave-induced seizure experiments to further test the theory (see below).

More complex physical modeling investigations requires determination of dielectric properties of tissues to enable construction of precise anisotropic models of particular biological systems, such as the eye, that need to be investigated. The development of a new sample holder, started in the previous report period, has progressed to the fabrication of one suitable for testing. The holder was designed to permit analysis of smaller samples of tissues and tissue-equivalent materials than previous was possible and will permit testing over a broader range of frequencies. This line of test development will continue as time permits.

C. Biodosimetry consists of scaling prompt responses to measure intensity of insult. For microwave bioeffects investigations, extensive use of the time to onset of a thermal seizure (seizure latency) during continuous high level exposure has provided a means of investigating energy absorption in rodents. Such an investigation was used to relate animal size to energy coupling from the field according to microwave frequency and the general orientation of the animal relative to the E-field plane of polarization (Schrot and Hawkins, 1975). The results of such investigations have consistently paralleled those derived from carefully controlled physical modeling studies (Gandhi, 1975) and are similarly related to whole-animal calorimetric dosimetry when these measurements have been available (Phillips et al, 1975).

In the present report period, seizure latency measurements were employed as a rapid validating procedure for antenna theory predictions of absorption enhancement with nearby reflectors (see above). In these experiments, 100 g rats were exposed while held without restraint in a nearly RF-transparent cage with the long axis of the cage (and almost always the long axis of the rat's body) parallel to the E-field plane. The frequency of 985 MHz is resonant for animals of their size. When flat or corner (90°) reflectors were placed behind the animal, it served as the dipole of the antenna so formed. With a 20 mW/cm² field density, which even at this resonance frequency is alone ineffective, the seizure latency was reliably shorter with the flat reflector placed 0.125λ behind the cage than when placed 0.25λ behind the cage, a result consistent with antenna theory and contrary to standing-wave theory. At 12 mW/cm², an average seizure latency of 995 sec (SD = +190 sec) was obtained at the shorter distance and none of the 3 rats tested at the 0.25λ distance convulsed during the 30 min test. (Postirradiation, the average colonic temperature was 41.3 °C). Antenna theory applied to the 90° corner reflector predicts a maximum gain near the corner at a distance of 0.4λ, and the results of the seizure tests were consistent with this. Seizures were induced at this distance within the 30-min test period with a field power level of 6 mW/cm².

In addition to providing confirmation of the physical modeling studies of absorption, the results of the biodosimetry investigations provide information useful for designing exposure arrangements for bioeffects investigations. When positioned at an enhancement position in front of a reflector, the animal is exposed to coherent microwaves but multilaterally. High rates of absorption can be achieved with lower levels power levels radiated from the source, and with multilateral exposures, the biological effects might be comparable to those obtained with multimodal cavity exposures.

2. Effects on Nervous and Sensory Systems

Investigations of microwave irradiation effects were continued on audiogenic seizure sensitivity and on blood-brain barrier permeability.

BEST AVAILABLE COPY

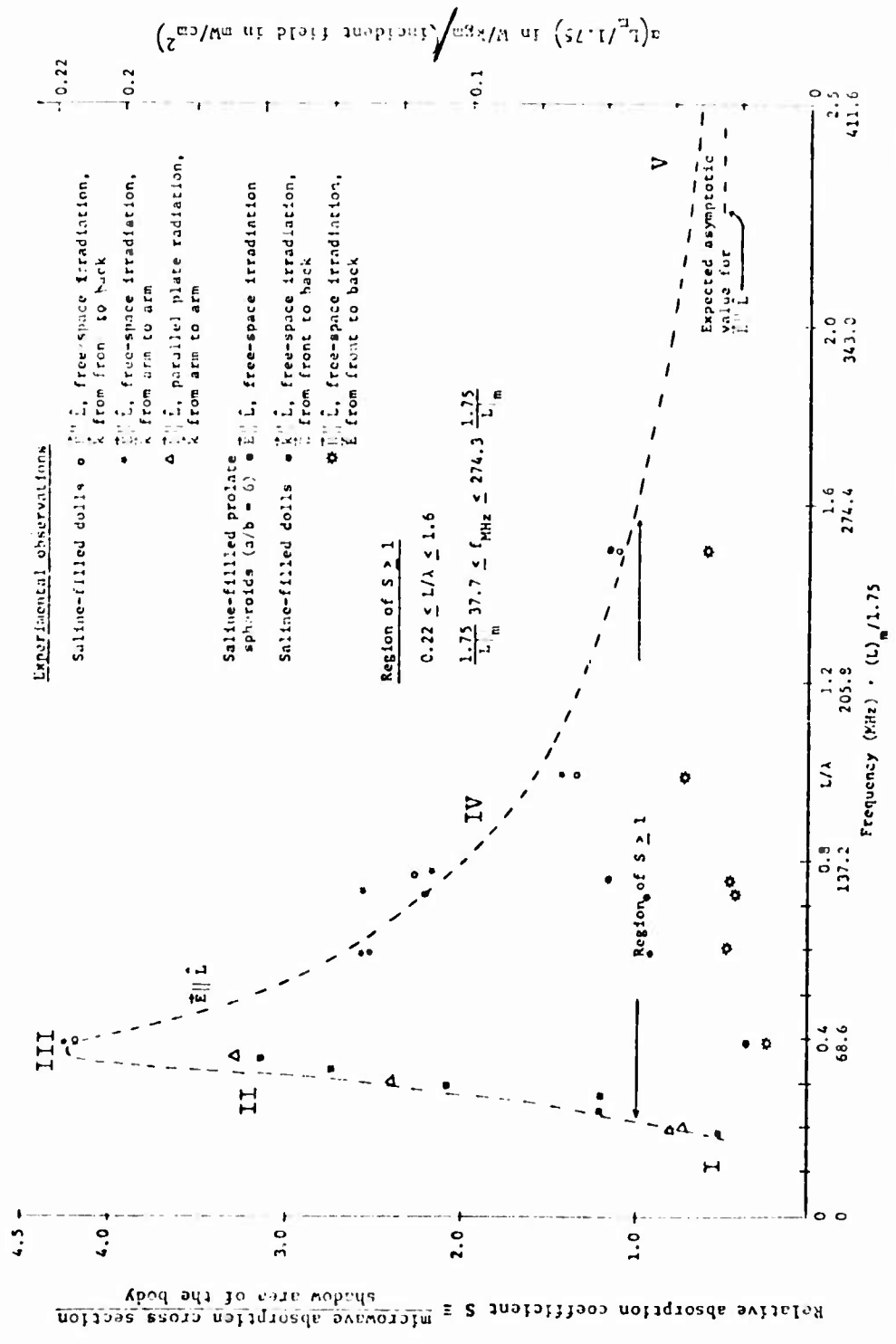


Fig. 1. Whole body absorbed power density and relative absorption coefficient S for humans without ground effects (obtained from experiments with saline-filled figurines and bodies of prolate spheroidal shape). (The value of S parameter, as shown, is not valid for $\vec{k} \parallel \vec{L}$ orientation.)

A. Audiogenic seizure sensitivity following acute exposures to continuous wave (CW), 2450 MHz microwave was not altered in seizure sensitive rat except at exposure levels that were thermalizing, and the effect found was a transient reduction in sensitivity (Hawkins and Hunt, 1975). During this report period, an experiment was conducted to evaluate effects of a 10 mW/cm^2 level of exposure to CW 2450 MHz radiation of four hours duration, repeated 5 days a week over a course of 10 weeks. Forty rats preselected from audiogenic seizure sensitive stock for being seizure prone, half of them irradiated and half sham-irradiated, were retested biweekly for seizure sensitivity. The seizure tests were designed to reveal either increases or decreases in seizure proneness. No changes in either direction were detected. These negative results are in contrast to the decreased sensitivity that was found following the single 30-min exposure at 75 mW/cm^2 . Future investigations will evaluate the effects of low level exposures ($\leq 10 \text{ mW/cm}^2$) to high peak power, pulsed fields when this capability is established. For these future investigations the genetic stock established for this type of study will be retained.

B. Blood-brain barrier permeability changes produced by low-level microwave irradiation in rats continued to be investigated in collaboration with Dr. K. Oscar, American University. The finding of an increased permeability following a single, 20 min exposure to 1.3 GHz was expanded by determining the relative effectiveness of CW and pulsed radiation, by attempting to determine unique pulse parameters responsible for producing the effect, by testing for permeability changes with larger molecules, and by testing for duration of the permeability change. Notably, it was found that following CW exposure increased permeability of the blood brain barrier increased with field power density up to about 1.0 mW/cm^2 and then decreased with further increases in field density. The effect was evident through this "power window," represented by an inverted "U" shaped function. Increases in blood brain barrier permeability were observed at average power densities as low as 0.03 mW/cm^2 . Several characteristics of the effect will be investigated further, particularly the "power window" phenomenon, and frequency dependence for the permeability change will also be investigated.

3. Effects on Behavior and Performance

Escape behavior from pulsed microwave fields at 1.2 (Frey et al, 1975) and 2.88 GHz (Hunt et al, 1975) have been reported to occur in rats with average field power densities below 10 mW/cm^2 . Investigations of this phenomenon with mice has been undertaken during this report period. The procedure employs a two-compartment shuttle box and a forced-trial design of conditioning the animal to discriminate the compartment in which exposure occurs using differential cues. The choice apparatus has been tested and environmental conditions established that reduce strong side preferences in mice given hour-long free choice tests. The investigation was delayed while procurement of a

pulsed source was completed. Suitably high peak power pulses can be obtained with this unit by using an ellipsoidal focusing dish to concentrate the radiator output in a 4-6 inch wide field.

Performance of a multiple schedule task has been reported to be altered in rats following long duration exposures to pulse microwaves (Thomas, 1975), and the results might be attributable to a disruption in the animal's discrimination of schedule components. A procedure has been developed that requires the animal to relearn a different sequence of responses in each session which provides a means for measuring development of discrimination learning within each session with repeated testing daily, if needed (Shrot et al, 1976). During this report period further testing of the procedure was undertaken to determine its sensitivity for detecting manipulations in schedule conditions, such as changing "time out" durations. It is anticipated that the constantly changing discriminative control of behavior generated by this procedure will be especially sensitive to effects of exposure to microwave radiation.

4. Stress Reactions to Microwave Irradiations

Ambient temperature and relative humidity variations alter the animal's capacity to dissipate heat and can be related to the animal's capacity to withstand heat loading during an acute, high level exposure to microwaves. Elapsed time to the onset of a thermal seizure was used to measure vulnerability under various combinations of ambient temperature (AT) and relative humidity (RH) to exposure₂ with 2450 MHz, CW microwave at a high field power density of 150 mW/cm². Rats 100-125 g in weight were individually exposed while held in a holder constructed of styrofoam and plexiglas rods, designed by open structure to maximally expose the animal to ambient conditions. AT's of from 62-94°F in combination with high medium and low RH conditions. A monotonic function of seizure latency with the temperature-humidity index (THI = $1.44(^{\circ}\text{C}) + 0.1 (\text{RH}) + 30.6$). A decrease in mean latency of 70% occurred with an increase in THI from the lowest value (58.6) to the highest (85.5). The microwave heating hazard is strongly influenced by the THI value of ambient conditions. These results provide useful information for determining ambient conditions suitable for some bioeffects studies.

5. Physiological and Biophysical Investigations

During this report period, effort was limited to program design and outfitting of suitable laboratory facilities to optically investigate changes during microwave irradiation in the structure of a nerve cell membrane. This work will utilize a giant nerve cell preparation from an marine animal for determining membrane events by flourescopic and birefringent microscopy.

6. Development of Tissue Enzyme Inactivation Systems

In collaboration with the Department of Medical Neurosciences, research engineering support is provided for design, fabrication and testing of microwave enzyme inactivation applicators, high power microwave sources (primarily by modifications) and associated instrumentation. In the refitting of the Varian source, the circuit modifications begun in FY 75 were completed. The unit was successfully tested on brain biochemical studies placed in operation during this report period.

In the use of inactivation systems for biochemical-anatomic investigations, the operational criteria equipment must meet to provide reliable results are very demanding such as that for uniform tissue heating, but they are essential for obtaining this type of information (Lenox et al, 1976). Planning and preliminary work on applicators for brain biochemical investigations in rats was undertaken. Tests have been made of two types of applicators that were designed by Professor Om P. Gandhi, University of Utah, for this WRAIR program. Presently, the design and procurement of more advanced sources and applicators has been undertaken.

7. Extramural Research Support

The WRAIR makes available the use of its microwave exposure facilities to extramural researchers. This type of support represents a cost in personnel equipment and facilities that directly augments the support with funds provided the extramural researcher by another sponsor, making WRAIR a co-sponsor of the research in fact. The need for this expanded use of the facilities by the microwave bioeffects research community will continue to increase, necessitating the need for an increased level of oversight and management of this support function.

A broader more public base than can be provided by in-house staff alone for furnishing protocol review and facilities scheduling for extramural researches has been designed in the form of a tri-partite agreement sponsored by WRAIR with the Naval Medical R and D Commands' EMR Project Office and with AFRRRI. The scheduling functions by the tri-partite committee for facilities use, operating now on a trial basis pending completion of engineering staffing at the WRAIR, informs extramural user of facilities use and provides them maximum flexibility in their own programming. The protocol review function of the tri-partite committee has not been used since only currently approved protocols are being honored pending completion of staffing.

During this report period, ten extramural research projects were provided this direct support, nine sponsored by the DoD of which two were Army funded projects. All of the DoD funded projects constitute identifiable research elements in the Tri-Service EMR Bioeffects Research Plan that is being instituted jointly by the medical R & D commands of the three services at the request of ODDR and E.

The protocol review function provided by WRAIR staff to-date has on 3 occasions failed to approve extramural proposals as not providing the promise of interpretable results. The staff has also provided active oversight as to the research operations that the WRAIR is supporting in this extramural program. It is anticipated that through tri-partite committee will actively function to provide such oversight.

Task Ø2 Microwave Radiation

Work Unit Ø57 Biological effects and hazards of microwave radiation

Literature Cited.

References:

1. Frey, A.H., Field, S.R., Frey, B.: Neural function and behavior: defining the relationship. Annals of the New York Academy of Sciences 247: 433-439, 1975.
2. Gandhi, O.P.: Conditions of strongest electromagnetic power deposition in man and animals. IEEE Trans. MTT, MTT-23, NO 12, 1021-1029, 1975.
3. Hawkins, T.D., Hunt, E.L.: Reduction in sensitivity to audiogenic seizure following a single, 2450 MHz, CW irradiation of rats. USRI 1975 Annual Meeting, 223, Oct 20-23.
4. Hunt, E.L., Phillips, R.D.: Absolute physical dosimetry for whole animal experiments. Digest of Papers, Joint U.S. Army/Georgia Institute of Technology Microwave Dosimetry Workshop, 74-77, June 1972.
5. Hunt, E.L., King, N.W., Loveley, R.H., Phillips, R.D.: "Avoidance" by rats of 2.88 GHz pulse microwave field. USRI 1975 Annual Meeting, 192, Oct 20-23.
6. Larsen, L.E., Moore, R.A., Acevedo, J.: A microwave decoupled brain temperature transducer. IEEE Trans. MTT, MTT-22, 438-444, 1974.
7. Lenox, R.H., Gandhi, O.P., Meyerhoff, J.L., Grove, H.M.: A microwave applicator for in-vivo rapid inactivation of enzymes in the central nervous system. IEEE Trans MTT, January 1976.
8. Oscar, K.J., Hawkins, T.D.: Electromagnetic radiation effects on the blood brain barrier system of rats. USRI 1975 Annual Meeting, 222, Oct 20-23.
9. Phillips, R.D., Hunt, E.L., King, N.W.: Field measurements, absorbed dose, and biological dosimetry of microwaves. Annals of the New York Academy of Sciences, 247:499-509, 1975.
10. Anonymous: A summary of the ERMAC work session on nervous system and behavioral effects of nonionizing electromagnetic radiations. Journal of Microwave Power, 10(2), 1975.

11. Schrot, J., Baren, J.J., Moerschbaecher, J.M.: Sequential reacquisition as a function of timeout from avoidance. *Journal of the Experimental Analysis of Behavior*, 1976, 25, 303-310.

12. Schrot, J., Hawkins, T.D.: Microwave frequency and E-field orientation interaction with animal size. USRI 1975 Annual Meeting 233, Oct 20-23.

Publications:

1. Gandhi, O.P.: Conditions of strongest electromagnetic power deposition in man and animals. *IEEE Trans. MTT*, 23, 1021-1029, 1975.

2. Larsen, L.E.: Sequential methods for parametric discriminant analysis. N.I. Burch (Editor) *Behavior and Brain Electrical Activity*, Plenum Press, New York, 1975, pp. 165-192.

3. Larsen, L.E., Bonney, C.W., Jacobi, J.H.: Memorandum Report to Tri-Service EMR Bioeffects Advisory Panel. Investigation of microwave frequency effects on lens and eye absorption in anesthetized rhesus and stump-tail monkeys, 1975.

4. Larsen, L.E., Evans, R.A.: An off-line image processing system for digital tomography. *Neuroradiology*, 9, No.5, 284-285, Sept. 1975.

5. Lenox, R.H., Gandhi, O.P., Meyerhoff, J.L., Grove, H.M.: A microwave applicator for in-vivo rapid inactivation of enzymes in the central nervous system. *IEEE Trans. MTT*, 24, 58-61, Jan 1976.

6. Schrot, J., Boren, J.J., Moerschbaecher, J.M.: Sequential reacquisition as a function of timeout from avoidance. *Journal of the Experimental Analysis of Behavior*, 25, 303-310, 1976.

Project 3A762759A829
MALARIA PROPHYLAXIS

Task 00
Malaria Investigations

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OB 6535	76 07 01	DD DR&E(AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ³	6 WORK SECURITY ⁴	7 REGRADING ⁵	8A DES'N INST'N	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 ⁶	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	62759A	3A762759A829		00	308		
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11 TITLE (Precede with Security Classification Code) ⁷							
(U) Biological Evaluation of Anti-malarial Drugs							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ⁸							
012600 Pharmacology 002600 Biology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
66 07		CONT		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
NA				PRECEDING			
A. DATES/EFFECTIVE:				FISCAL YEAR		B. FUNDS (in thousands)	
EXPIRATION				76		2.95	
B. NUMBER ⁹				CURRENT		105	
C. TYPE:				77		2.0	
D. KIND OF AWARD:				E. CUM. AMT.			
19 RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ¹⁰ Walter Reed Army Institute of Research				NAME: ¹¹ Walter Reed Army Institute of Research			
ADDRESS: ¹² Washington, DC 20012				ADDRESS: ¹³ Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: ¹⁴ Joy, COL. R.				NAME: ¹⁵ Davidson, D. E. LTC			
TELEPHONE: ¹⁶ 202-576-4551				TELEPHONE: ¹⁷ 202-576-2292			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: ¹⁸ Ketterling, L. L. CPT			
22 KEYWORDS (Precede EACH with Security Classification Code) ¹⁹							
(U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Biology; (U) Chemistry; (U) Pharmacodynamics; (U) Drug Metabolism; (U) Toxicology							
23. TECHNICAL OBJECTIVE, ²⁰ 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code) ²¹							
23. (U) To conduct in-house and contract studies in biology specifically related to the design, development and exploitation of new antimalarials for military use.							
24. (U) Close supervision will be maintained by providing guidance and an integrated evaluation of productivity, and by the redirection and coordination of objectives as dictated by feedback from clinical studies as candidate antimalarials.							
25. (U) 75 07 - 76 06 Compounds are tested for prophylactic or suppressive anti-malarial activity in approximately 25 test systems at six different laboratories. Of 10,900 compounds screened in the primary P. berghei suppressive test, 302 were active. Of these, 125 have undergone advanced studies including 23 compounds which have been tested against P. cynomolgi, P. vivax and/or P. falciparum in subhuman primates. Causal prophylactic and/or radical curative tests against sporozoite induced malarials in rodents and/or rhesus monkeys have been conducted for approximately 250 compounds. Five compounds with curative activity markedly superior to primaquine are undergoing special toxicologic studies. In vitro methods to study structure-activity relationships responsible for induction of hemolysis and methemoglobinemia by primaquine analogues are being developed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul - 30 Jun 76.							
Support in the amount of \$15,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

PII Redacted

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 308 Biological evaluation of antimalarial drugs

Investigators.

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

Associate: LTC Peter S. Loizeaux, VC, CPT Lyle L. Ketterling, MSC

1. Description

Candidate chemical compounds are tested for prophylactic or suppressive antimalarial activity employing some 25 animal and in-vitro test systems at seven different laboratories. Of 230,000 compounds tested during the 12 years of the program, about 3% (4500 compounds) were active. Approximately 200 of the most active were selected for advanced evaluation in simian test systems, and of these 40 have been selected for preclinical pharmacological and toxicological studies.

2. Progress

a. Suppressive Testing

The basic primary screening test for suppressive antimalarials utilizes trophozoite-induced Plasmodium berghei KBG 173 in ICR/HA Swiss mice. Active compounds lengthen survival time of mice when administered subcutaneously in peanut oil 72 hours after a standard supra-lethal inoculum of parasitized blood. Untreated controls survive only 6.2 ± 0.3 days. During this reporting period, 10,900 compounds were screened in the primary mouse test. Of these, 302 compounds had antimalarial activity, and 125 have been selected for secondary testing. Twenty-three compounds have undergone advanced suppressive testing against P. cynomolgi, P. vivax, or P. falciparum in sub-human primates.

b. Prophylactic Testing

Considerable progress has been made in establishing a sporozoite-induced P. berghei prophylactic test system capable of screening compounds in large numbers. Reference antimalarial compounds are being evaluated in this test system at the present time. Secondary rodent prophylactic test systems have been used to test approximately 200 compounds for causal prophylactic activity. Approximately 75 compounds have been tested for radical curative activity against Plasmodium cynomolgi in rhesus monkeys. Five compounds of the 8-

aminoquinoline class have been found which are at least 4 times as potent as primaquine and these are undergoing special toxicologic testing.

c. Antifolic Acid Assay

One-hundred compounds were tested for their ability to inhibit growth of three species of folate dependent bacteria. This information is utilized in the design and selection of antimalarials which act by interfering with the metabolism of folates.

d. Primaquine-Induced Hemolysis

Primaquine and certain other antimalarial drugs may induce hemolysis in glucose-6-phosphate-dehydrogenase (G-6PD) deficient individuals. G-6PD deficiency is a defect intrinsic to the erythrocytes of the affected person, and, although this hereditary condition is more common in Mediterranean and Oriental populations, approximately 10% of American Negro males are affected.

Welt, et al, studied the effect of primaquine on normal subjects by measuring the amount of $^{14}\text{CO}_2$ evolved during incubation of the subject's own erythrocytes and serum in the presence of glucose- $1\text{-}^{14}\text{C}$. The stimulation of CO_2 production through the pentose pathway by primaquine is believed to be related to its hemolytic potential.

Welt's method has been modified and is being used to screen antimalarial compounds. Whole blood is collected from normal human volunteers and defibrinated. One ml of defibrinated blood and one ml of test compound ($2.5 \times 10^{-3}\text{M}$) are mixed in a 25 ml Erlenmeyer Flask. To this mixture, 0.5 ml of glucose- $1\text{-}^{14}\text{C}$ is added. The flask is then sealed with a rubber stopper holding a disposable center well and incubated at 37°C for 90 minutes while shaking at 120 oscillations per minute. At the end of the first incubation period, 0.2 ml of 2N sodium hydroxide is injected into the center well and 0.5 ml of 3.7N Perchloric acid is injected directly into the incubation mixture. The vial is re-incubated at the same speed and temperature for an additional 30 minutes. At the end of the second incubation period the center well is removed and its contents placed into a scintillation vial containing 10 ml of Aquasol and 0.8 ml of water. After scintillation counting, the results for each test compound are compared with those of a primaquine standard.

To date, 61 compounds have been tested in this in vitro system. Of these, 33 were less potent in stimulating glucose- 1-C^{14} incorporation into CO_2 than primaquine. Structure-activity relationships of these compounds are being evaluated.

3. Summary:

More than 11,000 compounds have been tested for antimalarial activity in vitro or in animal models. More than 300 were active. Twenty-three highly promising suppressant antimalarials were tested against simian or human Plasmodia in subhuman primates. Five radical curative drugs were identified which were at least four times as active as primaquine in the simian model. Structure-activity relationships leading to primaquine-induced hemolysis are being studied in vitro.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	3. REPORT CONTROL SYMBOL ^c	
				DA OB 6536	76 07 01	DD-DR&E(AR)836	
4. DATE PREV. SUMRY	5. KIND OF SUMMARY	6. SUMMARY SCY ^d	7. WORK SECURITY ^e	8. REGRADING ^f	9. DIS'N INSTA ^g	10. SPECIFIC DATA CONTRACTOR ACCESS ^h	11. LEVEL OF SW ⁱ
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
12. NO. / CODES ^j	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62759A	3A762759A829	00	309			
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ^k							
(U) Determination of Pharmacological Effects of Antimalarial Drugs							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^l							
012600 Pharmacology		002600 Biology					
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD		
72 07	CONT	DA			C. In-House		
17. CONTRACT / GRANT				18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (in thousands)	
A. DATES/EFFECTIVE. NA				PRECEDING			
B. NUMBER ^m				76	4.5	252	
C. TYPE				CURRENT			
D. KIND OF AWARD:				77	9.0	390	
19. RESPONSIBLE GOV. ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Punish BEAR if U.S. Academic Institution)			
NAME: JOY, R. J. T., COL				NAME: HEIFFER, Dr. M. H.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3387			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				[REDACTED]			
22. ASSOCIATE INVESTIGATORS				NAME: EINHEBER, Dr. A.			
				NAME: ROZMAN, Dr. R. S.			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Pharmacodynamics; (U) Pharmacokinetics; (U) Toxicity; (U) Biotransformation; (U) Antimalarial Drugs; (U) Preclinical Pharmacology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number Precede last of each with Security Classification Code.)							
23. (U) The technical objectives are to develop and exploit animal models for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarial in man. The intended purposes of these studies are to provide a basis for predicting human response and to fulfill requirements for submission of IND for clinical trials of new antimalarials for military personnel in malarious areas.							
24. (U) The approach is to study both the effects of antimalarial drugs on healthy animals and the fate of these drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The effects of antimalarial drugs are being studied in infected animals. The handling of antimalarial drugs by diseased animals is being studied to determine the effects of malaria upon pharmacokinetics. This is in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).							
25. (U) 75 07 - 76 06 Technical management continued for 14 contracts in pharmacology. Two new IND applications and 13 supplements were written. Methods for determining human blood levels of 4 antimalarials utilizing extraction and high pressure liquid chromatography were developed. WR 142,490 was shown to maintain persistent levels for weeks in human plasma after oral dosing. WR 122,455 and WR 171,669 were both shown to be curative against P. falciparum malaria in humans. Absorption, distribution and excretion studies using radioactively labeled WR 180,409 were carried out in healthy mice. Cardiorespiratory evaluations in anesthetized dogs were made on WR 180,409. The mouse oral efficacy and toxicity and effects of microsomal modifiers on these continued to be intensively studied in primaquine analogs, both racemic mixtures and resolved optical forms. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 75 - 30 Jun 76. Support in the amount of \$92,000 from FY 77 funds is programmed for the period 1 Jul - 30 Sep 76.							

PII Redacted

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
996

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 309 Determination of pharmacological effects of anti-malarial drugs

Investigators.

Principal: Melvin H. Heiffer, Ph.D.

Associate: Dr. R. Rozman, Dr. A. Einheiser, 1LT J. Grindel, Dr. H. Chung, CPT D. Korte, Jr., MAJ R. Desjardins, 1LT V. Jimmerson, MAJ J. Rinehart, SP4 H. Gillum, P. Tilton, SP4 R. Keller, SP4 M. Neidig

1. Description.

The thrust of the pharmacological studies carried out by the department continue in two broad areas. First is the effect of the body or system on the drug, i.e. absorption, distribution, biotransformation and excretion. Second is the effect of the drug on the body or system. A considerable overlap exists between the two areas. This year a greater emphasis was placed on developing sensitive assay methods for several of the new antimalarial drugs in biological fluids than in the past.

2. Development and evaluation of a high pressure liquid chromatographic assay for WR 142,490 in blood, plasma and urine.

a. Background:

WR 142,490·HCl (Mefloquine·HCl), dl-erythro- α -(2-piperidyl)-2,8-bis (trifluoromethyl)-4-quinolinemethanol hydrochloride, is a radical curative agent for the treatment of drug-resistant falciparum malaria (Canfield and Rozman, 1974; Trenholme *et al.*, 1975) and has been shown to provide suppressive prophylaxis against mosquito-induced infections with *P. vivax* and *P. falciparum* in human volunteers (Rieckmann *et al.*, 1974; Clyde *et al.*, 1976). Due to the lack of information concerning the fate of mefloquine in man, a sensitive and specific high pressure liquid chromatographic assay for the parent drug in blood, plasma and urine was developed and evaluated.

b. Methods:

A high pressure liquid chromatograph equipped with a 280 nm absorbance monitor was utilized. Whole blood and plasma extracts were chromatographed on a column of 10 μ , fully porous silica bonded with a monomolecular layer of cyanopropylsilane (4 mm ID x 30 cm) using a mobile phase of isopropyl ether/p-dioxane/glacial acetic acid

(118:80:1 v/v/v) at 2 ml/min. Urine extracts were chromatographed on a column of 10 μ , fully porous silica bonded with a monomolecular layer of octadecylsilane (4 mm ID x 30 cm) using a mobile phase of methanol/0.1 M NaH_2PO_4 (3:2 v/v) at 2 ml/min.

A calibration curve was constructed using an internal standard. A solution of WR 184,806 was prepared in p-dioxane/methanol (7:3 v/v) (196 $\mu\text{g/ml}$). This solution was the solvent for the preparation of a stock solution of WR 142,490 (1028 $\mu\text{g/ml}$) from which dilutions were made to give solutions with concentrations of 10.2 to 205.6 $\mu\text{g/ml}$ of mefloquine and 1.96 $\mu\text{g/ml}$ of WR 184,806 (all concentrations expressed as the free base). Duplicate injections with each solution were made to yield data for the construction of a linear calibration curve relating the peak area ratio of WR 142,490/WR 184,806 vs amount of mefloquine injected.

For the general assay procedure, the internal standard solution (50 μl) containing 9.8 μg of WR 184,806, 5 ml of whole blood, plasma or urine and 5 ml of pH 7.4 phosphate buffer (0.065 M) were placed in a 45 ml glass conical tube with a teflon-lined screw cap and thoroughly mixed. 10 ml of ethyl acetate was added to each tube, the samples agitated at 100 strokes/min for 30 min on a reciprocating shaker bath, centrifuged at 2000 rpm for 10 min and the ethyl acetate layer carefully pipetted into 45 ml glass conical tubes with teflon stoppers. The extraction procedure was repeated twice, the combined ethyl acetate layers from each sample evaporated to dryness under a gentle stream of nitrogen at 40°C, and the residue stored overnight in a vacuum desiccator. The sample residues were reconstituted with 500 μl of the appropriate mobile phase, and 50 μl aliquots injected into the HPLC. Quantitation of WR 142,490 levels was achieved by measurement of peak area ratios for WR 142,490/WR 184,806 and relating the ratio to the linear calibration curve.

To quantitate recovery of WR 142,490 and WR 184,806, aliquots (25 ml) of human whole blood were spiked with a methanolic solution of mefloquine (250 $\mu\text{g/ml}$) to give drug concentrations of 1.00, 0.50 and 0.10 $\mu\text{g/ml}$. The samples were thoroughly mixed for 30 min and treated as described above except that the internal standard was not added until the extraction was completed. Aliquots of human blood were spiked with a methanolic solution of WR 184,806 (250 $\mu\text{g/ml}$) to give drug concentrations of 1.00, 0.50 and 0.10 $\mu\text{g/ml}$. The samples were thoroughly mixed for 30 min and treated as described above except that the internal standard was not added until the extraction was completed. Aliquots of human whole blood were spiked with a methanolic solution of WR 184,806 (250 $\mu\text{g/ml}$) to give drug concentrations of 1.00, 0.50 and 0.10 $\mu\text{g/ml}$. The samples were thoroughly mixed for 30 min and treated as described above except that the internal standard was not added.

To determine precision of the mefloquine assay, aliquots (25 ml) of human whole blood and plasma were spiked with a methanolic solution of mefloquine (250 $\mu\text{g/ml}$) to give drug concentrations of 5.00, 1.00, 0.50 and 0.05 $\mu\text{g/ml}$ while urine aliquots (25 ml) were spiked at 5.00, 1.00, 0.50 and 0.25 $\mu\text{g/ml}$. The samples were thoroughly mixed for 30 min and treated as described.

c. Results and discussion:

The choice of extraction solvent for the assay was based on solvating strength, transfer characteristics and ease of evaporation. Ethyl acetate fulfilled these criteria due to the excellent partitioning of mefloquine into it from pH 7.4 buffer (Mu et al., 1975), due to its good pipetting characteristics and due to its low boiling point which allowed quick evaporation under mild conditions.

The establishment of liquid chromatographic conditions was accomplished by examining the retention characteristics of mefloquine on normal phase and reverse phase partitioning columns. Comparative retention volumes for mefloquine and WR 184,806 are presented in Table 1. Optimal resolution of mefloquine and WR 184,806 from ethyl acetate extracts of blood and plasma was accomplished using a normal phase partitioning column with a mobile phase of isopropyl ether/p-dioxane/glacial acetic acid (3:2 v/v + 0.5%). These conditions, however, did not resolve mefloquine and WR 184,806 from urine extract peaks. Adequate resolution of the two compounds from urine extracts was achieved on a reverse phase partitioning column utilizing a mobile phase of methanol/0.1 M NaH_2PO_4 (3:2 v/v). Representative chromatograms are presented in Figures 1 and 2.

The selection of an internal standard for the assay was made on the basis of chromatographic resolvability and the possession of physico-chemical properties similar to that of mefloquine. WR 184,806 had the following properties: 1) a molar absorptivity at 280 nm nearly identical to that of mefloquine; 2) pKa, solvent partition coefficients and aqueous solubility similar to those of mefloquine; and 3) chromatographic resolution from mefloquine and from extracts of blood and urine under appropriate chromatographic conditions.

Recovery percentages from whole blood for both mefloquine and WR 184,806 using ethyl acetate as extraction solvent are presented in Table 2. The recoveries were greater than 95% for both compounds with standard deviations less than 10% at concentrations of 0.10-1.00 $\mu\text{g/ml}$. Precision data for the assay of blood, plasma and urine specimens spiked with known concentrations of mefloquine are presented in Table 3. The relative percent accuracy was + 3% of the amount added with relative standard deviations less than 10% over the range of 0.05 - 5.00 $\mu\text{g/ml}$ for blood and plasma and over the range of 0.25 - 5.00 $\mu\text{g/ml}$ for urine samples.

Daily calibration curves had a mean slope of 1.013 ($r^2 = 0.9995$) with a relative standard deviation of 3.9% for ten experiments over a three month period. The lower limit of sensitivity was 0.05 $\mu\text{g/ml}$ for blood and plasma samples and 0.25 $\mu\text{g/ml}$ for urine samples.

Based on the results of the foregoing experiments, an adult male was given 500 mg of mefloquine hydrochloride, and serial blood and plasma specimens were assayed for two months for mefloquine concentrations (Figures 3 and 4). Blood and plasma levels of mefloquine were readily detected 57 days after dosing. Random urine specimens were obtained on days 1 and 2 and assayed for free mefloquine content (Table 4). The levels, although low, were readily quantitated using the assay. These results established the applicability of the methodology for the study of mefloquine pharmacokinetics in man.

3. Development of a high pressure liquid chromatographic assay for WR 184,806 in whole blood.

a. Background:

WR 184,806·H₃PO₄, dl-2,8-bis(trifluoromethyl)-4-[1-hydroxy-3-(N-t-butylamino)propyl]quinoline phosphate, is a promising new antimalarial agent currently undergoing Phase I clinical evaluation. Due to the need for information concerning its fate in man, a sensitive and specific high pressure liquid chromatographic assay was developed to determine WR 184,806 levels in human whole blood specimens.

b. Methods:

A high pressure liquid chromatograph with a 280 nm absorbance monitor was utilized. Whole blood extracts were chromatographed on a column of 10 μ , fully porous silica bonded with a monomolecular layer of cyanopropylsilane (4 mm ID x 30 cm) using a mobile phase of p-dioxane/isopropyl ether/methanol/glacial acetic acid (117:80:2:1 v/v/v/v) at 2 ml/min.

A calibration curve was constructed using an internal standard. A solution of WR 142,490 was prepared in isopropyl ether/methanol (7:3 v/v) (100 $\mu\text{g/ml}$). This solution was the solvent for the preparation of a stock solution of WR 184,806 (1.00 mg/ml) from which dilutions were made to give additional concentrations of WR 184,806 (10 - 200 $\mu\text{g/ml}$) containing 100 $\mu\text{g/ml}$ of WR 142,490 (all concentrations are expressed as the free base). Duplicate injections with each solution were made to yield data for a linear calibration curve relating peak area ratio of WR 184,806/WR 142,490 vs amount of

WR 184,806 injected in the range of 25 ng - 5000 ng.

The general assay procedure used was as described for WR 142,490 except that only human whole blood was assayed and that 50 microliters (5.0 μ g) of WR 142,490 internal standard was added to each tube.

To determine precision for the WR 184,806 assay, aliquots (25 ml) of human whole blood were spiked with a methanolic solution of WR 184,806 (250 μ l/ml) to give drug concentrations of 5.00, 1.00, 0.50, 0.25 and 0.10 μ g/ml of blood. The samples were thoroughly mixed for 30 min and treated as described for the general assay procedure.

c. Results and discussion:

Based on the results of chromatographic (Table 1) and extraction (Table 2) experiments with both WR 184,806 and WR 142,490, it was determined that WR 142,490 would serve well as the internal standard for the WR 184,806 assay. Subsequently, experiments were performed to determine the accuracy and precision of the proposed internal standard method. The results of this experiment are presented in Table 5. The mean percent recovery was 100.1% with a standard deviation of 4.6%. The lower limit of quantitation for WR 184,806 is felt to be 100 ng/ml of whole blood. Based on blood level data for the mouse this level of quantitation should be adequate for the performance of pharmacokinetic studies in human subjects. The calibration curve was described by the following equation: $y = 1.9505 (\pm 0.1573) x + 0.0419 (\pm 0.0322)$, $r = 0.9949$.

4. Development of a high pressure liquid chromatographic assay for WR 177,602 in whole blood.

a. Background: WR 177,602-HCl, dl-threo- α -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride, is the diastereoisomer of WR 142,490. WR 177,602 has been shown to be as effective as WR 142,490 in curing *Aotus trivirgatus* monkeys infected with multi-drug resistant strains of malaria. Preliminary toxicological data suggest that WR 177,602 may be less toxic than WR 142,490. Due to the great similarity in physical properties expected of these two isomers, the information obtained from the WR 142,490 HPLC assay development was applied with a modification to the WR 177,602 project.

b. Methods:

Partition coefficients of WR 177,602 were determined. A stock solution of WR 177,602- ^{14}C -HCl (Lot 469a, 32.3427 $\mu\text{Ci}/\text{mg}$) labeled in the methanol carbon, was prepared in methanol (1 mg/ml). This solution was utilized to make a 500 ng (base)/ml solution in 0.065 M pH 7.4 Sorenson phosphate buffer. Two ml aliquots of the buffered drug solution were separately equilibrated for 2 hr against 2 ml of one of each of the following solvents: n-heptane, benzene, diethyl ether, ethyl acetate, chloroform and n-butanol. Samples were withdrawn from both the organic and aqueous layers and assayed for total ^{14}C . The ratio of the dpm (organic phase)/dpm (aqueous phase) was calculated for each solvent set (n = 4).

A high pressure liquid chromatograph with a 280 nm absorbance monitor was utilized. Whole blood extracts were chromatographed on a column of 10 μ , fully porous silica bonded with a monomolecular layer of cyanopropylsilane (4 mm ID x 30 cm) using a mobile phase of p-dioxane/isopropyl ether/methanol/glacial acetic acid (117:80:2:1 v/v/v/v) at 2 ml/min.

A calibration curve was constructed for WR 177,602. A solution of WR 184,806 was prepared in p-dioxane/methanol (7:3 v.v) (196 $\mu\text{g}/\text{ml}$). A stock solution of WR 177,602 was prepared in methanol at 0.98 (base) mg/kg. Appropriate amounts of this solution were added to 10 ml volumetric flasks, evaporated to dryness under N_2 and reconstituted in the internal standard solution to give concentrations of WR 177,602 of 200, 100, 50 and 20 $\mu\text{g}/\text{ml}$ containing 196 $\mu\text{g}/\text{ml}$ of WR 184,806. All concentrations are expressed as the free base. A calibration curve was constructed relating peak area ratio of WR 177,602/WR 184,806 vs amount of WR 177,602.

The general assay procedures were identical to that described for WR 142,490 except that only whole blood was analyzed. Precision determinations for the WR 177,602 assay were made. Aliquots (25 ml) of human whole blood were spiked with a methanolic solution of WR 177,602 (0.98 mg/ml) to give drug concentrations of 5.00, 1.00, 0.50 and 0.10 $\mu\text{g}/\text{ml}$. The samples were thoroughly mixed for 30 min and treated as described under the general assay procedure.

c. Results and discussion:

Since WR 177,602 is the diastereoisomer of WR 142,490, similarities in physical and chromatographic properties would be expected. A comparison of the spectroscopic properties of the four antimalarial drugs studied is presented in Table 6. The three quino-
linemethanols all have very similar molar absorptivity constants at

283 nm. Further, a comparison of the partition coefficients for WR 177,602 (Table 7), WR 142,490 (Mu et al., 1975), and WR 184,806 (Grindel et al., 1976) showed that ethyl acetate would be an excellent extraction solvent for all three drugs.

An evaluation of the retention volumes (R_V) for these three compounds using the chromatographic conditions detailed above showed that WR 177,602 ($R_V = 8.2$ ml) could be readily resolved from WR 184,806 ($R_V = 12.3$ ml) but not from WR 142,490 ($R_V = 7.8$ ml). Thus WR 184,806 was used as the internal standard for this assay.

Utilizing the above information an assay procedure for WR 177,602 was evaluated over the range 100 ng - 5000 ng/ml of blood. The results are presented in Table 8. The mean percent recovery was $102.8 \pm 6.2\%$. The linear calibration curve was described by the equation:

$$y = 1.1854x + (-0.0011), r = 0.9988.$$

5. Development of a high pressure liquid chromatographic assay for WR 180,409 in whole blood.

a. Background:

WR 180,409·H₃PO₄, dl-threo- α -(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate, has been shown to be effective in curing Aotus trivirgatus monkeys infected with multi-drug resistant strains of malaria. Its physicochemical characteristics are not unlike those observed for the quinoline-methanols; thus, the developmental work already cited was directly applied to this project.

b. Methods:

The chromatography procedures are as described for WR 177,602.

Calibration curves were constructed. An internal standard solution of WR 184,806 was prepared in p-dioxane/methanol (7:3 v/v) (196 μ g/ml). A stock solution of WR 180,409 (0.96 (base) mg/ml) was prepared in methanol. Appropriate aliquots of the stock solution were added to separate 10 ml volumetric flasks, evaporated to dryness under N₂ and reconstituted in the internal standard solution to give concentrations of WR 180,409 of 192, 96, 48 and 19 μ g/ml containing 196 μ g/ml of WR 184,806. Calibration curves were constructed as previously described using the ratio WR 180,409/WR 184,806.

The general assay procedure was identical to that described for WR 142,490 except that only whole blood was analyzed. Precision determinations for the WR 180,409 assay were carried out. Aliquots (25 ml) of human whole blood were spiked with a methanolic solution of WR 180,409 (0.96 mg/ml) to give drug concentrations of 4.800, 0.960, 0.480 and 0.096 μ g/ml. The samples were thoroughly mixed for 30 min and treated as described under the general assay procedure.

c. Results and discussion:

The physicochemical properties of WR 180,409 are reasonably similar to those of the quinolinemethanols studied. Thus, the molar absorptivity is approximately twice that seen for the quinoline-methanols (Table 6), its partition coefficients are very similar and its retention volume (8.00 ml) is nearly identical to those cited for WR 142,490 (7.8 ml) and WR 177,602 (8.2 ml).

Based on the foregoing data an assay procedure was evaluated for WR 180,409 over the range of 96 - 4800 ng/ml of whole blood (Table 9). The mean percent recovery was $97.9 \pm 4.6\%$. The linear calibration curve was described by the equation.

$$y = 1.8865 x + 0.0002, r = 0.9985.$$

6. Analysis of serum and whole blood specimens from human subjects.

a. Background:

An extension of the phase I clinical trials on mefloquine hydrochloride (WR 142,490-HCl) was performed at the Washington Hospital Center by Bio-Med, Inc. In a rising-dose, double-blind study design male subjects were administered mefloquine hydrochloride orally as 250 mg film-coated tablets or identical looking placebos. Serum or whole blood specimens were obtained as specified for analysis of drug content.

b. Methods:

The serum and whole blood specimens which were received from the Washington Hospital Center were stored at -20°C until analyzed. The method of analysis was as described above for WR 142,490.

Twelve adult male subjects, age 21-45 yrs and weighing 50-100 Kg, were dosed orally with mefloquine hydrochloride as 250 mg film-coated tablets (B-512) or identical-looking placebo tablets (B-513) on Days 1 and 8 of the study. Specimens for analysis were obtained on days 0, 2, 3, 7, 9, 10 and 14. Serum specimens were

submitted for groups 20 (1.00 g) and 21 (1.25 g) and whole blood specimens were submitted for group 22 (1.50 g).

c. Results and discussion:

The results of the analyses are presented in Table 10. Day 0 and placebo subject specimens exhibited no detectable levels of mefloquine. All specimens obtained after administration of drug demonstrated readily measurable levels of drug. The submission of whole blood specimens for group 22 instead of serum specimens as for groups 20 and 21 obscures detection of a dose/drug level relationship among these three groups.

7. The absorption, distribution and excretion of WR 180,409-¹⁴C.H₃PO₄ in mice.

a. Background:

DL-threo- α -(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate (WR 180,409.H₃PO₄) is a promising candidate antimalarial drug. The objective of this study was to determine the absorption, distribution and excretion of this compound in mice. In addition a number of physical properties of the drug was investigated as an aid to these studies.

b. Materials and methods:

Radioactive WR 180,409.H₃PO₄ (21.1179 μ Ci/mg; Lot No. 436a-3a) was synthesized by Dr. W. H. Yanko of Monsanto Research Corp. (Dayton Laboratory, Dayton, OH) with the ¹⁴C-label on the methanol carbon. The nonradioactive compound (Lot AC; bottle number BE 56685) was prepared by Ash Stevens, Inc. (Detroit, MI). A solution of WR 180,409-¹⁴C.H₃PO₄ with a specific activity of 2 μ Ci/mg was prepared by dissolving the appropriate amount of radioactive and nonradioactive compound in deionized water. Chemical and radiochemical purity of the solution were assayed by thin-layer chromatography. A premixed liquid scintillation solution, Hydromix (Yorktown Research Co. Hackensack, NJ) was used for radioassay. All other chemicals and solvents used were reagent grade quality.

Albino, ICR male mice from the Walter Reed Colony weighing about 25 g were used. The mice were fed D and G Laboratory diet (G. L. Baking Co., Frederick, MD) and were maintained in a temperature controlled room with a 12-hr light-dark cycle. The mice were fasted for about 18 hr prior to dosing but were permitted water ad libitum. A dosage of 20 mg/kg was administered to each mouse by oral intubation. The mice were then housed four to a modified Roth

metabolism cage and were allowed water ad libitum. Food was not allowed until four hours after dosing. Standard doses were taken for radioassay before, during and after dosing the animals.

Excreta was collected for analysis. Expired air was drawn through both base and acid scrubbers and analysis performed on consecutive 12 hr samples.

Urine samples were collected at 24 hr intervals for 10 days; aliquots of each sample were taken for total radioactivity determination. The samples were lyophilized to dryness and reconstituted in 5 ml phosphate buffer (pH 7.4). The samples were then extracted 3 times with 10 ml of diethyl ether. The pooled extract was evaporated to dryness and reconstituted with small amounts of absolute methanol for TLC study.

Fecal samples were collected at 24 hr intervals for 10 days, homogenized in absolute methanol in a Waring blender and extracted in glass columns with methanol. The eluates were measured and aliquots taken for gross radioassay. The methanol extracts were evaporated to dryness in a flash evaporator and reconstituted in 5 ml phosphate buffer (pH 7.4). The samples were then extracted with diethylether as described above.

Plasma and red blood cell levels were examined. At appropriate intervals after dosing the four mice in a group were anesthetized with ether and exsanguinated via a surgically exposed femoral artery. Aliquots of the heparinized pooled blood were used for radioassay along with aliquots for the hematocrit (Hct) determination (13,000 x g for 3 min). The remainder of each sample was centrifuged at 7,000 x g for 5 min to separate the plasma from the red blood cells. Aliquots of plasma were taken from each sample for radioassay. Red blood cell levels were calculated. The plasma and red blood cells were lyophilized and extracted by diethyl ether as described above.

Tissue levels of radioactivity were determined at appropriate intervals after dosing. All four mice in a group were anesthetized with ether, exsanguinated and the following tissues removed by dissection: submaxillary salivary glands, heart, lungs, liver, kidneys, spleen, gall bladder and bile, stomach and its contents, small intestine and its contents, cecum and its contents, and large intestine and its contents. Samples of abdominal fat and skeletal muscle were also taken. Each tissue type from each time interval group was pooled, placed in a preweighed container, weighed and sufficient methanol was added to cover the tissues.

For radioassay, fifteen ml of premixed scintillation fluid, Hydromix, was added to each sample. The samples were counted for 10 minutes in a Searle Mark II liquid scintillation counter. Quenching and counting efficiency were corrected by external standardization.

Thin layer chromatography was used extensively for isolation of drug. Appropriate amounts of samples were streaked on EM pre-coated silica gel F-254 TLC plates (EM Laboratories, Ltd., Elmsford, NY) and developed for 10 cm for the origin using either n-butanol:acetic acid:water (66:17:17 by volume) or benzene:methanol (3:1 by volume) as solvent systems. After air drying, the plates were visualized with UV light (254 nm) and scanned with a Varian Model 6000 Radio-scanner with integrator (time constant = 1 sec; speed = 4 in/hr; attenuation = 10 cps). A standard streak of WR 180,409- ^{14}C - H_3PO_4 was placed on each plate as a comparison standard.

c. Results and discussion:

The disposition of DL-threo- α -(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate (WR 180,409- H_3PO_4) was studied over a 240 hr period after 20 mg/kg of the C-14 labeled drug was administered orally to mice. About 84% and 5.5% of the drug-derived radioactivity was excreted via feces and urine respectively (Table 11). The excretion of the drug-derived radioactivity in urine and feces peaked at the time period between 48 and 72 hr. Approximately 4.4% of the administered radioactivity was recovered in the carcasses. A total of about 94% of the administered dose was recovered from the mice.

One mouse was dosed as described and placed into a small glass metabolism cage which was connected to acid and base air scrubbing columns to determine possible elimination via expired air. Samples of expired air trapping solutions were taken as described for 192 hr. A total of 0.32% of the administered radioactive dose was recovered in the expired air by 192 hr. Peak levels of radioactivity in the expired air were also found at the time period between 48 and 72 hr.

Radioactivity in urine and feces was analyzed by thin layer chromatography (TLC). TLC analysis showed that the fraction as parent drug decreased with time in both urine and feces (Table 12). When the data were plotted in a semi-log graph, linear regression analyzed and the slope of the line determined, apparent elimination half-lives of the parent drug ($T_{1/2}$) were calculated to be 21.1 hr and 25.9 hr for urine and feces respectively.

The concentration of ^{14}C -drug equivalents in the plasma and red blood cells (RBC) of the mouse was determined after a single oral

Each tissue pool, as well as the carcasses, was homogenized with methanol. Samples were taken from the homogenates for radioassay and the remaining homogenate was packed into a glass column. After the homogenate was thoroughly extracted with methanol, the volume of the methanol eluates was measured and aliquots were taken for radioassay. The remaining eluates were evaporated to dryness and reconstituted in small amounts of methanol for TLC analysis.

Hydrolysis of urine and fecal extracts was carried out. Representative aliquots of urine and fecal samples were placed in separate flasks with 5 ml of the following media: phosphate buffer (pH 7.4) as control; 700 units of β -glucuronidase in pH 7.4 phosphate buffer; β -glucuronidase/arylsulphatase, 29,000 units in pH 7.4 phosphate buffer; and 0.2 N HCl. The flasks were incubated at 37°C for 3 hrs. The samples were extracted 3 times with 15 ml diethyl ether each time. The extracts were evaporated to dryness and reconstituted in 0.5 ml of methanol. Aliquots of the extracts were taken for radioassay and the remainders analyzed by TLC.

Plasma protein binding was investigated using mouse plasma. Blood was collected from the mice as described previously. Aliquots of the pooled blood were taken for hematocrit determinations. The blood was centrifuged and the plasma was separated. An aliquot of a solution of WR 180,409- ^{14}C was added to phosphate buffer (pH 7.4) such that the final concentration of drug was 1,000 ng (base)/ml. From this stock solution, solutions with concentration of 50, 100, 500 and 1,000 ng (base)/ml were made. Duplicate aliquots (1 ml) of each solution were subjected to equilibrium dialysis against the plasma in multicavity dialysis cells (Model I Multicavity dialysis cells, National Scientific Co., Cleveland, OH). The samples were incubated for 20 hr at 25°C in a Dubnoff metabolic shaker at 100 strokes per min. Radioactivity was determined on both sides of the membrane and the percentage of drug bound to plasma proteins calculated.

Both the pKa and partition coefficients of the drug were measured. For the pKa determination, solutions of WR 180,409 $\cdot\text{H}_3\text{PO}_4$ were made by dissolving 10 mg of the drug in 10 ml of deionized water and titrating with 0.005 N sodium hydroxide solution. A Beckman expanded scale pH meter was used for pH measurement. A phosphate baseline curve was determined and subtracted from the drug curves. The pKa was determined graphically. For the partition coefficients, a solution of WR 180,409- $^{14}\text{C}\cdot\text{H}_3\text{PO}_4$ (4 $\mu\text{g}/\text{ml}$, 3.71 $\mu\text{Ci}/\text{mg}$) was prepared in phosphate buffer (pH 7.4). Aliquots of this solution were taken to determine the total radioactivity. Duplicate 1 ml aliquots of this solution were shaken with 1 ml of various organic solvents for 2 hr at 25°C. After centrifugation, aliquots of both phases were withdrawn for radioassay. K_p was calculated as the ratio of radioactivity in the organic phase to that in the buffer phase.

dose of the drug. The plasma and RBC concentration curves for WR 180,409-¹⁴C and total ¹⁴C-drug equivalents, expressed as μg of drug equivalents per ml, are summarized in Table 13. The highest levels of apparent parent compound and total radioactivity (0.86 $\mu\text{g}/\text{ml}$ and 2.03 $\mu\text{g}/\text{ml}$ respectively) were observed at 2 and 4 hr respectively. In RBC, the peak concentration of apparent parent compound and total radioactivity (2.96 $\mu\text{g}/\text{ml}$ and 3.71 $\mu\text{g}/\text{ml}$) were observed at 12 hr. The elimination $T_{1/2}$ of WR 180,409-¹⁴C in plasma and in RBC was estimated to be 26.2 hr and 27.4 hr respectively.

The distribution of WR 180,409-¹⁴C in selected tissues was studied. Of the organs investigated, the major sites of distribution of WR 180,409-derived radioactivity were the lungs, liver, kidneys, small intestine and contents, and residual carcasses (6.21%, 14.22%, 5.95%, 18.22% and 31.24% of the administered dose) two hr after oral administration of the drug (Table 14). Two hr after the administration of the drug, a total of about 61% of the dose was accounted for when adding the radioactivity in the submaxillary salivary glands, heart, lungs, liver, kidneys, spleen, gall bladder plus bile, plasma, RBC and residual carcasses after removal of the entire gastrointestinal tract with associated contents. This indicates that at least 61% of the dose was absorbed by 2 hr after oral administration of the drug. In terms of μg of drug per g of tissue, the major sites of deposition of drug derived radioactivity were the lungs, liver, kidneys and gall bladder plus bile 48 hr after oral administration of the drug (Table 15). A high percentage of unchanged WR 180,409-¹⁴C in these tissues was identified by TLC analysis (Table 16).

Several TLC solvent systems were tested for the metabolic profile of WR 180,409. Two were selected. These are n-butanol:acetic acid:water (66:17:17 by volume) and benzene:methanol (3:1 by volume). Table 17 shows the TLC profiles of urine and fecal extracts. The chromatography plates were developed two dimensionally with the two chosen solvent systems. The results show that four major bands were separated by these two solvent systems and that one of these bands was found to be identical to the parent drug.

The plasma protein binding of WR 180,409 was studied by equilibrium dialysis of fresh mouse plasma. Table 18 shows that 99% of the drug (concentration range from 100 ng/ml to 5,000 ng/ml) was bound to the mouse plasma.

Representative aliquots of urine and fecal extracts were subjected to β -glucuronidase, β -glucuronidase/arylsulphatase or 0.2 N HCl. These enzyme and acid hydrolysis conditions did not cause any significant changes in the chromatographic profile of the relative

composition of the radioactivity as analyzed by TLC.

The lipophilic characteristic of this drug was estimated by studying its partition between phosphate buffer (pH 7.4) and various organic solvents. The results of this study, presented in Table 19, indicate that WR 180,409 has high lipid solubility. The pKa of WR 180,409 was determined to be 8.1.

8. The cardiorespiratory effects of WR 180,409·H₃PO₄.

a. Background:

This investigation was undertaken to define the acute cardiorespiratory actions of the candidate antimalarial drug, DL-threo- α -(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate (WR 180,409·H₃PO₄) after intravenous administration to anesthetized dogs.

b. Materials and methods:

Ten mongrel dogs of either sex weighing between 15.0 and 22.7 kg were anesthetized with 30 mg/kg sodium pentobarbital administered intravenously. The first four animals were used to determine the basic cardiorespiratory activity of WR 180,409·H₃PO₄. The left femoral artery and vein were catheterized for measurement of arterial pressure and drug administration. An oral endotracheal tube was positioned and respiration monitored by a pneumotachometer. The Lead II electrocardiogram was recorded and its signal was fed into a cardiograph for measurement of heart rate. All measurements were recorded on a Hewlett-Packard 7700 series recorder. Body temperature was monitored via a rectal thermistor probe and maintained near normal by application of external heat. At the termination of the experimental procedures, animals still alive were euthanized with Lethane.

WR 180,409·H₃PO₄ was obtained from bottle number BE 99420, Lot AD. The drug was dissolved in a vehicle with the following composition: 6.25% propylene glycol, 12.5% absolute ethanol, and 81.25% dextrose 5% in distilled water. The drug concentration in the solution was 25 mg/ml. At this concentration, the drug remained in solution if kept at body temperature, 37°C.

The cumulative effect of intravenous WR 180,409·H₃PO₄ on cardio-respiratory responses was assessed in the first animal (Dog #1). The drug was administered as an iv bolus at 20 minute intervals in increasing doses and cardiorespiratory responses were observed. Progressive doses of 0.1 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 0.3 mg/kg, 1.0 mg/kg . . . were administered until death occurred

and the cumulative fatal dose was recorded. In a second animal (Dog #2) a 3 mg/kg dose of WR 180,409·H₃PO₄ was administered as an iv infusion over 40 minutes. A 3 mg/kg dose was then injected iv 20 minutes after termination of the infusion and again 20 minutes later. After vagotomy, an additional 3 mg/kg iv injection was made. Twenty minutes after the last injection, a 10 mg/kg dose of WR 180,409·H₃PO₄ was administered iv to determine whether vagotomy protected the animal from a larger dose of the drug. The ability of WR 180,409·H₃PO₄ to modify the cardiorespiratory effects of 1.0 µg/kg doses of epinephrine hydrochloride, isoproterenol hydrochloride, acetylcholine hydrochloride, histamine phosphate, and serotonin creatinine sulfate monohydrate, administered iv as the salt, was assessed in a third animal (Dog #3) before and after a 40 minute iv infusion of 3 mg/kg WR 180,409·H₃PO₄. Following these injections, 3 mg/kg WR 180,409·H₃PO₄ was injected as an iv bolus. In a fourth animal (Dog #4), after the iv bolus injection, and an additional 3 mg/kg dose of WR 180,409·H₃PO₄ was administered as an iv bolus.

The next series of four animals (Dogs #5-8) was used to evaluate the effects of a 60 minutes infusion of WR 180,409·H₃PO₄, 15 mg/kg. In addition to measuring the parameters described earlier, central venous pressure (CVP) and the pre-ejection period to left ventricular ejection time ratio (PEP/LVET) were monitored. CVP was measured from a catheter placed in the inferior vena cava via the left femoral vein. Drug infusions were made through a catheter placed in the right femoral vein. PEP/LVET has been used as a non-invasive method of assessing drug activity on myocardial function. An increase in the ratio is indicative of depressed myocardial function (Rinehart et al., 1974). For these studies, PEP was measured as the interval between the initiation of the R wave in the Lead II ECG and the beginning point of the rapid rise to systolic pressure observed in the arterial pressure curve. LVET was measured as the interval between the beginning point of the rapid rise to systolic pressure and the dicrotic notch observed in the arterial pressure curve. The standard 25 mg/ml solution of WR 180,409·H₃PO₄ was diluted with the vehicle so that the infusion consisted of 0.25 mg/kg WR 180,409·H₃PO₄/ 0.247 ml vehicle/min. Following the one hour infusion, the animals were observed for an additional one hour period. The paired t test of significance was used to compare baseline values with values obtained during and after the infusion period. Values were considered significant if the computed t exceeded the tabular t value for 5% level of significance.

Ascending aortic blood flow and myocardial contractility were monitored in the final two animals (Dogs #9 and 10) during and after infusing iv 15 mg/kg WR 180,409·H₃PO₄ over a 60 minute period. Blood pressure and heart rate were monitored as in previous animals.

The two animals were artificially respired with room air by positive pressure ventilation and the chest was opened by midline incision. The pericardial sac was then cut so as to expose the right ventricle and ascending aorta. The sac remained sufficiently intact to cradle the heart. The ascending aorta was cleared of connective tissue and an electromagnetic flow probe was placed around the ascending aorta. The impulse from the flow probe was calibrated so that the flow could be read in liters/min. A Walton-Brodie strain gauge arch was sewn onto the right ventricular muscle mass. Myocardial contractility was recorded as a percentage change in the baseline measurements. Total peripheral resistance (TPR) was calculated in peripheral resistance units (PRU), obtained by dividing mean arterial pressure (mm Hg) by aortic blood flow (ml/sec). A PRU value of less than one is associated with vasodilation or a decrease in resistance while a value greater than one denotes vasoconstriction or an increase in peripheral resistance.

c. Results and discussion:

The cardiorespiratory responses to repeated administration of increasing doses of WR 180,409·H₃PO₄ are given in Table 20. In this animal, 0.1 and 0.3 mg/kg had little effect upon the measured parameters. WR 180,409·H₃PO₄, 1 mg/kg, produced a bradycardia, a slight hypotension, and a brief period of apnea followed by tachypnea. Increasing the dosage to 3 mg/kg produced a more profound hypotension. Arterial pressure returned to control values within the 20 minute interval between injections. Heart rate returned to control levels within the 20 minute interval except prior to injection of 10 mg/kg. Beginning with the 1 mg/kg dose, there were marked changes in respiration. The tachypnea following the postinjection apnea had not returned to control levels within the 20 minute interval between injections. A summary of the cardiorespiratory responses to an iv bolus injection of WR 180,409·H₃PO₄, 3 mg/kg, is given in Table 21. The hypotension, bradycardia, and respiratory depression observed after WR 180,409·H₃PO₄ is similar to the Bezold-Jarish response observed with the injection of veratrum alkaloids. Administration of veratrum alkaloids stimulates afferent vagal fibers in the coronary sinus and left ventricle resulting in a brief period of hypotension and bradycardia. Veratrum alkaloids also depress respiration by an action on pulmonary stretch receptors (Nickerson, 1971). The effect of vagotomy upon the cardiorespiratory actions of WR 180,409·H₃PO₄ is given in Table 22. Vagotomy reversed the chronotropic response, attenuated the hypotension, abolished the period of apnea, and markedly reduced the tachypnea observed after injection of WR 180,409·H₃PO₄. These responses are qualitatively similar to those obtained with veratrum alkaloids after vagotomy.

Dog #1 died after a cumulative dose of 18.8 mg/kg of WR 180,409-H₃PO₄. Dog #2 was administered 9 mg/kg of WR 180,409-H₃PO₄, in three 3 mg/kg doses, was vagotomized and given an additional 3 mg/kg dose of WR 180,409-H₃PO₄. Finally, a 10 mg/kg dose was administered which resulted in death of the animal. Vagotomy, although it modified cardiorespiratory responses, did not appear to protect the animal since a cumulative dose of 22 mg/kg WR 180,409-H₃PO₄ was fatal to Dog #2. In Dogs #1 and #2, the pH and hematocrit of the venous blood were monitored at different intervals. The control value for pH of venous blood averaged 7.37 while the hematocrit averaged 37%. The pH values decreased with increasing doses of WR 180,409-H₃PO₄ and the final value, obtained prior to the 10 mg/kg dose, averaged 7.15. During the same period the hematocrit increased to 56% with definite hemolysis present in the two dogs. Autopsy of Dogs #1 and #2 revealed a marked pulmonary congestion. Rapid, repeated intravenous injections of WR 180,409-H₃PO₄ produced profound alterations in the cardiorespiratory systems resulting in a loss of vascular fluid into the lungs. This could explain the hemoconcentration and acidosis observed in these two animals.

Infusing the 3 mg/kg dose of WR 180,409-H₃PO₄ over a 40 minute period produced a decrease in heart rate but no change in blood pressure or respiratory rate (Table 23). WR 180,409-H₃PO₄, 3 mg/kg, infused over a 40 minute period, did not appear to modify the magnitude or duration of the cardiorespiratory responses following iv injections of epinephrine, isoproterenol, acetylcholine, histamine and serotonin (Table 24).

The maximum oral dose of WR 180,409-H₃PO₄ that will be administered to humans is projected, at this time, to be one gram. Based on a 70 kg man, this is a dose of approximately 15 mg/kg. But doses of 3 mg/kg, when administered as an iv bolus injection, produced marked cardiorespiratory alterations in the dog. Consequently, an experiment was designed to approximate the gastric absorption of an oral dose of WR 180,409-H₃PO₄. A dose of 15 mg/kg of the drug was infused slowly over an hour while recording the appropriate cardiorespiratory responses. The results of these studies are given in Table 25. WR 180,409-H₃PO₄, 15 mg/kg, administered in an iv infusion over one hour, produced a significant decrease in heart rate throughout the entire observation period following the infusion and produced a significant increase in PEP/LVET for 30 minutes following termination of the infusion. These results indicate a depression of myocardial function. However, PEP/LVET values during the remainder of the observation period were returning to baseline levels. The infusion had little effect on mean arterial pressure, central venous pressure, or respiration.

Results of the WR 180,409·H₃PO₄ infusion studies in open chest animals are given in Table 26. The initial decrease in arterial pressure recorded in these animals may be attributed to a decrease in peripheral resistance as ascending aortic flow remained unchanged during the initial portion of the infusion. The decrease in arterial pressure observed after peripheral resistance had returned to baseline values may be attributed to the progressive decrease in myocardial contractility which resulted in a decrease in aortic flow. The decrease in arterial pressure observed in the open chest animals was in contrast to the relatively stable blood pressure obtained in the closed chest experiments even though those animals also had depressed myocardial function as indicated by the PEP/LVET ratios. These differences in blood pressure responses may be due to the experimental model since opening the chest and artificial ventilation may disrupt reflex mechanisms which would normally work to maintain constant aortic pressure. These experiments indicate that WR 180,409·H₃PO₄, 15 mg/kg, has negative inotropic activity when infused over a 60 minute period in both closed and open chest animals. However, this depression of myocardial function observed with infusion of WR 180,409·H₃PO₄ may not be clinically relevant since the closed chest animals were able to maintain a stable blood pressure and appeared to be recovering from the drug effects during the observation period.

9. Studies on the oral efficacy and toxicity of candidate anti-malarials and the effect of modification of host drug-metabolizing capacity on these parameters.

a. Background:

On the basis of the rationale and methodology outlined in previous Annual Progress Reports, investigations of candidate anti-malarials of the 8-aminoquinoline class, as well as of those classes offering promise against drug-resistant malaria, have continued in mice.

Prior to projected investigations on possible interactions between candidate antimalarials and other drugs, and on the impact of such interactions on efficacy and toxicity, it is usually necessary to conduct preliminary investigations. Such pilot studies not only provide specific data required for planning and conducting more detailed and systematic studies but also often provide unique information beyond this. Some of the investigations herein described are in this category.

b. Comparison of the oral curative activities of racemic 4-methylprimaquine (WR 181,023) and its dextro (WR 221,033) and levo (WR 221,036) enantiomers against trophozoite-induced P. berghei infections in mice:

Recently, the dextro and levo forms of racemic 4-methylprimaquine became available for study in limited quantities. The oral efficacy of racemic 4-methylprimaquine was shown to be greater than that of primaquine against trophozoite-induced infections in mice; cures could be obtained with apparently non-toxic doses of WR 181,023, whereas doses of primaquine in the lethal range were required to effect cures under the same conditions. Moreover, while the lethal toxicity (LD-50, 7-day endpoint) of WR 181,023 was approximately one-half that of primaquine qualitative differences in toxicity resulting from these two agents were observed. Notable among these was the gross liver damage that occurred in mice after large doses of 4-methylprimaquine but not after similar doses of primaquine.

Accordingly, it was important to learn whether differences in oral antimalarial efficacy and oral toxicity were experimentally discernible among 4-methylprimaquine and its optical isomers.

Table 27 shows the results of two identically performed experiments in which 4 groups of *P. berghei*-infected mice were treated once by gavage on Day 3 of infection with either agent vehicle (MCT) alone, WR 181,023, WR 221,033, or WR 221,036. The agents were given in equivalent doses of base. 194.5 mg/kg was used since WR 181,023 at this level was well tolerated and cured 16 of 21 mice.

In the first experiment (Table 27, Experiment A), all control animals died of malaria whereas 100% of the mice were cured by treatment with WR 181,023 or its optical isomers. However, in this experiment percentage parasitemia was considerably below the usual Day 3 level. In the repeat experiment (Table 27, Experiment B), percentage parasitemia was at a higher, more desirable level at the time of treatment on Day 3. The results show again that all mice given vehicle control died of malaria and that a majority of cures was achieved with each of the agents; both the racemate and the levo isomer effected a cure rate of about 75% while the dextro isomer effected a cure rate of 92%. These differences are not statistically significant, however. The cure rate afforded by WR 181,023 is in close agreement with the 76% cure rate previously observed using this same dose under similar conditions.

Both experiments, therefore, strongly suggest that the dextro and levo forms are no less efficacious in curing trophozoite-induced infections in mice than is the parent racemate. The relative parasitemia-suppressing activity of these 3 agents when given in smaller, subcurative doses remains to be determined, however. Additional experiments will also be required to ascertain whether or not the dextro isomer possesses a greater antimalarial activity.

It is noteworthy, as footnoted in Table 27, that of the uninfected mice (toxicity controls) run in parallel with the infected mice in each experiment and treated with either of the agents, only those given the dextro isomer suffered toxic deaths (3 of a total of 12 mice) during the course of the experiment. This suggested that WR 221,033 might be less well tolerated than the other two agents.

c. Comparison of the acute oral toxicity of 4-methylprimaquine racemates with that of its optical isomers in uninfected mice:

The results of Experiment A in Table 28 show that toxic deaths did not result from oral administration of 194.5 mg base/kg of WR 181,023 or its levo isomer, while one death did result after this dose of the dextro isomer. These findings are consistent with those obtained with the uninfected toxicity controls in the foregoing study (Table 27). Combining the latter data with those of Experiment A, Table 28, the overall 7-day mortality for the groups dosed with the racemate or the levo form is the same, viz., 0% (0/22), while that for the dextro form is 18% (4/22).

Experiment B in Table 28 contrasts the cumulative mortalities following administration of each of the agents at a dosage of base equivalent to the estimated LD-50 (7-day endpoint) of WR 181,023, viz., 423 mg base/kg. The data shown were pooled from two identical experiments since the results from each were similar. At this higher dose, each of the agents produced a significant mortality by 7 days; the 60% mortality caused by the racemate was intermediate between that caused by the dextro isomer (80%) and the levo isomer (30%). Only the difference in mortality between the levo and dextro forms is statistically significant, however.

It is noteworthy that in a previous more intensive comparison of the acute oral toxicities (7-day endpoint) of racemic primaquine and its dextro and levo forms, the dextro form is the most toxic and the levo form the least toxic of the three agents. In regard to the order of toxicity, then, there is a tentative correspondence among the respective forms of primaquine and those of its 4-methyl analogue, WR 181,023.

It is noteworthy, that, at the dose levels studied, neither WR 181,023 nor its dextro or levo forms produced facial edema or gross skeletal muscle changes in the mice as seen previously with racemic primaquine and its dextro form.

Gross examination of the livers of non-surviving mice and of 7-day survivors revealed that all 3 forms of 4-methylprimaquine produced signs of hepatotoxicity when given at a dose of 423 mg base/kg. Gross liver abnormalities were not seen in 7-day survivors that received these agents at a dose of 194.5 mg base/kg, however.

d. Comparison of the oral suppressive efficacy of primaquine and 3-methylprimaquine (WR 211,814) against trophozoite-induced infection, when given once to mice in equivalent doses of base:

Peters, on the basis of results from his mouse causal prophylaxis model, has reported that WR 211,814, the 3-methyl analogue of primaquine ". . . has proved to be one of the most active 8-aminoquinolines we have encountered . . ." and that the fully active dose was less than 0.1 mg/kg subcutaneously. Since a dose of 30 mg/kg was non-toxic, he indicated that this compound clearly has a high therapeutic ratio in his rodent model system. More recently, he reported that the minimum fully active dose of this "outstandingly active" 8-aminoquinoline was between 0.1 and 0.3 mg/kg s.c. (Peters, 1975b).

It, therefore, became of interest to examine 3-methylprimaquine's activity against trophozoite-induced infection (Table 29) and also its toxicity (Table 30) when given once by gavage instead of parenterally. The effects of 3-methylprimaquine were compared in parallel with those of primaquine.

The results in Table 29 show clearly that 3-methylprimaquine either as the diphosphate (Group C) or as the dihydrochloride (Group D) exhibits blood schizontocidal activity and that this activity is significantly greater than that of primaquine (Group B). However, while the dose of primaquine used was deliberately not curative, neither was the same dose of 3-methylprimaquine of either salt form.

Two groups of uninfected mice of 5 each (not shown in Table 29), serving as toxicity controls, were treated on Day 3 with either of the salt forms of 3-methylprimaquine precisely as were infected Groups C and D. These control mice appeared in good health 19 days after treatment, when all infected mice (Groups A to D) were dead. Upon killing and autopsy, none of these controls showed any gross abnormalities as a consequence of the 3-methylprimaquine they had received.

Thus, 3-methylprimaquine given orally at a dose of 20.7 mg salt/kg (11.4 mg base/kg) caused no toxic deaths or other apparent drug-related abnormalities in uninfected mice; in infected mice it exhibited greater parasitemia suppressing activity than an equivalent dose of primaquine, but, like the latter, did not effect any cures.

e. Comparison of the acute oral toxicities of primaquine and 3-methylprimaquine (WR 211,814) in uninfected mice:

Table 30 shows that 3-methylprimaquine (Groups II to IV), like primaquine (Group I), can evoke overt swelling of the snout and tongue of mice during the first 6 hours after oral treatment. However, these effects of 3-methylprimaquine generally occurred in fewer mice per group, developed more slowly, and were considerably less severe than after the standard dose of primaquine used. Moreover, as compared to primaquine, which caused swelling of the tongue and snout to an equally severe degree, 3-methylprimaquine appeared to cause more pronounced swelling of the tongue than of the snout, especially in individual mice.

From the cumulative mortality results in Table 30 it is evident that the lethal toxicity of 3-methylprimaquine is greater than that of primaquine. Since 91.2 mg base/kg of primaquine is approximately one-half of the LD-50 (7-day endpoint) it is estimated that the lethal toxicity of WR 211,814 is approximately four fold greater than that of primaquine.

Hindleg weakness and prostration (oftimes lasting for hours but with retention of good eye color and wink reflex) were observed in mice destined to die after treatment with 3-methylprimaquine. Necropsy of such animals usually revealed gross evidence of liver abnormality; livers appeared mottled due to swirls of pallor, or were uniformly pale and presumably fatty, often with reddish speckling. Abnormal appearing livers with focal lesions were also seen upon killing and autopsy of 3-methylprimaquine-treated mice that survived 7 days, but not in any of the primaquine-treated 7-day survivors. In contrast, 8 of 10 of the latter mice showed necrotic lesions of the diaphragm and/or tongue, whereas none of the former (a total of 6 mice) showed such changes. In view of the limited number of mice available for examination in this study, we can only tentatively conclude that gross skeletal muscle changes, akin to those seen after primaquine, do not occur after 3-methylprimaquine.

3-methylprimaquine (WR 211,814), but not 4-methylprimaquine (WR 181,023) causes acute overt facial swelling in mice, as does primaquine. It would be of interest to learn whether this toxic property of 3-methylprimaquine racemate is ascribable solely to its dextro component as is the case with primaquine racemate.

f. The effect of prior modification of host-drug-metabolizing capacity (by phenobarbital stimulation and SKF 525-A inhibition) on the parasitemia suppressing activity of WR 184,806 AJ:

WR 184,806 is the most recent quinolinemethanol antimalarial to be formulated and submitted for clinical trials. It joins two already highly successful predecessors of this chemical class, WR 30,090 and WR 142,490.

Since we had previously investigated the effects of prior stimulation (phenobarbital pretreatment) and inhibition (SKF 525-A pretreatment) of host drug metabolism on the oral antimalarial efficacy of WR 30,090 and WR 142,490 in mice, it was of interest to examine similarly the third member of this series, WR 184,806.

Table 31 presents a comparison of 6 groups of mice that were studied side-by-side and subjected to the experimental and control manipulations indicated. The results may be summarized briefly as follows: First, none of the mice, regardless of type of pretreatment regimen or treatment on Day 3, were cured of malaria. Second, all mice given control treatment (water only) on Day 3 (Groups I to III), irrespective of pretreatment regimen, behaved similarly regarding the course of malaria infection. Third, the oral dose of WR 184,806 AJ used, viz., 5 mg salt/kg, was clearly suppressive (Group IV vs Groups I, II, or III). Fourth, phenobarbital pretreatment significantly reduced the suppressive efficacy of this dose of WR 184,806 AJ (Group V vs Group IV) to essentially control levels (Group V vs Groups I, II or III). Fifth, SKF 525-A pretreatment significantly increased the efficacy of WR 184,806 AJ, this was reflected by the significantly lower parasitemias on Days 6, 8 and 10 (Group VI vs Group IV).

The findings that modification of host drug metabolism by both pretreatment regimens (Groups V and VI) significantly affected the oral efficacy of WR 184,806 AJ, and that phenobarbital stimulation and SKF 525-A inhibition decreased and increased efficacy, respectively, suggest that: the liver ordinarily processes this antimalarial; this processing serves to terminate its parasitemia-suppressing activity which is, therefore, probably due to the administered parent chemical and not to metabolic by-products; and finally, medications known to alter drug metabolism may affect the activity of this antimalarial.

The pattern of change in the oral antimalarial efficacy of WR 184,806 AJ observed after the pretreatment regimens is identical to that found in our previous studies with the other two quinolinemethanols (WR 30,090 and WR 142,490).

10. Evaluation of WR 122,455 and WR 171,669 for antimalarial activity in man.

a. Background:

During World War II two phenanthrenemethanols were shown to have blood schizontocidal activity against P. vivax in man. In the late 1960's another phenanthrenemethanol, WR 33,063, was shown to be curative in man for both chloroquine-resistant and -sensitive strains of P. falciparum. These results led to the selection for clinical trials of two more phenanthrenemethanols, WR 122,455 and WR 171,669.

b. Methods:

Subjects for these investigations were healthy male inmates of the Kansas City Corrections Institute. Subjects were carefully advised of the nature and risks of these studies before written consent was granted. A double blind two by two rising dose design was used for Phase I studies. One day before and 1, 7 and 14 days after drug administration, extensive patient interviews, physical examinations, and clinical and laboratory tests were carried out. For Phase II studies clinical malaria was induced by intravenous injection of 10^6 - 10^7 parasitized red cells. Subjects were treated soon after parasitemia developed and followed for 8 weeks after parasite clearance. Two strains of P. falciparum were used: African Uganda I, which is sensitive to all common antimalarials and Vietnam Smith, which is chloroquine-resistant.

c. Results:

Phase I: Tolerance and toxicity. WR 122,455 in single oral doses was tolerated up to and including 800 mg. A multiple dose regimen of 240 mg twice daily was tolerated for 3 to 5 days but symptoms developed in subjects receiving drug for 6 days. Intolerance to WR 122,455 was manifested by abdominal cramps and diarrhea.

WR 171,669 was tolerated in single oral doses of 750 mg, the highest dose tested. A multiple dose trial of 420 mg 3 times per day was tolerated for one but not 2 and 3 days. A regimen of 250 mg every 6 hours for 19 doses induced symptoms in one of 2 subjects. Intolerance to WR 171,669 was also manifested by gastrointestinal symptoms of abdominal pain, cramps, and diarrhea. No significant alterations in clinical or laboratory findings were noted in any case.

Phase II: Antimalarial efficacy. Single oral doses of 440-880 mg WR 122,455 promptly cleared parasitemia and fever in five subjects, but recrudescences developed in all subjects. However,

WR 122,455 at 480 mg/day for 3 to 6 days cured 9/9 cases of chloroquine-resistant and 4/4 cases of chloroquine-sensitive P. falciparum. Parasite and fever clearance times in subjects cured with WR 122,455 were 3.1 days and 66 hours, respectively. WR 171,669 at one gram/day for 3 days cured 6/6 cases of chloroquine-resistant and 3/3 cases of chloroquine-sensitive infections. In patients cured with WR 171,669 parasite and fever clearance times were 3.5 days and 42 hours, respectively.

d. Discussion:

Intolerance to WR 122,455 and WR 171,669 was manifested by gastrointestinal symptoms, although no gastrointestinal blood loss, laboratory abnormalities or weight loss were seen in symptomatic subjects. Phase II efficacy studies showed that WR 122,455 and WR 171,669 are highly effective drugs for treatment of chloroquine-resistant and -sensitive P. falciparum infections. The full potential of these agents must be determined by further studies against naturally acquired malaria infections in areas of known drug resistance.

11. Development of new antimalarial drugs.

a. Background:

The Department of Pharmacology is also charged with the responsibility of writing Notice of Claimed Investigational Exemption for New Drug (IND) submissions. These include planning and designing the experiments, and assembling, evaluating, coordinating and correlating the data required for both the initial submission and all supplementary submissions for each drug. The data must be continuously monitored and evaluated from both in-house and contract sources, as well as proprietary and open literature sources.

b. Investigational New Drug submissions:

Two new IND applications were written. They were WR 30,090 free base in oil and WR 180,409·H₃PO₄.

Thirteen supplements to IND submissions were written. They were for 10 single drugs and 3 combinations.

c. Technical monitoring of contracts necessary for data generation:

Fourteen active contracts were closely guided by the Department. These ranged from pharmacological areas such as toxicology,

drug metabolism and bioavailability of the drugs to those of their formulation and development of methods to determine blood levels of drugs.

Table 1

Retention Volumes (V_R) for Mefloquine and WR 184,806
upon Reverse Phase or Normal Phase Partition Chromatography

Reverse Phase Partition Chromatography ^a		
Mobile Phase	V_R (ml)	
	WR 142,490	WR 184,806
Methanol/0.1 M NaH_2PO_4		
(3:2 v/v)	5.4	4.0
(2:1 v/v)	4.6	2.9
(4:1 v/v)	3.0	2.2
Normal Phase Partition Chromatography ^b		
Mobile Phase	V_R (ml)	
	WR 142,490	WR 184,806
Isopropyl ether/p-dioxane/ glacial acetic acid		
(3:2 v/v + 0.5%)	4.4	10.3
(2:3 v/v + 0.5%)	3.7	6.5
(1:4 v/v + 0.5%)	20.5	42.5

^a μ Bondapak C_{18} column (4 mm ID x 30 cm), Waters Associates.

^b μ Bondapak CN column (4 mm ID x 30 cm), Waters Associates.

Table 2

Recovery of Mefloquine and WR 184,806 from
Spiked Whole Blood Specimens^a

Mefloquine Added, $\mu\text{g/ml}$ ^b	Percent Recovery \pm S.D.	
	Mefloquine	WR 184,806
1.00	95.4 \pm 2.4	95.5 \pm 1.3
0.50	99.1 \pm 8.3	96.3 \pm 4.9
0.10	105.2 \pm 13.6	95.3 \pm 9.3
MEAN	99.9 \pm 9.3	95.7 \pm 5.4

^aChromatographic conditions are described under methods.^bFour samples at each concentration level.

Table 3

Precision of the Method for the Determination of Mefloquine
in Spiked Whole Blood, Plasma and Urine Specimens^a

Mefloquine Added, $\mu\text{g/ml}^b$	Assayed Mefloquine Concentration, $\mu\text{g/ml}^c$		
	Whole Blood	Plasma	Urine
5.00	4.96 \pm 0.25	5.20 \pm 0.11	4.76 \pm 0.14
1.00	1.04 \pm 0.02	1.00 \pm 0.06	0.97 \pm 0.06
0.50	0.54 \pm 0.04	0.47 \pm 0.07	0.48 \pm 0.05
0.25			0.25 \pm 0.04
0.05	0.05 \pm 0.004	0.05 \pm 0.004	
Mean Percent Recovery \pm R.S.D.	102.9 \pm 6.7%	99.5 \pm 9.5%	97.1 \pm 9.4%

^aChromatographic conditions are described under methods.

^bThe concentration of internal standard was 1.96 $\mu\text{g/ml}$.

^cMean \pm S.D. for four replicates at each level.

Table 4

Mefloquine Excretion in the Urine of an Adult Male
after a Single Oral Dose of 500 mg of Mefloquine·HCl^a

Day ^b	Mefloquine Excretion		
	μg/ml	μg/Sample	% Dose
1	3.03	527.92	0.12
2	0.76	127.01	0.03

^aChromatographic conditions are described under methods.

^bA random urine sample was obtained on each of both days.

Table 5
Precision Data for the Determination
of Mefloquine in Spiked Whole Blood Specimens^a

WR 184,806 Added, $\mu\text{g}/\text{ml}$ ^b	Assayed WR 184,806 Levels ^c	
	$\mu\text{g}/\text{ml}$	% Recovery
5.00	5.22 \pm 0.19	104.4 \pm 3.8
1.00	0.96 \pm 0.03	96.3 \pm 3.0
0.50	0.47 \pm 0.02	94.0 \pm 4.0
0.25	0.26 \pm 0.02	104.0 \pm 8.0
0.10	0.10 \pm 0.001	100.0 \pm 1.0
	MEAN	100.1 \pm 4.6

^aChromatographic conditions are described under methods.

^bThe concentration of internal standard was 1.00 $\mu\text{g}/\text{ml}$.

^cMean \pm S.D. for four replicates at each level.

Table 6

Comparative Molar Absorbance Values for Four Antimalarial Drugs

Compound	λ max (nm) (Solvent)	ϵ
WR 142,490 ^a	283.0 (water)	5620 (\pm 0.6%)
WR 184,806 ^b	283.0 (MeOH)	6060 (\pm 0.8%)
WR 177,602 ^c	282.5 (EtOH)	5850 (\pm 1.0%)
WR 180,409 ^d	278.0 (MeOH)	11,000 (\pm 0.4%)

^aLot AH, BE 16387.^bLot AJ, BE 19520.^cLot AD, BE 77728.^dLot AC, BE 56685.

Table 7
Partition Coefficients of WR 177,602^a

Organic Phase	K _p
Ethyl Acetate	139.9
Ethyl Ether	98.8
n-Butanol	87.1
Chloroform	72.0
Benzene	41.0
n-Heptane	1.4

^aK_p is expressed as the ratio of the concentration of drug in the organic phase to that in the aqueous phase, Sorensen phosphate buffer (pH 7.4). Data are averages of four determinations.

Table 8

Precision Data for the Determination of WR 177,602
in Spiked Whole Blood Specimens^a

WR 177,602 Added, $\mu\text{g/ml}$ ^b	Assayed WR 177,602 Levels ^c	
	$\mu\text{g/ml}$	% Recovery
5.000	5.430 \pm 0.114	108.6 \pm 2.3
1.000	1.034 \pm 0.062	103.4 \pm 6.2
0.500	0.506 \pm 0.016	101.2 \pm 3.2
0.100	0.098 \pm 0.010	98.0 \pm 10.0
	MEAN	102.8 \pm 6.2

^aChromatographic conditions are described under methods.

^bThe concentration of internal standard was 1.96 $\mu\text{g/ml}$.

^cMean \pm S.D. for four replicates at each level.

Table 9

Precision Data for the Determination of WR 180,409
in Spiked Whole Blood Specimens^a

WR 180,409 Added, $\mu\text{g/ml}$ ^b	Assayed WR 180,409 Levels ^c	
	$\mu\text{g/ml}$	% Recovery
4.800	4.617 \pm 0.133	96.2 \pm 2.8
0.960	0.933 \pm 0.069	97.2 \pm 7.2
0.480	0.442 \pm 0.015	92.1 \pm 3.0
0.096	0.102 \pm 0.008	105.9 \pm 8.4
	MEAN	97.9 \pm 4.6

^aChromatographic conditions are described under methods.

^bThe concentration of internal standard was 1.96 $\mu\text{g/ml}$.

^cMean \pm S.D. for four replicates at each level.

Table 10

Human Serum and Blood Concentrations of Mefloquine
after Dosing with Mefloquine on Days 1 and 8

Group 20		Serum Concentration ($\mu\text{g/ml}$)						
Subject No	Dose	Day 0	Day 2	Day 3	Day 7	Day 9	Day 10	Day 14
85	1.00 g	0.00	0.64	0.77	0.42	1.18	1.17	1.27
86	Placebo	0.00	0.00	0.00	0.00	0.00	0.00	0.00
87	1.00 g	0.00	0.70	0.85	0.35	1.00	1.13	1.32
88	Placebo	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Group 21		Serum Concentration ($\mu\text{g/ml}$)						
Subject No	Dose	Day 0	Day 2	Day 3	Day 7	Day 9	Day 10	Day 14
89	Placebo	0.00	0.00	0.00	0.00	0.00	0.00	0.00
90	1.25 g	0.00	1.00	0.85	0.65	2.15	1.75	1.00
91	Placebo	0.00	0.00	0.00	0.00	0.00	0.00	0.00
92	1.25 g	0.00	1.25	1.25	1.00	2.55	2.06	1.25
Group 22		Whole Blood Concentration ($\mu\text{g/ml}$)						
Subject No	Dose	Day 0	Day 2	Day 3	Day 7	Day 9	Day 10	Day 14
93	Placebo	0.00	0.00	0.00	0.00	0.00	0.00	0.00
94	1.50 g	0.00	1.20	1.14	1.16	2.04	1.96	1.82
95	1.50 g	0.00	1.34	1.53	0.90	2.42	2.12	1.26
96	Placebo	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 11

Percent of WR 180,409-¹⁴C Derived Radioactivity Recovered in the Urine and Feces of Mice after Oral Administration of the Drug^a

Time (hr)	Percent ¹⁴ C Recovered ^b	
	Urine	Feces
0-12	0.19	7.79
12-24	0.79	8.56
24-48	1.06	13.54
48-72	1.89	19.73
72-96	0.33	11.91
96-120	0.35	8.15
120-144	0.21	5.53
144-168	0.25	3.21
168-192	0.22	2.63
192-216	0.14	1.92
216-240	<u>0.02</u>	<u>0.85</u>
Total Recovery (%) ^c	5.47	83.82

^aWR 180,409-¹⁴C was administered 20 mg/kg orally.

^bAverage of two cages of 4 mice each.

^cTotal percent ¹⁴C recovery including residual carcasses (4.41% of total ¹⁴C) was 93.70%.

Table 12

TLC Analysis of Total Radioactivity in Urine and Feces After Oral Administration of WR 180,409-¹⁴C to Mice^a

Time (hr)	Urine		Feces	
	D ^b	M ^c	D ^b	M ^c
0-12	78.3	21.7	69.5	30.5
12-24	57.1	42.9	54.5	45.5
24-36	37.9	62.1	42.5	57.5
36-48	26.9	73.1	38.8	61.2
48-60	15.0	85.0	25.6	74.4
60-72	16.0	84.0	25.3	74.7
72-84	12.6	87.4	14.2	85.8
84-96	11.6	88.4	17.0	83.0

^aWR 180,409-¹⁴C was administered 20 mg/kg orally.

^bThe area of radioactivity with R_f value corresponding to the standard for WR 180,409-¹⁴C on TLC.

^cAll radioactive peaks not included under (b).

Table 13

Concentration of ^{14}C -Drug Equivalents in the Plasma and RBC of the Mouse after a Single Oral Dose of WR 180,409- ^{14}C ^a

Time (hr) ^b	Drug Equivalents ($\mu\text{g}/\text{ml}$)			
	Plasma		Red Blood Cells ^c	
	Total	WR 180,409 ^d	Total	WR 180,409 ^d
2	1.45	0.86	2.52	2.17
4	2.03	0.77	3.28	2.31
12	1.54	0.65	3.71	2.96
24	1.99	0.47	2.87	1.67
48	1.73	0.17	2.55	0.59
72	1.31	0.16	1.79	0.49
96	0.99	0.06	1.20	0.23
120	0.67	0.04	0.89	0.14

^aWR 180,409- ^{14}C was administered 20 mg/kg.

^bFour mice per time period with a single determination from their pooled blood.

^cRadioactivity for plasma samples was determined directly, for red blood cells it was calculated from the following equation:

$$\text{RBC } \mu\text{g}/\text{ml} = \frac{\text{Whole blood } (\mu\text{g}/\text{ml}) - [\text{Plasma } (\mu\text{g}/\text{ml}) \times (1-\text{Hct})]}{\text{Hct}}$$

^dArea of radioactivity on TLC plate corresponding to the reference sample of WR 180,409 after development in n-butanol: glacial acetic acid:water (66:17:17; V:V:V).

Table 14

Percent of Dose of Total Radioactivity Derived from WR 180,409-¹⁴C,
Recovered from Selected Tissues, Excreta and Carcasses of Mice^a

Tissue	% ¹⁴ C Recovered			
	2 hr ^b	4 hr ^b	24 hr ^b	48 hr ^b
Submaxillary Salivary Glands	1.40	1.60	0.91	0.90
Heart	0.92	0.96	0.58	0.38
Lungs	6.21	5.47	6.24	4.00
Liver	14.22	11.27	13.36	9.70
Kidneys	5.95	7.58	3.15	3.74
Spleen	0.66	0.69	0.67	0.45
Gall Bladder + Bile	0.17	0.22	0.05	0.19
Stomach + Contents	8.69	6.09	3.45	2.51
Small Intestine + Contents	18.22	17.63	13.83	0.27
Cecum + Contents	1.45	2.20	2.59	3.20
Large Intestine + Contents	2.57	4.58	3.69	4.11
Plasma	0.12	0.11	0.25	0.21
Red Blood Cells	0.22	0.22	0.31	0.16
Feces	-	0.11	21.69	35.19
Urine	0.029	0.25	2.39	1.31
Carcasses	31.24	30.45	23.51	15.39
Total Recovery (%)	90.07	89.43	96.67	91.71

^aWR 180,409-¹⁴C was administered 20 mg/kg orally.

^bHours postdose at sacrifice, 4 mice per time period.

Table 15

Distribution of Radioactivity Derived from WR 180,409-¹⁴C in
Tissues of Mice after a Single Oral Dose of the Drug^a

Tissue ^b	μg/g			
	2 hr ^c	4 hr ^c	24 hr ^c	48 hr ^c
Submaxillary Salivary Glands	33.69	66.43	29.43	22.03
Heart	38.06	34.98	21.38	13.82
Lungs	192.82	171.88	214.23	104.03
Liver	99.92	71.64	40.06	68.13
Kidneys	105.08	127.22	44.80	47.50
Spleen	74.37	64.81	50.56	28.18
Gall Bladder + Bile	56.39	37.32	47.38	69.33
Stomach + Contents	227.22	89.50	20.30	28.59
Small Intestine + Contents	98.39	83.32	35.88	30.81
Cecum + Contents	42.02	54.62	34.53	45.47
Large Intestine + Contents	32.09	54.02	41.07	40.73
Abdominal Fat	13.20	12.62	10.99	8.95
Skeletal Muscle	26.82	25.42	16.18	11.05

^aWR 180,409-¹⁴C was administered 20 mg/kg orally.

^bMethanolic extracts.

^cHours postdose at sacrifice, 4 mice per time period.

Table 16

TLC Analysis of the Methanolic Extract of Radioactivity in Selected Tissues after Oral Administration of WR 180,409-¹⁴C to Mice^a

Tissue	% Unchanged WR 180,409 ^b			
	2 hr ^c	4 hr ^c	24 hr ^c	48 hr ^c
Submaxillary Salivary Glands	92.8	89.5	79.3	62.6
Heart	66.8	90.3	61.9	46.5
Lungs	90.5	90.6	87.4	89.4
Liver	96.0	96.6	79.1	63.9
Kidneys	97.1	92.6	88.3	64.4
Spleen	90.0	86.5	82.5	76.6
Gall Bladder + Bile	63.4	77.0	70.0	39.0
Stomach + Contents	93.5	92.7	72.7	54.0
Small Intestine + Contents	86.7	82.9	82.2	60.6
Cecum + Contents	53.4	48.7	46.5	31.6
Large Intestine + Contents	78.1	67.3	53.8	43.0
Abdominal Fat	75.5	68.0	67.0	62.6
Skeletal Muscle	89.5	95.0	67.3	52.5
Plasma	58.4	37.9	23.8	10.0
Red Blood Cells	86.2	70.3	58.4	23.1
Feces	-	-	54.5	38.8
Urine	-	-	67.2	25.5
Carcasses	91.9	92.6	79.4	89.7

^aWR 180,409-¹⁴C was administered 20 mg/kg orally.

^bThe area of radioactivity with the R_f value corresponding to standard for WR 180,409-¹⁴C in TLC.

^cTime period postdose at sacrifice, four mice per time period.

Table 17
Relative R_f Values for WR 180,409 and
Metabolites as Determined by TLC

Solvent System ^a	R_f				
	WR 180,409	M_1^b	M_2^c	M_3^c	M_4^c
A	0.70	0.70	0.92	0.97	0.83
B	0.37	0.37	0.30	0.62	0.81

^aA = n-BuOH:HOAc:H₂O; 66:17:17 (V:V:V).

B = Benzene:MeOH; 3:1 (V:V).

^bTentatively identified as WR 180,409.

^cUncharacterized metabolites, listed from greatest proportion to the least.

Table 18

Percent Binding of WR 180,409-¹⁴C-H₃PO₄ to Mouse Plasma
as Determined by Equilibrium Dialysis

Concentration of WR 180,409- ¹⁴ C (ng/ml) ^a	% Bound to Plasma ^b
100	99
500	99
1,000	99
5,000	99

^aConcentration of WR 180,409-¹⁴C (base) in the plasma solution prior to dialysis.

^bAverage of two determinations.

Table 19
Partition Coefficients (K_p) of WR 180,409^a

Organic Phase	K_p^b
Chloroform	151
Ethyl Acetate	147
Diethyl Ether	125
n-Butanol	70
Benzene	66
n-Heptane	6

^a K_p is expressed as the ratio of the concentration of drug in the organic phase to that in the aqueous phase (pH 7.4 phosphate buffer).

^bAverage of three determinations.

Table 20

Dose of WR 180,409·H ₃ PO ₄ ^a	Mean Arterial Pressure (mmHg)		Heart Rate (bpm)		Respiratory Rate (#/min)	
	Baseline	After Drug	Baseline	After Drug	Baseline	After Drug ^c
0.1 mg/kg	100	100	100	100	4	5
	100	100	100	100	5	5
0.3 mg/kg	100	100	100	100	5	6
	100	100	100	100	6	6
1 mg/kg	100	85	100	80	6	*
	100	85	100	80	45	*
3 mg/kg	95	65	100	83	45	*
	95	60	107	88	60	*
10 mg/kg ^b	95	35	125	110	72	*

^aDoses were administered as an iv bolus at 20 minute intervals. Each dose was administered twice. "Baseline" readings were made immediately prior to drug injections; "After Drug" readings were made 1 minute after drug injection.

^bDog died 5 minutes after drug administration. He had received a cumulative dose of 18.8 mg/kg.

^c=Response of apnea lasting approximately one minute followed by tachypnea.

Table 21
 Cardiorespiratory Effects of WR 180,409·H₃P0₄(3 mg/kg) in Three Dogs^a

	Baseline ^b	WR 180,409·H ₃ P0 ₄ 3 mg/kg	% of Control
Heart Rate - bpm ($\mu \pm$ SE)	139.0 <u>+7.3</u>	93.0 <u>+3.7</u>	66.9
Mean Arterial Pressure - mmHg ($\mu \pm$ SE)	93.3 <u>+2.4</u>	36.7 <u>+4.4</u>	39.3
Respiratory Rate ^c - #/min ($\mu \pm$ SE)	16.0 <u>+4.5</u>	90.3 <u>+7.8</u>	564.0
Postinjection Apnea (Sec) ($\mu \pm$ SE)	-	72.0 <u>+8.3</u>	

^aData from Dogs #2, 3 and 4.

^bBaseline readings were made immediately prior to injection of WR 180,409·H₃P0₄. The drug readings were made 1 minute after WR 180,409·H₃P0₄ administration.

^cRespiratory response to the injection was a period of apnea followed by tachypnea. The postinjection respiratory rate refers to the tachypnea.

Modification of Cardiorespiratory Effects of WR 180,409·H₃P0₄
by Vagotomy in Two Dogs^a

	Baseline ^b	WR 180,409·H ₃ P0 ₄ 3 mg/kg	% of Control
Heart Rate - bpm			
Control Injection	154.0	95.5	62.0
After Vagotomy	150.0	188.0	125.3
Mean Arterial Pressure -mmHg			
Control Injection	97.5	32.5	33.3
After Vagotomy	97.5	60.0	61.5
Respiratory Rate ^c - #/min			
Control Injection	20.5	98.0	478.0
After Vagotomy	18.5	23.5	127.0
Postinjection Apnea - Sec			
Control Injection	-	78.0	-
After Vagotomy	-	0	-

^aDogs #2 and #4.

^bBaseline readings were made immediately prior to injection of WR 180,409·H₃P0₄. The drug readings were made 1 minute after WR 180,409·H₃P0₄ administration.

^cRespiratory response to the control injection was a period of apnea followed by tachypnea. The postinjection respiratory rate refers to the tachypnea.

Table 23

Cardiorespiratory Effects of a 40 Minute Infusion of
WR 180,409- H_3PO_4 , 3 mg/kg, in Three Dogs^a

	Minutes ^b			
	0	20	40	60
Heart Rate - bpm ($\mu \pm SE$)	181.7 +12.4	174.3 + 6.8	171.3 +12.7	163.0 +13.2
Mean Arterial Pressure - mmHg ($\mu \pm SE$)	98.3 + 6.0	103.0 + 6.8	96.7 +4.4	103.3 + 3.3
Respiratory Rate - #/min ($\mu \pm SE$)	16.0 + 3.8	13.0 + 4.2	13.3 + 3.9	13.7 +5.0

^aDogs #2, #3, and #4.

^bInfusion was begun at time 0 and was terminated after 40 minutes. The 0 minute observation was taken immediately prior to the start of the infusion and the 40 minute observation was taken immediately after termination of the infusion.

Table 24

Cardiorespiratory Responses of Two Dogs^a to Vasoactive Compounds^b both Before and After a 40 Minute Infusion of WR 180,409-H₃PO₄ (3 mg/kg)

	Responses Before WR 180,409-H ₃ PO ₄				Responses After WR 180,409-H ₃ PO ₄			
	Baseline ^c	Postinjection	% Control		Baseline	Postinjection	% Control	
Mean Arterial Pressure-mmHg								
Epinephrine	107.5	122.5	114.0		105.0	130.0	123.8	
Isoproterenol	105.0	55.0	52.4		102.5	60.0	58.5	
Acetylcholine	105.0	70.0	66.7		105.0	71.5	73.8	
Histamine	97.5	82.5	84.6		102.5	85.0	82.9	
Serotonin	107.5	97.5	90.7		105.0	100.0	95.2	
Heart Rate-bpm								
Epinephrine	163.5	150.5	92.0		146.5	125.0	85.3	
Isoproterenol	158.5	215.5	136.0		146.5	215.0	146.8	
Acetylcholine	166.0	195.5	117.8		146.5	177.5	121.2	
Histamine	167.5	172.0	102.7		173.0	183.5	106.1	
Serotonin	172.0	183.5	106.7		154.0	155.0	100.1	
Respiratory Rate #/min								
Epinephrine	6.5	7.5	115.4		11.0	11.5	104.5	
Isoproterenol	8.5	15.5	182.4		9.0	14.0	155.6	
Acetylcholine	8.5	10.0	117.6		11.0	12.0	109.1	
Histamine	9.5	12.0	126.3		12.0	13.5	112.5	
Serotonin	10.5	11.0	104.8		9.0	10.0	111.1	

Footnotes to Table 24

^aDogs #3 and #4.

^bDrugs given as a rapid iv injection of the salt at a dose of 1 μ g/kg 10 minutes apart.

^cBaseline responses were recorded immediately prior to injections. Postinjection responses were recorded for the peak effect.

Table 25

Cardiorespiratory Responses of 4 Dogs^a to a 60 Minute Infusion of
WR 180,409·H₃PO₄(15 mg/kg)^b

	Time - minutes								
	WR 180,409·H ₃ PO ₄ , 15 mg/kg infusion								
	0	15	30	45	60	75	90	105	120
Mean Arterial Pressure mmHg ($\mu \pm$ SE)	115.0 +4.0	117.5 +3.2	118.7 +5.1	121.7 +6.3	123.0 +7.2	120.5 +6.6	120.0 +6.1	119.5 +6.0	122.0 +4.7
Heart Rate bpm ($\mu \pm$ SE)	144.0 +10.3	134.0 +11.2	131.0 +9.2	133.0 +9.9	128.0* +9.1	127.0* +8.4	127.0* +5.8	127.0* +5.8	128.0* +5.8
Respiratory Rate #/min ($\mu \pm$ SE)	9.5 +2.1	9.0 +1.7	9.5 +1.4	10.5 +1.4	8.7 +1.1	8.2 +0.9	9.0 +1.3	9.5 +1.3	9.5 +1.6
Central Venous Pressure mmHg ($\mu \pm$ SE)	0.5 +1.2	0.9 +1.3	1.0 +1.6	1.2 +1.5	0.8 +1.6	0.9 +1.6	0.9 +1.6	0.9 +1.7	1.0 +1.5
PEP/LVET ($\mu \pm$ SE)	0.68 +0.06	0.74 +0.07	0.75 +0.08	0.77 +0.08	0.80 +0.08	0.82* +0.07	0.82* +0.07	0.80 +0.07	0.79 +0.07

^aDogs #5-8.

^b*=Statistically significant value from 0 time reading at 5% level of significance, paired t test of significance.

Table 26

Cardiovascular Responses of Two Open-Chest Dogs^a to a 60 Minute Infusion of WR 180,409·H₃P0₄ (15 mg/kg)

	Time - minutes									
	0	15	30	45	60	75	90	105	120	
WR 180,409·H ₃ P0 ₄ , 15 mg/kg infusion										
Mean Arterial Pressure mmHg (mean)	92.5	65.0	75.0	77.5	75.0	75.0	75.0	67.2	62.5	57.5
Heart Rate bpm (mean)	177.0	169.0	173.0	173.0	173.0	162.0	169.0	173.0	173.0	168.0
Myocardial Contractility % Control (mean)	100.0	83.3	83.3	83.3	76.7	84.0	72.2	70.0	74.0	74.0
Aortic Flow L/min (mean)	4.5	4.5	4.4	3.7	3.5	3.8	3.5	2.9	2.9	2.9
Total Peripheral Resistance pru - (mean)	1.23	0.87	1.02	1.26	1.29	1.18	1.16	1.29	1.29	1.19

^aDogs #9 and #10.

Table 27

Comparison of the Curative Activities of Racemic WR 181,023 and Its Optical Isomers WR 221,033 (+) and WR 221,036 (-) When Given Once Orally in Equivalent Doses of Base (194,5 mg base/kg) to P. berghei (Trophozoite) - Infected Mice^a

Group	Treatment on Day 3 ^b	Median % Parasitemia on Day 3 ^c	No. of Mice Used ^d	No. of Mice Alive on Day 28 ^e	% Malaria Cures (No. cured/No. used) ^f
Experiment A					
I	Control (MCT) ^a	<1	12	0	0 (0/12)
II	WR 181,023	<1	11	11	100 (11/11)
III	WR 221,033	<1	10	10	100 (10/10)
IV	WR 221,036	<1	12	12	100 (12/12)
Experiment B					
I	Control (MCT)	2	12	0	0 (0/12)
II	WR 181,023	2	11	8	73 (8/11)
III	WR 221,033	2	12	11	92 (11/12)
IV	WR 221,036	2	12	10 ^e	75 (9/12)

^aOn Day 0, Groups I-IV were inoculated i.p. with ca 500,000 P. berghei parasitized (drug-sensitive KBG 173 strain) RBC. On Day 3, after smears were taken, mice were given, p.o., one of the 3 agents prepared in 0.2% methylcellulose and 0.4% Tween 80 in sterile 0.9% saline (MCT), or MCT alone as control (Group I). The injection volume was 1% of the body weight. In each experiment 3 additional uninjected groups of 6 mice each (not shown) were treated p.o. on Day 3 with either of the 3 agents; these served as toxicity controls. Of these, only mice given WR 221,033 suffered toxic deaths during the first week after treatment, viz. 2 mice in Exp. A and one mouse in Exp. B; all other toxicity controls survived in apparent good health until Day 28 (25 days post-treatment).

Footnotes to Table 27 (cont.)

^bWR 181,023, the diphosphate, is 58% base; the dextro and levo isomers are sesquiphosphates and each 65% base.

^cIn Exp. A, smears were taken on Days 3,7,10,14 and 27 and in Exp. B on Days 3,6,8,10,14 and 27.

^dGroups I to IV each consisted of 12 mice initially. Mice were excluded from the study when the cause of early death (by Day 9) could not be ascertained (Exp. A, Group II) or appeared related to causes other than malaria (Exp. A, Group III; Exp. B Group II). One 28-day survivor (Exp. A, Group III) was excluded because its Day 3 smear taken just before treatment was negative for parasites.

^eOne 28-day survivor (Exp. B, Group IV) had malaria. All other 28-day survivors appeared healthy, had negative smears, and upon autopsy after exsanguination presented no gross visceral abnormalities indicative of malaria; all of the latter had shown positive smears on Day 3 before treatment.

^fTwenty-eight day survivors, apparently malaria-free (See footnote "e"), were exsanguinated under light ether anesthesia. Each mouse's total bleeding volume (0.6 to 1.0+ ml) was separately collected in a heparin-moistened syringe and was promptly injected i.p. into a previously normal recipient. Recipients alive 28 days after subinoculation, with negative smears, healthy appearance, and absence of signs of malaria upon autopsy were taken as evidence that their corresponding donors had been cured of malaria.

Table 28

Comparison of the Acute Toxicity of 4-Methylprimaquine Racemate (WR 181,023) with That of Its Dextro (WR 211,033) and Levo (WR 221,036) Isomers when Given Once Orally (Day 0) in Equivalent Doses of Base to Uninfected Mice^a

Group	Agent	Dose, mg/kg as:		Cumulative Mortality, %			
				Days After Dosing			
		Salt	Base	1	2	3	7
Experiment A (N=10)							
I	WR 181,023	334	194.5 ^b	0	0	0	0
II	WR 221,033	229	194.5 ^b	0	0	10	10 ^c
III	WR 221,036	299	194.5 ^b	0	0	0	0
Experiment B (N=20)							
IV	WR 181,023	729 ^c	423	0	20	35	60 ^e
V	WR 221,033	650	423	5 ^d	10	45	80
VI	WR 221,036	650	423	5 ^d	10	15	30 ^f

^aAll agents were prepared in MCT and given in a volume equivalent to 1% of the body weight.

^bSame dose as was used in the efficacy study (Table 27).

^cThis dose, with confidence limits of 671 to 794 mg salt/kg, is the estimated LD-50 (7-day endpoint) for WR 181,023 previously reported.

^dOne mouse died within 30 min. after dosing; death was not due to misinjection.

^eIncludes one mouse that died several minutes after the 7-day endpoint.

^fAs calculated by Chi-square, using Yates' correction, only the difference in mortality on Day 7 between Groups V and VI is statistically significant ($p < 0.05$).

Table 29

Comparison of The Parasitemia Suppressing Activities of Primaquine (WR 2975) and Two Salts of 3-Methylprimaquine (WR 211,814) When Given Once Orally in Equivalent Doses of Base to *P. berghei* (Trophozoite)-Infected Mice^a

Group	Treatment ^b		Median % Parasitemia ^c (No. of Mice)				
	Agent	Dose, mg base/kg	Day of Infection				
			Before Oral Rx		After Oral RX		
			3	6	8	10	14
A	Vehicle (Control)	(1% BW) ^a	1.8 (10)	51.2 (10)	69.7 (4)	- (1)	- (0)
B	WR 2975 AG	11.4	2.1 (10)	0.0+ (10)	8.7 (10)	27.9 (6)	56.5 (4)
C	WR 211,814 AA	11.4	2.0 ^d (10)	0.0+ ^d (10)	0.7 ^e (10)	18.2 ^d (10)	43.7 ^d (5)
D	WR 211,814 AB	11.4	1.7 ^{d,f} (10)	0.0+ ^{ef} (10)	0.8 ^{ef} (10)	24.1 ^{df} (10)	- (2)

^aOn Day 0, Groups A to D were inoculated i.p. with ca 500,000 *P. berghei*-parasitized (drug-sensitive KBG 173 strain) RBC. On Day 3, after smears were taken, mice were given, p.o., one of the 3 agents prepared in MCT vehicle (See Table 27), or MCT alone (Group A). The injection volume was 1% of the body weight. To serve as toxicity controls, two additional uninfected groups of 5 mice each (not shown) were treated the same, respectively, as Group C and D on Day 3. All of these controls survived in apparent good health through Day 22 (19 days post-treatment) and were killed and autopsied. By this time all mice from Groups A to D were dead from malaria. Autopsy of the controls revealed no abnormal changes.

^bWR 2975 AG is the diphosphate salt of primaquine (57% base); WR 211,814 AA and WR 211,914 AB are, respectively, the diphosphate sesquihydrate (55% base) and dihydrochloride (79% base) salts of 3-methylprimaquine. All are racemates.

Footnotes to Table 29 (cont.)

^cPercentage parasitemia is routinely based on the examination of 250-300 RBC on a Giemsa-stained thin blood film. When this initial examination reveals no parasites, the blood film and its replicate are thoroughly scanned. Percentage parasitemia is designated 0.0+% when this secondary search reveals parasites and 0.0% when it does not. Values for fewer than three mice are not presented. The Mann-Whitney rank test was used for all statistical comparisons. Statistical significance was set at $p \leq 0.05$. The dose of 11.4 mg base/kg was chosen because prior experience with WR 2975 at this level indicated it to be appropriately suppressive but subcurative in all mice tested.

^dDifference from Group B is not statistically significant.

^eDifference from Group B is statistically significant.

^fDifference from Group C is not statistically significant.

Table 30

Comparison of Acute Toxic Swelling and Cumulative Mortality in Uninfected Mice Given Single Oral Doses (Day 0) of WR 2975 AG (Primaquine, 2H₃PO₄) or WR 211,814 AA (3-Methylprimaquine • 2H₃PO₄ • 1.5H₂O)

Group (No. of Mice Used)	Dose, ^a mg base per kg	Mean Degree of Overt Swelling of Snout/Tongue ^b						Cumulative Mortality, %									
		Time Postchallenge						Time Postchallenge									
		Day 0		Day 0		Day 0		Day 0	Day 1	Day 2	Day 3	Day 7					
		3 hr	4 hr	5 hr	6 hr	6 hr	Day 0	Day 1	Day 2	Day 3	Day 7						
A. Challenge (Day 0): Primaquine ^a , p.o.																	
I (10)	91.2	0.7/0.7 (4) ^b	1.9/1.4 (8)	2.7/2.6 (10)	2.6/2.7 (10)	0	0	0	0	0	0	0					
B. Challenge (Day 0): 3-Methylprimaquine ^a , p.o.																	
II (10)	45.6	0.0/0.0 (0)	0.2/0.0 (1)	0.2/0.1 (1)	0.1/0.0 (1)	0	10	20	50	60							
III (10)	68.4	0.0/0.0 (0)	0.1/0.0 (1)	0.8/0.8 (6)	1.2/1.4 (7)	0	30	40	80	100							
IV (20)	91.2	0.0/0.0 (0)	0.1/0.0 (1)	0.3/0.4 (7)	0.4/0.4 (8)	0	20	65	80	90							
V (10)	182.4	0.0/0.0 (0)	0.3/1.1 (3)	0.3/1.3 (4)	0.2/1.5 (5)	0	60	90	90	90							
VI (10)	364.8	0.0/0.0 (0)	0.0/0.0 (0)	0.1/0.4 (3)	0.3/1.1 (5)	0	80	90	90	100							

^aEach agent was prepared in the vehicle MCT. All doses were delivered in a volume equivalent to 1% of the body weight. WR 2975 AG and WR 211,814 AA are, respectively, 57% and 55% base. Both are racemates. The dose of WR 2975 used, which is approximately one-half of the acute oral LD-50 (7-day endpoint) was chosen because it reliably elicits uniform toxic reactions yet kills only rarely.

Footnotes to Table 30 (cont.)

^bEach mouse was gently raised by the scruff of the neck and examined under a surgical lamp. Swelling of the snout (including lips) and tongue were scored independently on an arbitrary scale of 1, 2, or 3 for the former and 1, 2, 3, and 4 for the latter, always by the same observer. Scores intermediate between integral scores were denoted at intervals of 0.5. The absence of swelling was scored "0". Zero scores were included in calculating the mean values shown. In parentheses is the number of mice per group, at each time period, that displayed swelling of the snout and/or tongue.

Table 31

Effect of Phenobarbital or SKF 525-A Pretreatment on the Oral Antimalarial Activity of WR 184, 806 AJ Given Once to *P. berghei* (Trophozoite) - Infected (Day 0) Mice

Group	Pretreatment ^a	Median % Parasitemia ^b (No. of Mice)				
		Day of Infection				
		Before Oral Rx 3	6	8	10	
					14	
A. Vehicle Control, 1% BW p.o on Day 3 ^c						
I	Vehicle (Control)	4.3 (10)	60.3 (9)	- (1)	- (1)	- (0)
II	Phenobarbital	3.5 ^d (10)	53.3 ^d (10)	56.3 (6)	- (2)	- (1)
III	SKF 525-A	3.0 ^{d,e} (10)	57.6 ^{de} (10)	- (2)	- (0)	- (0)
B. WR 184,806 AJ ^f , 5 mg salt/kg p.o on Day 3						
IV	Vehicle (Control)	3.9 (10)	5.4 (10)	13.8 (10)	30.2 (9)	43.59 ⁽⁵⁾
V	Phenobarbital	2.3 ^h (10)	52.1 ^j (10)	52.1 ^j (5)	- (2)	- (1)
VI	SKF 525-A	3.2 ^{h,i} (10)	0.0+ ^j k(10)	4.0 ^j k(10)	20.1 ^j (9)	67.7 ^h (6)

Footnotes to Table 31

- a Pretreatment regimens consisted of either three daily i.p. injections of 100 mg/kg phenobarbital sodium in sterile water, water alone (controls) for three days (Days 0,1,2) before oral dosing on Day 3, or a single i.p. injection of 50 mg/kg of SKF 525-A in 0.9% saline on Day 3, one hour before oral dosing.
- b On Day 0, Groups I to VI were inoculated i.p. with ca 500,000 P. berghei-parasitized (drug-sensitive KBG 173 strain) RBC. Percentage parasitemia is routinely based on the examination of 250-300 RBC on a Giemsa-stained thin blood film. When this initial examination reveals no parasites, the blood film and its replicate are thoroughly scanned. Percentage parasitemia is designated 0.0+% when this secondary search reveals parasites and 0.0% when it does not. Values are not presented for fewer than three mice. The Mann-Whitney rank test was used for all statistical comparisons. Statistical significance was set at $p < 0.05$.
- c The agent vehicle, in which WR 184,806 AJ was prepared, was commercial pyrogen-free water intended for human i.v. use. The volume of all injections was 1% of the body weight (BW).
- d Difference from Group I is not statistically significant.
- e Difference from Group II is not statistically significant.
- f WR 184,806 AJ is the monophosphate salt of which 80% is the base.
- g All mice from all groups were dead from malaria by Day 28 except for one mouse in Group IV which had a positive smear at this time. It was exsanguinated into a heparin-moistened syringe and the collected blood (0.53 ml) was injected i.p. into a previously normal recipient mouse; the latter died of malaria 6 days later.
- h Difference from Group IV is not statistically significant.
- i Difference from Group V is not statistically significant.
- j Difference from Group IV is statistically significant.

Footnotes to Table 31 (cont.)

kDifference from Group V is statistically significant.

Representative Chromatograms (A) of a Whole Blood Extract Containing 0.98 μg of Internal Standard (WR 184,806) and (B) of a Whole Blood Extract Containing 0.05 μg of Mefloquine and 0.98 μg of Internal Standard (WR 184,806)

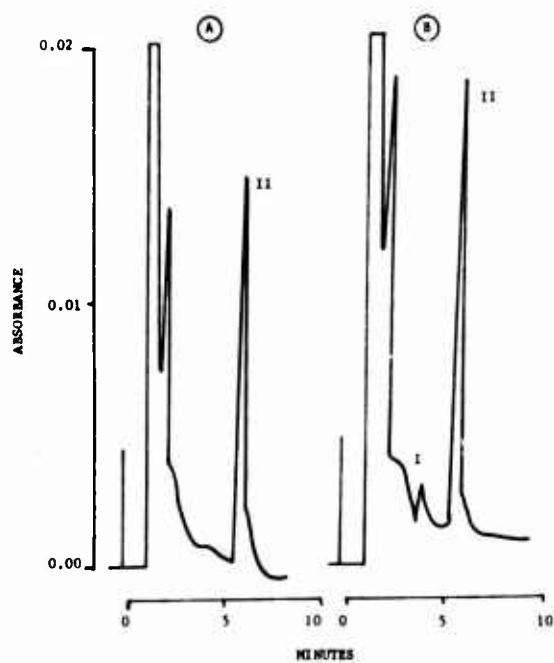


Figure 1

Representative Chromatograms (A) of a Urine Extract Containing 0.98 μg of Internal Standard (WR 184,806) and (B) of a Urine Extract Containing 0.125 μg of Mefloquine and 0.98 μg of Internal Standard (WR 184,806)

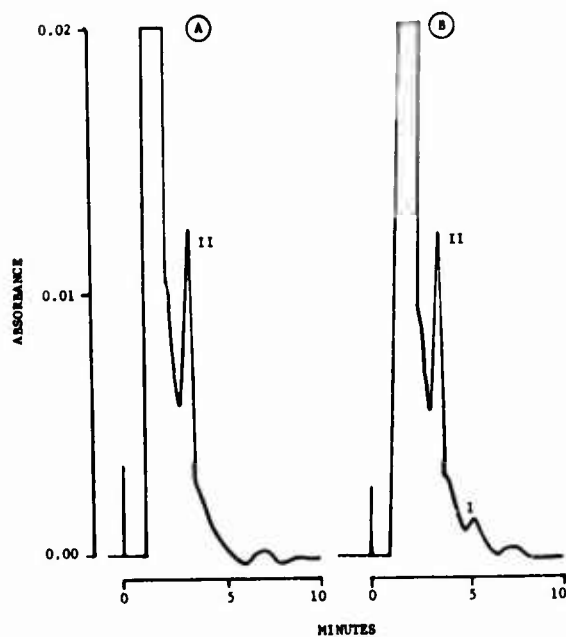


Figure 2

Whole Blood Concentrations of Mefloquine in a Man Following a Single Oral Dose of 500 mg of Mefloquine-HCl

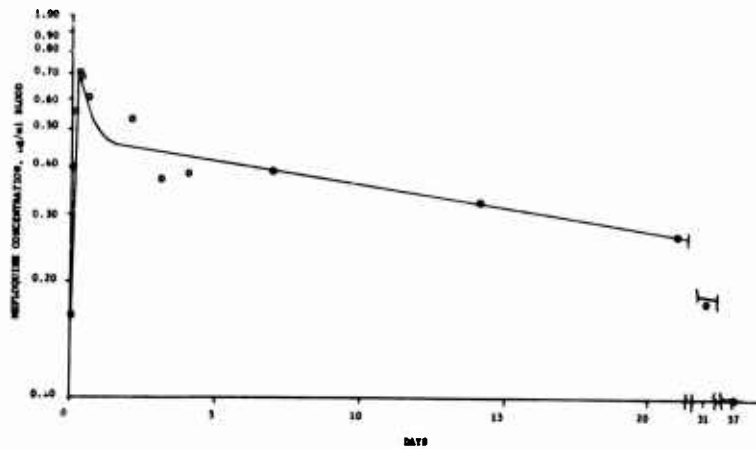


Figure 3

Plasma Concentrations of Mefloquine in a Man Following Single Oral Dose of 500 mg of Mefloquine·HCl.

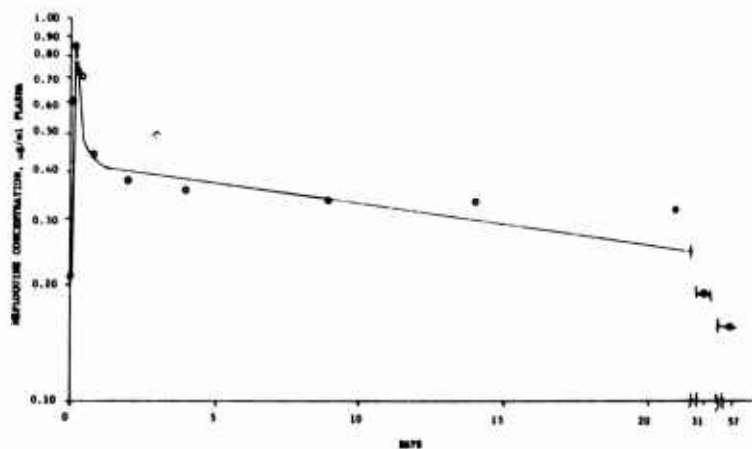


Figure 4

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 309 Determination of pharmacological effects of anti-malarial drugs

Literature Cited.

References:

1. Canfield, C. J. and Rozman, R. S.: Clinical testing of new antimalarial compounds. *Bull. Wld. Hlth. Org.* 50:203-212, 1974.
2. Trenholme, G. M., Williams, R. L., Desjardins, R. E., Frischer, H., Carson, P. E., Rieckmann, K. H. and Canfield, C. J.: Mefloquine (WR 142,490) in the treatment of human malaria. *Science.* 190:792-794, 1975.
3. Rieckmann, K. H., Trenholme, G. M., Williams, R. L., Carson, P.E., Frischer, H. and Desjardins, R. E.: Prophylactic activity of mefloquine hydrochloride (WR 142,490) in drug-resistant malaria. *Bull. Wld. Hlth. Org.* 51:375-377, 1974.
4. Clyde, D. F., McCarthy, V. C., Miller, R. M. and Hornick, R. B.: Suppressive activity of mefloquine in sporozoite-induced human malaria. *Antimicrob. Agents Chemother.* 9:384-386, 1976.
5. Mu, J. Y., Israili, Z. H. and Dayton, P. G.: Studies of the disposition and metabolism of mefloquine-HCl (WR 142,490), a quinolinemethanol antimalarial, in the rat. Limited studies with an analog, WR 30,090. *Drug Metabol. Disp.* 3:198-210, 1975.
6. Grindel, J. M. and Tilton, P. F.: Quantitation of the anti-malarial, mefloquine, in blood, plasma and urine using high pressure liquid chromatography. *J. Pharm. Sci.* (Submitted).
7. Rinehart, J. J., Lewis, R. P. and Balcerzak, S. P.: Adriamycin cardiotoxicity in man. *Ann. Int. Med.* 81:475-478, 1974.
8. Nickerson, M.: Antihypertensive agents and the drug therapy of hypertension. In, *The Pharmacological Basis of Therapeutics* (Goodman, L. S. and Gilman, A., eds.) The MacMillin Company, New York, N.Y., 1971, pp. 734-735.
9. Peters, W.: Chemotherapy of rodent malaria. Drug action against exo-erythrocytic stages and drug resistant strains. 1st

Quarterly Technical Report. Contract No. DAJA 37-75-C-1723. 1 Nov 1974 to 31 Mar 1975.

10. Peters, W.: Chemotherapy of rodent malaria. Drug action against exo-erythrocytic stages and drug resistant strains. Final Technical Report. Contract No. DAJA 37-75-C-1723, October 1975.

11. Einheber A., Palmer, D. M. and Aikawa, M.: Plasmodium berghei: phase contrast and electron microscopical evidence that certain antimalarials can both inhibit and reverse pigment clumping caused by chloroquine. *Exper. Parasit.*, in press.

12. Rinehart, J., Arnold, J. and Canfield, C.: Evaluation of two phenanthrenemethanols for antimalarial activity in man: WR 122,455 and WR 171,669. *Amer. J. Trop. Med. Hyg.*, in press.

Publications:

1. Grunewald, G. L., Grindel, J. M., Vincek, W. C. and Borchardt, R. T.: Importance of the aromatic ring in adrenergic amines. Non-aromatic analogs of phenylethanolamine as substrates for phenylethanolamine-N-methyltransferase. *Mol. Pharmacol.* 11:694-699, 1975.

2. Trenholme, G. M., Williams, R. L., Desjardins, R. E., Frischer, H., Carson, P. E., Rieckmann, K. H. and Canfield, C. J.: Mefloquine (WR 142,490) in the treatment of human malaria. *Science.* 190:792-794, 1975.

3. Grindel, J. M., Rozman, R. S., Leahy, D. M., Molek, N. A. and Gillum, H. H.: The absorption, distribution and excretion of a quinoline-methanol antimalarial, 2,8-bis(trifluoromethyl)-4-[1-hydroxy-3-(N-t-butylamino)propyl]quinoline phosphate (WR 184,806). *Drug Metab. Disp.* 4:133-139, 1976.

4. Grunewald, G. L., Grindel, J. M., Patil, P. N. and Salman, K. N.: Importance of the aromatic ring in adrenergic amines. 2. Synthesis and adrenergic activity of some non-aromatic six- and eight-membered ring analogs of β -phenylethanolamine. *J. Med. Chem.* 19:10-16, 1976.

5. Chung, H., Gillum, H. H. and Rozman, R. S.: The absorption, distribution and elimination of threo- α -(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate (WR 180,409.H₃PO₄) in mice. *Fed. Proc.* 34:328, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	3 REPORT CONTROL SYMBOL ^a	
				DA OA 6506	76 06 30	DD-DR&E(AR)636	
5 DATE PREV SUMMARY	4 KIND OF SUMMARY	3 SUMMARY SCTY ^a	4 WORK SECURITY ^a	7 AGRAS/AG ^a	8A DDB/IN INSTR ^a	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUB A WORK UNIT
75 07 01		U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10 NO CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	62759A	3A762759A829	00	315			
6. PRIMARY							
7. CONTRIBUTING							
8. CONTROLLING: CARDS 114F							
11 TITLE (Precede with Security Classification Code) ^a							
(U) Blood level Assay for Antimalarial Drugs							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002300 Biochemistry 002900 Biology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
64 07		76 06		DA		C. In-House	
17 CONTRACT/GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING			
b. NUMBER ^a				FISCAL YEAR		c. FUNDS (in thousands)	
c. TYPE				75		3	
d. KIND OF AWARD:				76		3	
e. CUM. AMT.							
20 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME ^a : Walter Reed Army Institute of Research				NAME ^a : Walter Reed Army Institute of Research			
ADDRESS ^a : Washington, DC 20012				ADDRESS ^a : Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Punish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME ^a : Demaree, LTC G.E.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2211			
21 GENERAL USE				21 SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Sleeman, H.K., Ph.D.			
				NAME: Demaree, LTC G.E. DA			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Antimalarials; (U) Pharmacokinetics; (U) Pharmacodynamics							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) The technical objective of this work unit is to study the pharmacokinetics of antimalarial drugs and to correlate these findings with pharmacodynamics in animal models in order to predict the chemotherapeutic and toxic effects of these drugs in military personnel.							
24. (U) Instrumental, chemical and immunological techniques for analysis and identification of experimental antimalarial drugs in biological materials will be developed and evaluated in animal model experiments for application to clinical specimens. These findings will be correlated with studies on the pharmacological and toxic effects of the drugs in animal models.							
25. (U) 75 07 - 76 06 Chromatographic systems, TLC, GC, and HPLC, have been developed for the analysis of antimalarial drugs in biological fluids. TLC lacks sensitivity. GC with extraction and derivatization and HPLC are promising. Organic extracts of blood and derivatization permitted the detection by GC of 25 ng/ml of mefloquine. Preliminary results with HPLC of spiked plasma samples gave results similar to those found with GC. Attempts to produce antibodies to the antimalarials have been unsuccessful. The injection into rabbits of chloroquine conjugated to polylysine produced no antibodies detectable by complement fixation or a globulin filtration system. For technical report, see the Walter Reed Army Institute of Research Annual Progress Report, 1 Jun 75 - 30 Jun 76.							

PII Redacted

DD FORM 1498 1 MAR 68

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

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 315 Blood level assays for anti-malarial drugs

Investigators.

Principal: LTC Gale E. Demaree, MSC

Associate: H. Kenneth Sleeman, Ph.D.; Betty J. Boone, Ph.D.;
Leo Kazyak, B.S.; Elvio A. Levri, M.S.; Ann R. Berman,
B.S.; SP5 Omayya A. El-Soukkary; SP5 Cynthia D. Kindell;
CPT Horace K. Webster, MSC; John A. Kintzios, B.S.;
Jean E. Matusik, B.S.; SFC Evelyn Moore.

The major effort under this work unit involved attempts to develop methods for the analysis of experimental antimalarials in biological materials, and the extension of the studies on the biochemical effects of antimalarials on the host. Specific studies reported for FY 76 include:

1. Development of chromatographic methods for analysis of antimalarials in biological materials.
2. Synthesis of conjugated haptens of antimalarials.
3. Production of antisera to antimalarial haptens.
4. Biochemical effects of antimalarials.

1. Development of chromatographic methods for analysis of anti-malarials in biological materials.

A method was developed for the analysis of α -(2-piperidyl)-2,8 Bis (Trifluoromethyl)-4-quinoline methanol hydrochloride (Mefloquine·HCl, WR 142490) in biological materials by gas chromatography (GC) using an electron capture detector. The optimum conditions for drug extraction, derivatization, and chromatography were established. The method was evaluated using Mefloquine·HCl fortified human blood and plasma, and rat whole blood, plasma and erythrocytes after ingestion of the drug.

The distribution of Mefloquine·HCl between aqueous and organic phases was determined with Mefloquine- ^{14}C ·HCl. Aqueous solutions of the drug and serum with added drug were extracted with various organic solvents, ethyl acetate, diethyl ether, benzene containing 1.5% isopentanol, hexane containing 1.5% isopentanol, n-butanol, or chloroform containing 4.0% ethanol. The recovery of Mefloquine·HCl was maximum with ethyl acetate, 101% from water and 93% from serum. However, ethyl acetate also extracted many other polar compounds from biological materials, which interfered with GC analysis, so chloroform:ethanol

(96:4) was the solvent of choice for the extraction of biological samples (88% and 68% extraction of drug from water and serum, respectively).

One milliliter of the specimen was diluted with 3 ml of 0.05 M phosphate buffer, pH 8.0, and extracted (2 min) with 25 ml chloroform: ethanol. The organic phase was separated, evaporated to dryness over low heat with a stream of nitrogen. The walls of the flask were washed with methanol and again evaporated to dryness. The residue was dissolved in dimethyl formamide (50 to 250 μ l) and reacted with a 10-fold excess of N, O, Bis (Trimethyl silyl) acetamide (TMS) for 30 min at 25°C. (The optimum time and temperature for silylation was determined experimentally.) Volumes of 1 μ l were injected onto the column for analysis. A standard curve was prepared by injecting on column 6 levels (0.25 to 15.00 ng) of Mefloquine·TMS.

TABLE 1
MEFLOQUINE STANDARDS

Mass Injected (ng)	Mean* Integrator Response	Range Integrator Response	Coefficient** of Variance (%)
0.25	3769	2899 - 4970	14.73
0.50	7097	5787 - 8150	10.30
1.00	15293	13270 - 18760	11.28
5.00	61429	54770 - 69780	6.82
10.00	106325	101000 - 114600	4.14
15.00	152331	140700 - 163600	4.29

*Mean of 17 samples

**Coefficient of variance (%) = $\frac{\sigma_s}{\mu}$ = $\frac{\text{Standard Deviation}}{\text{Mean}}$

The gas chromatograph was a Hewlett-Packard Model 7600A with auto-sample injector, an electron capture detector (^6Ni), and a digital integrator interfaced to a 2166C Hewlett-Packard computer. The column was packed with 3% OV-17 on Gas Chrom Q (80/100 mesh). Other column packings investigated were 3% OV-1, OV-225, silar 5CP, EGSP-Z, and QF-1, each on Gas Chrom Q. A column temperature of 155° was used. The retention time of Mefloquine·TMS was 14.80 min on 3% OV-17.

The results of animal experiments indicate that analysis of drug from biological fluids can be performed quantitatively. The amount of drug present in the blood was dose related, and the highest levels of drug were found in the washed erythrocyte fractions.

TABLE 2

GC ANALYSIS OF RAT BLOOD FOLLOWING DRUG INGESTION

mg Drug/Kilo	Whole Blood ng/ml	Washed Cells* ng/ml	Plasma ng/ml
25	854.2	789.4	86.4
25	782.0	631.4	68.1
10	244.0	236.2 230.4	22.31
10	235.1	266.1	21.8
5	210	237.0 228.8	18.40
5	Animal died as a result of surgery		
Vehicular Control	0	0	0

Animal: Rat: Sprague-Dawley
Weighed and given drug/kilo body weight mg/kilo
Animal sacrificed 24 hrs after dosing

*Cells corrected for HCT.

The Mefloquine-TMS structure was confirmed by mass spectrometry. Two additional peaks were found in the erythrocytes of rats which were given Mefloquine at retention times of 2.97 and 7.79 min respectively. These peaks may represent metabolites of Mefloquine. Further studies of these possible metabolites are required.

The method developed is specific, sensitive, and quantitative for Mefloquine. It was shown to be useful in detecting low drug levels in biological specimens.

2. Synthesis of antimalarial haptens for protein conjugation.

Chloroquine or Primaquine were conjugated to polylysine through an α -bromo-t-butyl acetate bridge. Ultraviolet analysis of the complex showed that 140 μ g Chloroquine or 90 μ g of Primaquine were contained in each milligram of polylysine-drug conjugate.

A method for the conjugation of WR 142490 to protein has been investigated without success. Treatment of WR 142490 with succinic anhydride or α -bromo-t-butyl acetate formed respectively an imide and a lactone, both unsuitable for protein conjugation. Attempts to oxidize the secondary alcohol to a ketone with meta-chloroperbenzoic acid produced cleavage of the α -piperdyl ring but no ketone. The use of milder oxidizing conditions to produce a ketone (i.e., chromium trioxide) are now in progress. Even the use of a milder oxidation procedure will probably open the piperdyl ring but hopefully will not cleave it from the molecule. The synthesis of the molecule with a reactive functional group is another possible approach.

3. Production of antisera to antimalarial haptens.

The Chloroquine-polylysine and Primaquine-polylysine conjugates were dissolved in saline, mixed with Freund's complete adjuvant and injected either subcutaneously (multiple sites) or into the footpads of rabbits. Two rabbits each were injected with conjugate which contained 10, 25, 50, and 100 μ g of the hapten. Booster injections of the same amount of hapten were given 14 days after the initial injection, and the rabbits bled for antibody analysis at 10 days and twice weekly for 2 weeks after the booster injection.

The rabbit sera were tested for antibody titer using two methods, the complement fixation test and a millipore filter system (method described in Annual Report, Department of Biological Chemistry). No antibody production was found in these sera using these tests. Again booster injections of conjugate which contained 10 μ g hapten were given subcutaneously to four rabbits that had been injected previously with 50 and 100 μ g of hapten subcutaneously. The rabbits were bled twice weekly for two weeks and the sera tested as before. The tests were negative for hapten antibodies.

Studies will be continued using other hapten-protein conjugates and other methods for antibody detection. Antibody isolation prior to testing for specific antibodies will be investigated.

4. Biochemical effects of antimalarials.

Studies were continued on the biochemical effects of antimalarials. The objectives of these studies were to develop a test to monitor adverse biochemical reactions, and to determine the mechanism of drug action. This report covers primarily the biochemical effects of the antimalarial WR 142490.

Male Sprague-Dawley rats (250-300 g) were administered by gastric intubation vehicle, 5, 25 or 125 mg (free base) per kilogram body weight of WR 142490. The suspending vehicle was methyl cellulose, 0.5%, and Tween 80, 0.1%. Four groups of six rats per group were given drug

daily for 14 days (125 mg/kg group; 10 days because of drug toxicity), anesthetized with sodium pentobarbital, bled from the abdominal aorta, and selected tissues removed.

The rats were weighed prior to starting drug administration and again prior to sacrifice. The vehicle control and the 5 mg/kg groups increased weight normally, the 25 mg/kg group had no weight increase, and the 125 mg/kg group lost weight. The 125 mg/kg exhibited signs of toxicity, diarrhea, red exudate around nostrils, ruffled fur, and rapid breathing. The organ weights (wt/100 g body weight) of the adrenals, lungs, spleen, kidneys and liver were similar with all drug doses and controls. The thyroid weight was decreased in 25 mg/kg and 125 mg/kg groups.

The blood hematocrit and hemoglobin content was not affected by any drug dose. The WBC of the 125 mg/kg group was increased 34% over the vehicle control group.

Selected serum enzyme activities were studied to assess possible drug related enzyme induction and/or tissue damage. The LDH activity was increased in the 25 and 125 mg/kg groups. The 25 mg/kg group showed a pronounced increase in LDH isoenzymes 1 and 5 (electrophoretic bands), while the 125 mg/kg group showed a major increase in LDH isoenzymes 1 and 5 and some increase in LDH isoenzymes 2, 3, and 4. The SGOT activity in the 125 mg/kg group showed a 4-fold increase over the vehicle control group with an increase in all the SGOT isoenzymes. The SGPT activity in the 125 mg/kg group was increased 2-fold over vehicle control with an increase in one isoenzyme. These preliminary plasma enzyme results suggest that the liver and kidney may be affected by the drug.

The biochemical effects of WR 142490 on serum corticosterone and T_3 and T_4 were investigated. The serum corticosterone levels were not affected by the administration of this drug. However, WR 142490 administration produced a dose related decrease in serum T_4 (3.6 $\mu\text{g}/100\text{ ml}$ in untreated rats to 1.8 $\mu\text{g}/100\text{ ml}$) in the 125 mg/kg group. T_3 and T_3 uptake (the relative saturation of thyroxine binding protein) were not affected. Results suggest a mild depression of thyroid function, or interference with thyroxine binding to carrier protein.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 315 Blood level assays for anti-malarial drugs

Literature Cited.

Publications:

1. Rice, K., Boone, B.J., Rubin, A., Paulo, T.: The synthesis and biological properties of benzo-(h)-quinoline methanols. J. Med. Chem. 19: 887, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1 AGENCY ACCESSION ⁶	2 DATE OF SUMMARY ⁷	REPORT CONTROL SYMBOL DD-DR-2 (AR) 36	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ⁸	6 WORK SECURITY ⁹	7 REGRADING ¹⁰	8A DISP INSTR ¹¹	8B SPECIFIC DATA- CONTRACTOR ACCESS		9 LEVEL OF SUM A. WORK UNIT
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		
10 NO / CODES ¹²	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62759A	3A762759A829		00	318			
b. CONTRIBUTING								
c. CONTRIBUTING	CARDS 114F							
11 TITLE (Precede with Security Classification Code) ¹³								
(U) Biological Studies of Insect Infection and Disease Transmission								
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ¹⁴								
002600 Biology								
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD		
65 07		CONT		DA		C. In-House		
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19A PROFESSIONAL MAN YRS		19B FUNDS (In \$000s)
a. DATES/EFFECTIVE ^{17A} NA				PRECEDING				
b. NUMBER ^{17B}				FISCAL YEAR		4.5		21
c. TYPE				CURRENT		4.3		21
d. KIND OF AWARD				f. CUM. AMT.				
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION				
NAME ^{19A} Walter Reed Army Institute of Research				NAME ^{20A} Walter Reed Army Institute of Research				
ADDRESS ^{19B} Washington, DC 20012				ADDRESS ^{20B} Washington, DC 20012				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish MAN (U.S. Academic Institution))				
NAME Joy, COL R.J.T.				NAME ^{20C} Eldridge, COL B. F.				
TELEPHONE: 202-576-3551				TELEPHONE 202-576-3719				
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]				
21 GENERAL USE				ASSOCIATE INVESTIGATORS				
Foreign intelligence not considered				NAME: Schneider, Dr. I.				
				NAME: Wilkinson, MAJ R. N.				
22 KEYWORDS (Precede EACH with Security Classification Code) ¹⁵								
(U) Anopheles; (U) Mosquitoes; (U) Malaria; (U) Immunization; (U) Plasmodium								
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
<p>23. (U) Development of physiological means of interrupting malaria transmission through an understanding of factors influencing susceptibility of anophelene vectors to malaria and of the various factors affecting gametocyte infectivity in vivo and in vitro. Test systems are developed for studying the mechanisms underlying sporozoite induced immunity for the eventual prevention and control of malaria in military troops.</p> <p>24. (U) Studies are conducted to determine quantitatively such parameters as the minimum number of sporozoites required for the initial immunizing dose as well as the spacing, number and strength of the subsequent boosters. Attempts to develop one or more serological assays for demonstrating the course of the immune response in the blood serum of animals immunized against the sporozoite stage. Evaluation of the effects of various environmental stresses upon the infectivity of the gametocytes. Isolation of these forms from the other blood stages on density gradients for subsequent use in culture systems.</p> <p>25. (U) 75 07 - 76 06. Congenitally athymic mice were completely susceptible to sporozoite challenge after either a single dose of -irradiated Plasmodium berghei sporozoites or a series of 5 such doses, whereas immunized normal mice were completely protected. This demonstrates that the ability of mice to mount a sporozoite-induced protective response is T-cell dependent. A technique for the isolation of P. cynomolgi ookinetes from mosquitoes was developed. Such ookinetes were found to develop to the oocyst stage when cultured in vitro. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$48,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.</p>								

PII Redacted

DD FORM 1498
1 MAR 68

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

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 318 Biological studies of insect infection and disease transmission

Investigations

Principal: Imogene Schneider, Ph.D.

Associate: COL Bruce F. Eldridge, MSC; MAJ Rowland N. Wilkinson, MSC; CPT Anthony B. Bosworth, MSC; Jerome E. Freier, Ph.D.; Talmadge Neal, B.S.; Raymond Fleming, B.S.; SP5 Lawrence Macken; SP5 Amy Federman; PFC Caesar Butkiewicz; PVT Cynthia Skelton

Description

The major objectives of this work unit have been (1) understanding the mechanisms underlying sporozoite-induced immunity in Plasmodium berghei malaria and (2) isolating and concentrating P. cynomolgi ookinetes on density gradients for subsequent utilization in an in vitro culture system. Studies have also been undertaken to determine the feasibility of isolating gametocytes on density gradients for initiating the sporogonic cycle of the malaria parasite in vitro. In addition, preliminary attempts have been made to evaluate the use of a cell line derived from tissues of the tsetse fly, Glossina morsitans, to support the growth of the insect cycle of Trypanosoma brucei.

Progress

1. Immunization against P. berghei malaria with γ -attenuated sporozoites

Previous studies at WRAIR and at other institutions have shown that immunization of rodents with repeated injections of γ -irradiated P. berghei sporozoites results in extensive and often complete protection against a subsequent challenge of infectious, homologous sporozoites. In an attempt to determine the relationship between protection afforded by immunization of mice with P. berghei sporozoites and cellular induced factors, Balb/c congenitally athymic and Balb/c homozygous mice were immunized with either a single dose of 7.5×10^4 γ -irradiated sporozoites or, in addition, received four boosters of 1×10^3 or 1×10^4 sporozoites at 2-week intervals. The mice were maintained in a specific pathogen free environment throughout the course of the experiments. All 3 groups received intravenous challenges consisting of 1×10^4 homologous sporozoites 2 weeks after the last immunization. In each instance following challenge, the immunized nude mice developed a parasitemia and died whereas both the heterozygous and homozygous mice never developed patent infections. By contrast, the course of sporozoite-induced infections in non-immunized nude and homozygous mice did not differ significantly from each other in the length of the prepatent period, the subsequent parasitemia or the mean day of death (Table 1). Thus, the ability of Balb/c mice

to mount a sporozoite-induced protective response appears to be T-cell dependent but once the infection has been established, no appreciable difference exists between a fully immunocompetent or T-cell deficient mouse in the ability to handle the infection.

Following this study, attempts were made to demonstrate immunity in mice by adaptive transfer of sensitized cells. One or 2 spleen or peritoneal cell equivalents from mice 3, 5, 7 and 10 days post immunization were inoculated IV or IP to syngeneic recipients and the latter challenged 2-4 hours later with 1×10^4 sporozoites. In no instance was any detectable immunity demonstrated. Similar results were obtained with peritoneal exudate transfers 7 days post-immunizations.

Currently, transfer experiments are being carried out with fractionated cell subpopulations in an attempt to determine whether the above results are due to an activated macrophage or a T-cell suppressor phenomenon.

2. Isolation of P. cynomolgi parasites on density gradients and the cultivation of the ookinete stage in vitro.

The successful demonstration of sporozoite-induced immunity in rodent, primate and human malaras has necessitated an urgent requirement for the mass production of sporozoites. Attempts to define the nature of the immune response has been hampered by the limited and variable development of malaria parasites in mosquitoes. The main goal of this research has been to devise a method of obtaining large numbers of sporozoites for use as antigens while minimizing the role of the mosquito. Research efforts were directed at isolating ookinetes from mosquito midguts, placing these parasites in culture and attempting to maintain the cultures until the oocysts matured and sporozoites formed.

Prior to isolation and cultivation, it was necessary to determine the optimal period for infecting A. stephensi mosquitoes with P. cynomolgi from rhesus monkeys. Since mosquito infections are dependent upon the presence of viable gametocytes, a detailed investigation was made on the influence of various physical and chemical factors on gametocyte infectivity. P. cynomolgi blood infections demonstrate parasitemia cycles with a periodicity of approximately 7 days. The daily course of blood induced P. cynomolgi infections was observed on thin smears and the number of trophozoites, schizonts and gametocytes per 10,000 erythrocytes determined. Whether the parasites were in the early, middle or late phase of each stage was also recorded. Mosquitoes were fed each day on infected monkeys and the incidence and degree of mosquito infection determined by counting oocysts on the midgut 7 days after a blood meal. Attempts were made to correlate parasitemia factors with mosquito infections to establish indicators of optimal periods to initiate sporogony. The mean values for 16 infections in rhesus monkeys showed that during the course of an 18 day infection (Figs. 1-3) each parasite stage had at least 2 complete cycles. From this study it was concluded

that first peak oocyst infections in mosquitoes can be predicted on the basis of changes in gametocyte population density. The greatest number of oocysts was found in mosquitoes fed during the increasing phase of the gametocyte cycle just prior to the peak when 6 ± 2 gametocytes/ 10^4 RBC were observed. However, second peak mosquito infections did not always correspond to the rise in gametocytemia. Frequently, peak infections occurred 24 to 48 hours after a detectable increase in the number of gametocytes. The only constant factor for second peak infections was that they occurred 7 days after the first peak. This observation was used to predict the best time to infect mosquitoes during the second cycle (Table 2).

Erythrocyte and leukocyte numbers per mm^3 and packed cell volume were also determined in conjunction with parasitemia observations. Results showed (Fig. 4) that while the number of erythrocytes/ mm^3 and the packed cell volume steadily and synchronously declined, the leukocyte number displayed two complete cycles with a periodicity and a rate of change identical with the mean gametocytemia curve (see Fig. 3). Thus, leukocyte counts may also be of value in predicting subsequent mosquito infections.

After determining the periods of optimal parasite infectivity, an attempt was made to quantitatively study the conditions affecting gametocyte infectivity. A miniature, constant temperature blood feeding chamber was designed to bioassay gametocyte response to temperature changes, centrifugal force and chemicals such as anticoagulants. Mosquitoes from the same batch were fed on infected blood through the membrane which was composed of sheep mesentery. Membrane fed mosquitoes were fed both before and after treatment of the blood. Additional mosquitoes were fed on the same donor monkey to serve as controls. Preliminary experiments compared the infectivity of gametocytes fed on monkeys to those fed via a membrane and without any special treatment. Results from these experiments showed that the number of oocysts in mosquitoes fed through a membrane was similar to those in mosquitoes fed directly on a monkey (Table 3). The cyclic pattern of mosquito infectivity was also shown in the oocyst infections from the membrane fed group. Frequently, the number of developing oocysts was greater in mosquitoes fed through the membrane. This may have been due to the gametocytes settling and concentrating near the surface of the membrane and thus becoming more readily available to mosquitoes than those obtained from host capillaries.

Further experiments with the bioassay system evaluated the ability of gametocytes to remain viable after centrifugation. Forces of $1000 \times g$ resulted in a small loss of infectivity compared with the controls (Table 4). After centrifugation, the plasma layer was replaced with an equal volume of unsupplemented Medium 199 (M199) and the suspension fed to mosquitoes. The presence of oocysts was observed on days 17 and 18 after a sporozoite induced infection but feeding on subsequent days failed to produce oocysts as well as oocytes even though numerous females were engorged with the M199-blood cell mixture.

Proposals for cultivating the sporogonic cycle from the gametocyte stage usually involve the use of density gradients for the isolation of the gametocytes. Therefore, a series of experiments were performed to determine the effect of density gradient media on gametocyte viability. Results from these experiments showed that 10-15% Ficoll (a high molecular weight polymer of sucrose), Renografin (a polyiodobenzoic acid isopaque solution) and bovine serum albumin (BSA) all adversely affected gametocyte viability (Tables 5 - 7, respectively). Incubation of gametocytes in Ficoll or Renografin resulted in no oocyst development even when the medium was supplemented with BSA. However, BSA alone was able to maintain viability for up to 60 min. (Table 7).

The influence of temperature on gametocyte development was studied by withdrawing blood from monkeys and feeding half the blood immediately to mosquitoes while the remainder was incubated at either 4, 24, or 37 C for 60 min. Almost invariably, a complete loss of gametocyte infectivity resulted after this interval regardless of the temperature maintained (Tables 5-7).

All experiments, up to this point, used Locke's-Heparin solution (4 units heparin/ml); however, the effect of this anticoagulant and others upon gametocytes was unknown. Infective blood solutions containing either 0.25% sodium citrate; 50 mM EDTA; heparin (70 units/ml); or mechanically defibrinated blood were tested to determine the effect of each method on parasite viability. Tested solutions were incubated with infected blood for 30 min at 24 C. Results of two trials showed heparin to be the most effective in maintaining gametocyte infectivity (Table 8). Sodium citrate, EDTA and defibrination each significantly reduced the level of parasite infectivity in mosquitoes.

Since in vitro cultivation of gametocytes did not appear feasible due to their ephemeral nature, attention was shifted to the mass isolation of P. cynomolgi ookinetes and the cultivation of these parasites to mature oocysts with sporozoites. Preliminary experiments established that ookinetes are present in the midgut of A. stephensi from 16-20 hours after an infective blood meal. The procedure for isolating ookinetes from midguts was as follows: midguts from mosquitoes were dissected 24 hrs after the blood meal and gently homogenized. The homogenate was centrifuged at 50 x g and the supernatant suspension filtered through a 25 μ filter. The filtrate was centrifuged at 1,100 x g and the residue layered onto a Renografin (5-40%)-bovine serum albumin (Path-O-Cyte-V, 17.5-35%) continuous density gradient and spun at 16,000 x g for 90 min. Gradients were fractionated from the top, the refractive index of selected samples determined and each fraction diluted with 5 volumes of M199. The diluted fractions were centrifuged at 1,100 x g for 10 min and the pellet resuspended in M199 supplemented with 3% BSA. Aliquots (5 μ l) were spread on somatic cell counting slides (Bellico), air dried, fixed with absolute methanol and stained with Giemsa.

Thirty isolations of P. cynomolgi ookinetes have been performed to date and a representative profile of one isolation is shown in Fig. 5. An estimated 3×10^5 ookinetes were recovered from the 400 mosquito sample. Recoveries averaging 300-800 ookinetes per midgut were frequent. Ookinetes isolated daily from mosquitoes during an infective cycle correspond in magnitude to the numbers of oocysts that subsequently develop on the mosquito midgut (Table 9). Refractive index values for each ookinete fraction on the density gradient were converted to density values and the overall mean density of ookinetes recovered was 1.1156 g/ml (Table 10). Comparisons were made between the number of ookinetes recovered from 24 and 48 hr postfed mosquitoes after both groups are simultaneously fed on the same monkey. Results showed that ookinete recoveries from 48 hr postfed mosquitoes were substantially less (<5%) than the number isolated from 24 hr postfed mosquitoes. Hence, all subsequent ookinetes recoveries have been confined to mosquitoes infected 24 hrs before the isolation.

After the ookinetes were recovered from the gradient, they were washed and resuspended in M199 with 3% BSA. Ookinete containing fractions on each side of the recovery peak were combined and the parasites sedimented and resuspended in the final oocyst culture medium which was composed of M199, 10% fetal bovine serum, 500 units/ml penicillin, 500 µg/ml streptomycin, 200 µg/ml gentamycin and 250 µg/ml Fungizone (amphotericin B). Continuous flow cultures were established using a chemotaxis chamber (Bellco) with a 0.22 µ Millipore membrane separating the culture chamber into two sections, an upper culture area and a lower effluent chamber with a siphon. Isolated ookinetes were injected into the chamber with a syringe needle through a side port in the culture chamber. An opposite port was fitted with a needle to admit a continuous flow of culture medium and antibiotics from an elevated reservoir using gravity pressure to maintain flow. Cells were maintained in culture for 48 hrs (72 hrs after the infective blood meal). Cultures examined after this incubation period showed the presence of developing oocysts. After the cultures were terminated, mosquitoes from the same ookinete source were killed and fixed in Carnoy's, embedded in wax, sectioned at 6µ and stained with Giemsa/Colophonium. Morphological comparisons were made between 72 hr oocysts growing in vitro and oocysts of the same age developing in vivo. Results showed that in both groups of parasites the nuclear chromatin within the 7-8 µ spherically-shaped cells was distinct and, in some cases, the chromatin had divided two or more times.

3. Cultivation of Trypanosoma brucei in a cell line of Glossina morsitans

Studies on T. brucei and other African pathogenic trypanosomes have been hampered by the inability of the infectious forms to grow in vitro. Once placed in culture, development usually ceases at the trypomastigote stage (i.e., that found in the midgut of the tsetse fly). Development of the epimastigote and metatrypanosome (infective) stages does not take place. Since a limited amount of success has been achieved in cultures containing tsetse-fly tissues, a cell line derived from such tissues was

established at Walter Reed to determine whether such cells might act as a "feeder layer" for the growth and development of T. brucei in vitro.

In very preliminary experiments, blood-stream form trypanosomes at a concentration of 1×10^3 /ml were inoculated into T-30 flasks containing monolayers of G. morsitans cells. Following a lag phase of approximately 24 hrs, the number of parasites increased about 50 fold by day 4. Mice injected IP with an 0.25 ml suspension from such cultures developed parasitemias after a prepatent period of 4-5 days. By contrast, trypanosomes cultured in medium alone rapidly declined in number and had disappeared by day 3. Studies are currently under way to subculture both cells and trypanosomes in an attempt to increase the numbers of the latter without loss of infectivity.

Conclusions and recommendations

1. Original plans called for a two-sided approach to gain an understanding of the mechanisms of immunity in sporozoite-immunized experimental animals; namely, to determine the relationship between protection and both humoral and cellular induced factors. However, since the evidence for the former has, as yet, been inconclusive whereas that implicating T-cell dependency of sporozoite antigens has now been documented, emphasis should be shifted to a thorough understanding of cell mediated immunity in the P. berghei system. Studies on the role, if any, played by serum antibody may be undertaken if time and resources permit.

2. Bioassay experiments have shown quite conclusively that the gametocyte stage of the malaria parasite is extremely sensitive to environmental changes and quickly loses the ability to infect mosquitoes. The failure of gametocytes to survive in either Ficoll- or Renografin-BSA solutions precludes the use of these materials for the mass isolation of viable parasites of this stage. By contrast, the feasibility of isolating ookinetes on density gradients has been shown and their subsequent inclusion in a culture system has led to the formation of early stage oocysts. Additional studies are warranted to determine under what conditions the oocysts will continue development in vitro to the sporozoite stage.

3. The use of a cell line of Glossina morsitans to serve as a "feeder layer" for the growth of Trypanosoma brucei in vitro appears quite promising. Additional studies are needed to find the upper limits of parasite multiplication without loss of infectivity. Studies on the metabolism of the epimastigote and metatrypanosome forms of T. brucei may eventually be feasible.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 318 Biological studies of insect infection and disease
transmission

Literature Cited

References: None

Publications:

1. Bosworth, A. B., I. Schneider and J. E. Freier. 1975 Mass isolation of Anopheles stephensi salivary glands infected with malarial sporozoites. J. Parasitol. 61: 769-772.
2. Hockmeyer, W. T., A. B. Bosworth, I. Schneider, S. M. Phillips and F. E. Hemphill. 1976 T-cell dependence of sporozoite induced immunity in mice. Amer. Soc. Microbiol. Abs. Ann. Meeting. p. 86.

TABLE 1

Median survival time and length of prepatent period of challenged non-immunized nude (DN) and Balb/c (DNC) mice, immunized nude (ISN) and Balb/c (ISB) mice and immunized, boosted nude (NIS) and Balb/c (NIB) mice. Non-immunized Balb/c (ISBC, NIBC) mice served as challenge controls for the immunized and immunized, boosted groups

Animal Group (No.)	Median Survival (Days)	Median Patency Time (days)	CSP-SNA* Activity
<u>Non-immunized</u>			
DNC - Homozygotes (8)	21.0	5.0	
DN - Nudes (2)	27.5	4.5	
<u>Immunized**</u>			
ISB - Homozygotes (10)	Immune	Immune	+
ISBC - Homozygotes (1)	24.0	3.0	
ISN - Nudes (10)	23.0	4.0	-
<u>Immunized***</u>			
NIB - Homozygotes (10)	Immune	Immune	+
NIBC - Homozygotes (10)	21.5	4.0	
NIS - Nudes (9)	23.5	4.0	-

* CSP-SNA, circumsporozoite precipitation and serum neutralizing antibodies

** Immunized with 7.5×10^4 sporozoites

*** Immunized as above plus 4 boosters of 1×10^3 or 1×10^4 sporozoites

TABLE 2

Relationship between parasite dose, delivered by intravenous blood passage, and the time period for the development of peak infections

Monkey Number	Parasite Dose	Parasite Stage	Days After Blood Infection	
			First Peak	Second Peak
R 166	5.8 X 10 ⁷ P/Kg *	Asexual	7	14
		Sexual	7	13
		Oocyst	6 (127.8)**	13 (153.8)
P 872	3.4 X 10 ⁶ P/Kg	Asexual	9	14
		Sexual	9	13
		Oocyst	8 (216.2)	14 (111.1)
P 882	9.6 X 10 ⁷ P/Kg	Asexual	6	14
		Sexual	7	13
		Oocyst	3 (7.9)	15 (9.0)
P 972	8.1 X 10 ⁷ P/Kg	Asexual	7	13
		Sexual	8	14
		Oocyst	5 (145.8)	13 (460.6)
R 167	1.2 X 10 ⁷ P/Kg	Asexual	8	13
		Sexual	9	13
		Oocyst	8 (106.6)	13 (86.2)
R 161	4.4 X 10 ⁷ P/Kg	Asexual	6	13
		Sexual	5	14
		Oocyst	6 (323.3)	14 (132.7)
P 973	1.4 X 10 ⁸ P/Kg	Asexual	6	13
		Sexual	7	12
		Oocyst	7 (175.6)	13 (120.6)
M 756	1.5 X 10 ⁸ P/Kg	Asexual	6	14
		Sexual	7	13
		Oocyst	5 (180.5)	13 (367.9)

* Blood taken from rising or falling parasitemia (asexual)

** Mean oocyst number

TABLE 3

Comparison of the infectivity of *P. cynomelgi* gametocytes fed to *A. stephensi* via donor monkeys and via a membrane during a 5-day course of blood infection

Days after Infection	Percentage Parasitemia	(Gametocytes)*	Mean Oocyst Number (Range)	
			Fed on donor monkey	Fed via membrane
16	1.42	(2)	7.7(0-17)	9.7(0-34)
17	4.80	(0)	34.7(0-102)	76.7(1-174)
18	6.78	(8)	30.0(1-52)	77.9(0-201)
19	3.62	(8)	134.5(36-300)	284.4(64-500)
20	3.50	(6)	18.4(1-77)	93.7(4-242)

* Gametocytes per 10,000 erythrocytes counted

TABLE 4

Effect of centrifugation (10 min, 24°C) of gametocytes and the replacement of the plasma layer with Medium 199 (incubation 15 min, 24°C) on the infectivity of gametocytes

Days After Infection	Percentage Parasitemia	(No. Gametocytes)*	Mean Coeyst Number (Range)			
			Fed on donor monkey		Fed via membrane	
			Control (0 min)	Control (15 min, 24°C)	Centrifuged 1,000 X G	Plasma Replacement
15	0.60	(0)	0.0	0.2(0-2)	0.0	0.0
16	1.00	(0)	6.2(0-23)	9.8(0-22)	0.0	0.0
17	2.64	(2)	9.1(0-24)	13.7(1-40)	1.7(1-4)	4.4(2-7)
18	5.92	(2)	44.9(6-137)	60.4(5-135)	40.9(4-90)	27.0(6-64)
19	3.46	(4)	9.9	1.3(0-6)	0.1(0-1)	0.0**
20	1.38	(6)	132.1(9-300)	54.1(4-600)	51.4(1-95)	0.0**
21	0.74	(0)	6.6(0-15)	31.5(2-155)	5.9(0-21)	0.0**

* Gametocytes per 10,000 erythrocytes

** Females nongravid

TABLE 5
Effect of 10% Ficoll on *P. cynomolgi* gametocyte infectivity

Days After Infection	Z Parasitemia (No. Gametocytes)*	Mean Number Oocysts (Range)		
		Fed on donor monkey	Control (60 min, 4°C)	10% Ficoll (60 min, 4°C)
12	1.32 (0)	0.0	0.0	0.0
13	3.80 (0)	0.8(0-3)	0.0	0.0
14	6.56 (4)	1.9(0-7)	0.0	0.0
15	8.00 (8)	7.6(0-55)	0.2(0-3)	0.0
16	2.42 (12)	14.2(0-63)	1.6(0-15)	0.0
17	2.30 (8)	0.2(0-2)	0.0	0.0
18	0.22 (0)	0.0	0.0	0.0

* Gametocytes per 10,000 erythrocytes counted

TABLE 6

Effect of 15% Ficoll on *T. cynomolgi* Gametocyte infectivity

Days After Infection	\bar{x} Parasitemia	(No. Gametocytes)*	Mean Number Oocysts (Range)			
			Fed on donor monkey	Control (0 min)	Control (60 min, 4°C)	15% Ficoll (60 min, 4°C)
15	4.54	(2)	53.4 (15-107)	15.3 (0-80)	0.0	0.0
16	10.40	(10)	0.9 (0-3)	1.4 (0-6)	0.0	0.0
17	6.30	(8)	37.9 (3-169)	41.8 (10-105)	0.0	0.0
18	3.32	(4)	0.7 (0-4)	1.0 (0-10)	0.0	0.0
19	0.71	(4)	0.2 (0-1)	0.0	0.0	0.0

* Gametocytes per 10,000 erythrocytes counted

TABLE 7

Effect of temperature and density gradient media (Ficoll, Path-O-Cyte-V, and Renografin)
P. cynomolgi gametocyte infectivity

Days After Infection	% Parasit.	(No. ♂)*	Fed on Donor Monkey	Mean Number Oocysts (Range)												
				Control (0 min)	24°C (60 min)	37°C (60 min)	Ficoll (15 min) (60 min)	Ficoll (60 min)	Fed via Membrane Ficoll + Path-O-Cyte - V (15 min) (60 min)	Path-O-Cyte - V (15 min) (60 min)	Path-O-Cyte - V + Renografin (15 min) (60 min)					
5	1.38	(2)	67.0(5-194)	1.8(0-11)	0.5(0-4)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
6	5.63	(4)	55.2(19-208)	49.6(14-136)	7.9(0-63)	0.0	0.0	0.0	0.0	0.0	3.6(0-33)	0.3(0-3)	0.0	0.0		
7	12.34	(12)	139.3(17-400)	65.9(8-165)	5.7(0-59)	0.0	0.0	0.0	0.0	0.0	0.0	5.9(0-28)	N.A.	0.0	0.0	
8	9.68	(10)	13.5(0-56)	3.8(0-37)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	4.02	(10)	0.5(0-7)	0.2(0-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* Gametocytes per 10,000 erythrocytes counted
 N.A. - Data not available

TABLE 8

Effect of anticoagulants on *P. cynomolgi* gametocyte infectivity

Days after Infection	Parasit. %	(No. G)*	Mean Number Oocysts (Range)					
			Fed on donor monkey	0 min.	Heparin	Fed via membrane Na Cit	EDTA Defib.	
5	1.06	(2)	46.9(8-189)	32.1(3-79)	37.1(1-61)	12.8(0-76)	0.1(0-1)	0.1(0-1)
6	6.34	(10)	180.5(58-419)	3.0(0-16)	15.4(0-58)	4.6(0-22)	0.1(0-1)	2.8(0-24)
7	7.60	(34)	0.4(0-4)	0.0	0.2(0-2)	0.0	0.0	0.0
8	5.61	(11)	0.5(0-7)	0.0	0.0	0.0	0.0	0.0
15	0.42	(2)	21.4(0-66)	0.0	1.6(0-14)	0.4(0-6)	0.1(0-1)	0.0
16	0.59	(2)	10.0(0-42)	0.0	0.0	0.0	0.0	0.0
17	0.22	(0)	3.3(0-17)	0.0	4.8(0-24)	0.3(0-2)	0.0	0.0

* Gametocytes (G) per 10,000 erythrocytes counted

TABLE 9

Results of density gradient isolations of P. cynomolgi ookinetes from A. stephensi midguts

Days After Infection	Percentage Parasitemia	(No. Gametocytes)	Mean Number Oocysts (Range)	No. ookinetes Isolated	$\frac{\text{No. ookinetes}}{\text{Mosquito}}$
5	1.67	(0)	33.2(5-105)	3,150	7.9
6	3.10	(0)	139.6(19-246)	8,700	21.8
7	8.63	(0)	145.9(5-320)	61,050	197.6
8	12.18	(0)	35.4(0-74)	22,500	56.2
13	1.53	(4)	190.7(10-385)	205,800	514.5
14	2.39	(10)	460.6(347-600)	204,200	510.5
15	1.78	(11)	174.7(0-425)	291,600	729.0

TABLE 10

Density of ookinetes isolated on Path-O-Cyte-V: Renografin
density gradients

Days After Infection	Ookinete* Peak Density (g/ml)	Density Range (g/ml)
5	1.1050	1.1010 - 1.1270
6	1.1250	1.1105 - 1.1390
7	1.1240	1.0975 - 1.1600
8	1.098	1.0700 - 1.1440
13	1.1200	1.0865 - 1.1490
14	1.1150	1.0750 - 1.1500
15	1.1225	1.0750 - 1.1550

* Mean density of ookinete peaks = 1.1156

CAPTIONS FOR FIGURES

- Figure 1. Mean parasitemia values from 16 Rhesus monkeys infected with P. cynomolgi. Trophozoite cycles versus subsequent oocyst numbers.
- Figure 2. Mean parasitemia values from 16 Rhesus monkeys infected with P. cynomolgi. Schizont cycles versus subsequent oocyst numbers.
- Figure 3. Mean parasitemia values from 16 Rhesus monkeys infected with P. cynomolgi. Gametocyte cycles versus subsequent oocyst numbers.
- Figure 4. Mean hematological values from 16 monkeys infected with P. cynomolgi.
- Figure 5. Number and density of P. cynomolgi ookinetes isolated on a Renografin-bovine serum albumin gradient.

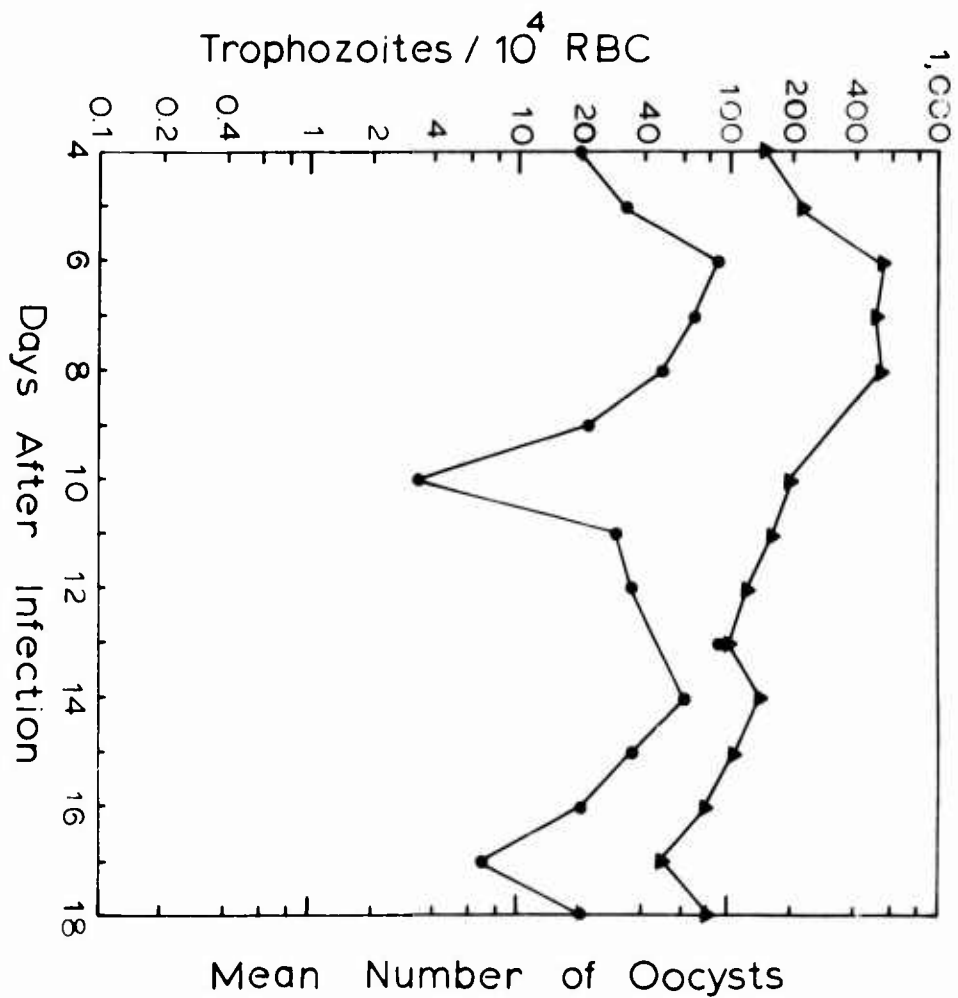


Figure 1. Mean parasitemia values from 16 Rhesus monkeys infected with *P. cynomolgi*. Trophozoite cycles versus subsequent oocyst numbers.

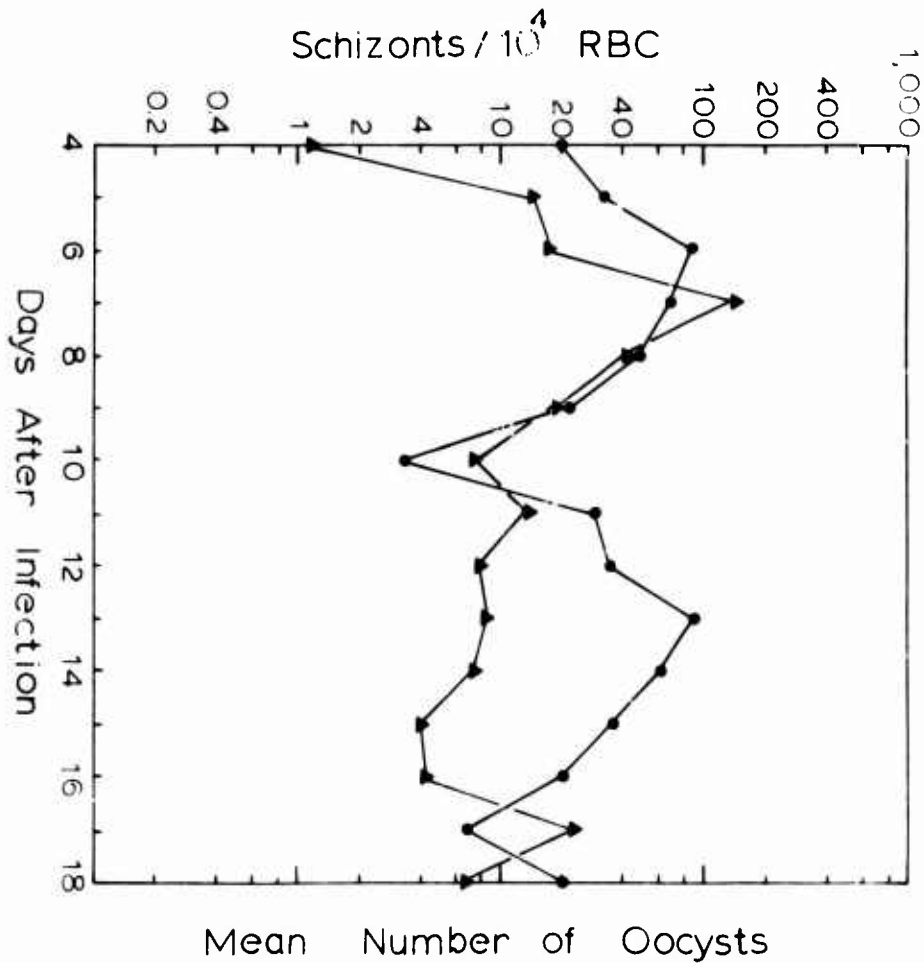


Figure 2. Mean parasitemia values from 16 Rhesus monkeys infected with *P. cynomolgi*. Schizont cycles versus subsequent oocyst numbers.

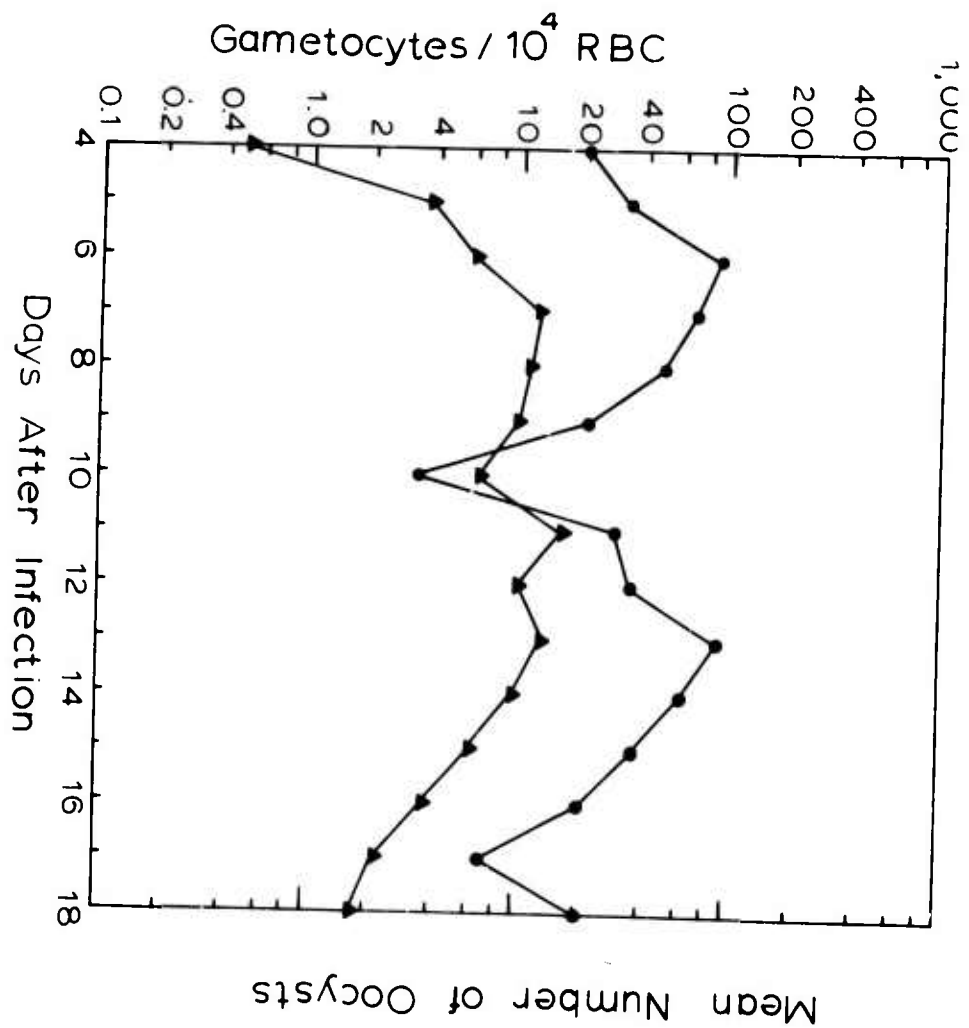


Figure 3. Mean parasitemia values from 16 Rhesus monkeys infected with *P. cynomolgi*. Gametocyte cycles versus subsequent oocyst numbers.

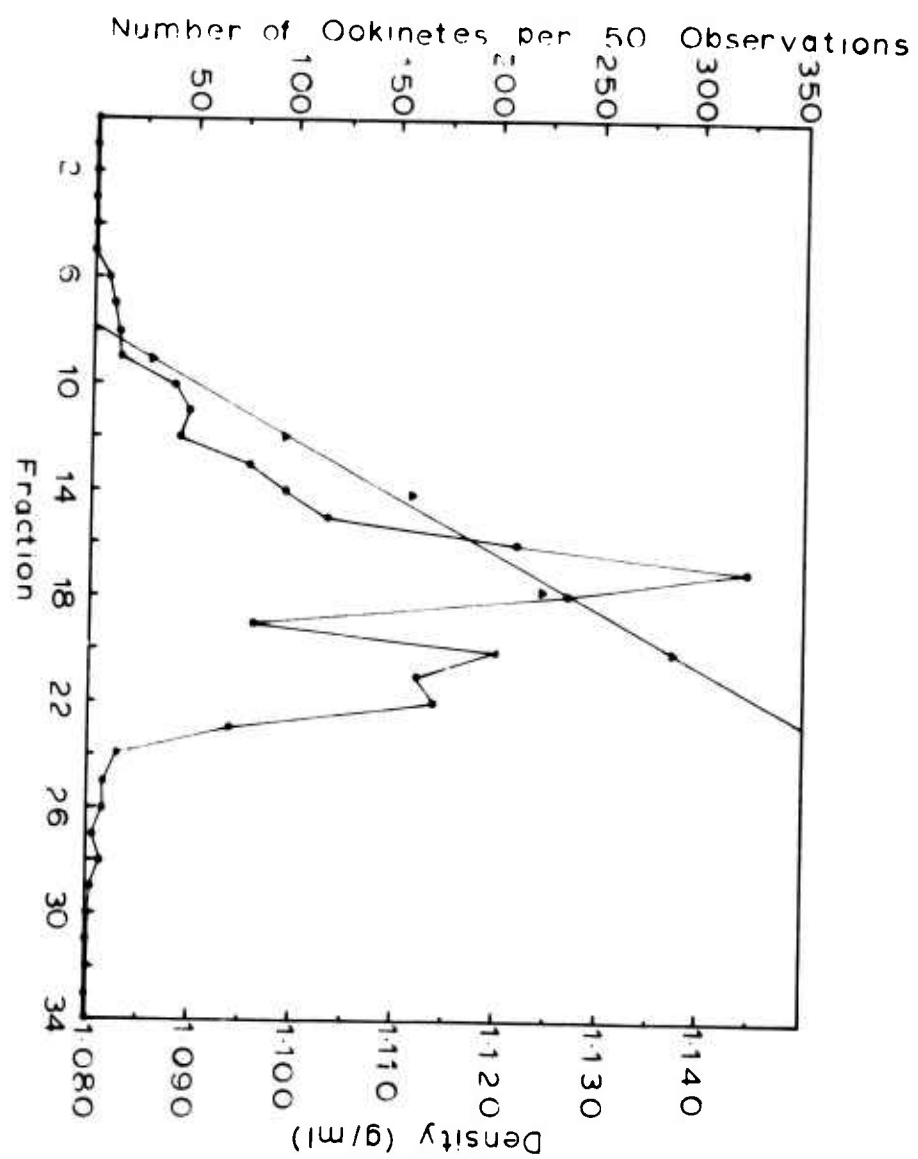


Figure 4. Mean hematological values from 16 monkeys infected with *P. cynomolgi*.

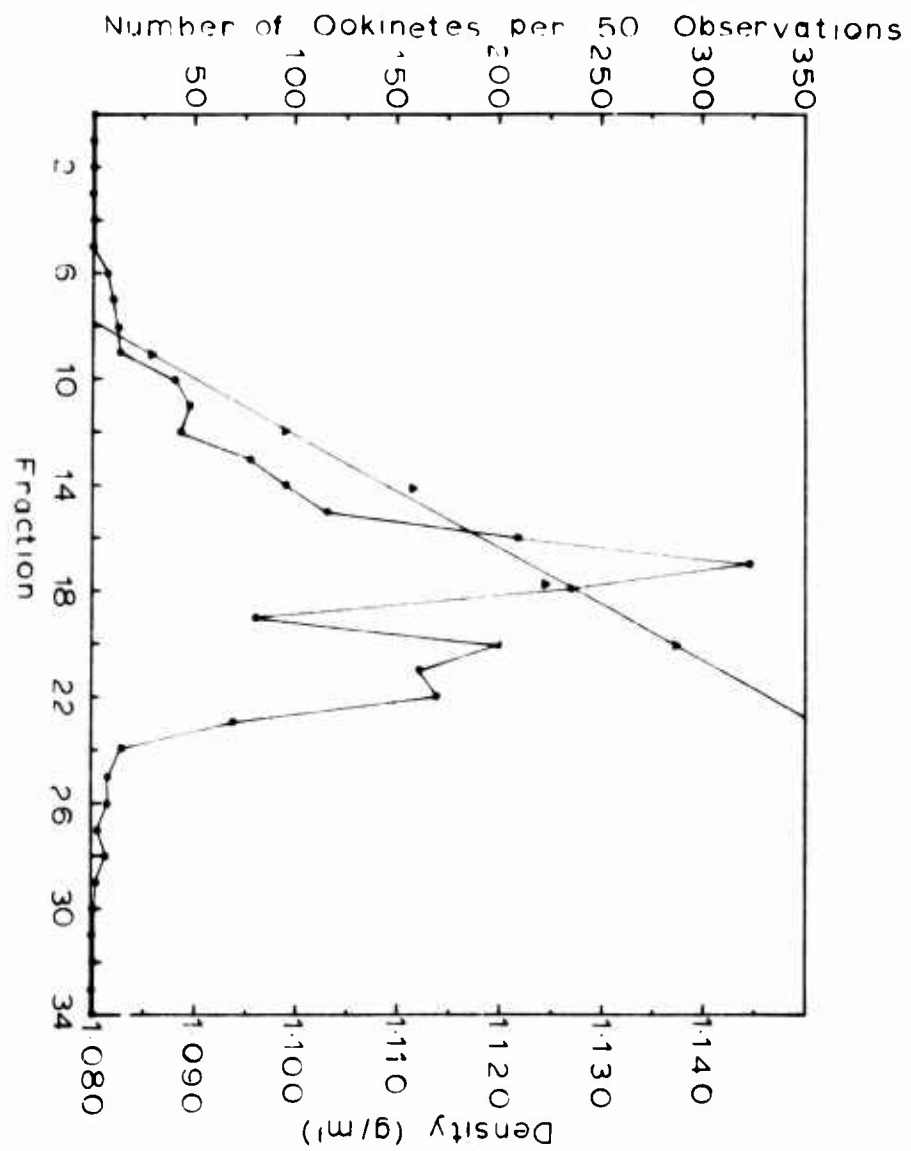


Figure 5. Number and density of *P. cynomolgi* ookinetes isolated on a Renografin-bovine serum albumin gradient.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
					DA OA 6520	76 07 01	DD-DR&L-AR/636	
3. DATE PREV. SUPPLY	4. KIND OF SUMMARY	5. SUMMARY SCTY.	6. WORK SECURITY	7. REGRADING ⁷	8A. DR&PH INSTR ⁸	9. SPECIFIC DATA - CONTRACTOR ACCESS		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
11. NO./CODES ¹¹		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER
A. PRIMARY		62759A		3A762759A829		00		324
B. CONTRIBUTING								
C. XXXXXXXX		CARDS 114F						
11. TITLE (Precede with Security Classification Code) ¹¹								
(U) Host Responses to Malaria								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ¹²								
002600 Biology								
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
64 07		CONT		DA		C. In-House		
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)
A. DATES/EFFECTIVE: NA				B. EXPIRATION		C. PRECEDING		
D. NUMBER ¹⁷				E. AMOUNT:		FISCAL YEAR		
E. TYPE:				G. CUM. AMT.		CURRENT		
F. KIND OF AWARD:						76		4
						77		4
								187
								239
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				
				ADDRESS: Washington, DC 20012				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME if U.S. Academic Institution)				
NAME: JOY, R.J.T., COL				NAME: BURKE, COL J. C.				
TELEPHONE: (202) 576-3551				TELEPHONE (202) 576-2273				
				SOCIAL SECURITY ACCOUNT NUMBER				
				ASSOCIATE INVESTIGATORS				
				NAME: MOON, A. P. DA				
				NAME:				
21. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Irradiation; (U) Cellular Immunity; (U) Lymphocyte; (U) Tolerance; (U) Serology								
22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23. (U) To study the physiopathology, immunology and serology of malaria--a disease of prime concern to troops stationed in tropical areas, causing considerable loss of man-days due to severe morbidity and mortality.								
24. (U) Evaluation of cellular and humoral immunity in rodent malaria by passive-transfer protection studies with lymphocytes, macrophages and serum. Study of mechanisms of immune response to antigens of rodent malaria through induction of antigen-specific unresponsiveness (tolerance) to P. berghei.								
25. (U) 75 07 - 76 06 Two lines of the 17X strain of P. berghei yoelii were used to demonstrate protective immunity against murine malaria. Both lines were invariably lethal to normal BALBc mice. Gamma irradiated P. yoelii infected mouse erythrocytes were used as an experimental immunogen. Although both lines were immunogenic, the one derived from the rapidly-killing strain elicited a more efficient response than that of the slow-to-kill strain as shown by reduced parasitemias and by prolonged survival. Once crisis was passed these animals either exhibited only low grade parasitemia or they remained subpatent for the duration of the experiment. Other mice were treated with irradiated P. berghei infected mouse RBC's and cyclophosphamide to determine specific suppression of the protective immune response to malaria. Specific suppression occurred in excess of drug related specific suppression to both malaria and the control antigen. The immune response to malarial immunogens thus appears to occur in a manner analogous to that of non-protective immunogens. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 75 - 30 June 76. Support in the amount of \$62,000 from FY 7T funds is programmed for the period 1 Jul - 30 Sep 76.								

PII Redacted

DD FORM 1498
1 MAR 66

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

1097

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaric. Investigations

Work Unit 324 Host responses to malaria

Investigators

Principal: COL James C. Burke, MSC

Associate: COL C. L. Diggs, MC; MAJ L. K. Martin, MSC; MAJ D. R. Stutz, MSC; MAJ R. A. Wells, MSC

1. An inbred model for protective immunization against malaria in BALB/c mice.

Several different approaches have been used in attempts to induce protective immunity against rodent malaria. Drug-modified infections of P. berghei have resulted in protection of mice against challenge. Protection by in vitro attenuation of a previously virulent strain of P. berghei was accomplished by Weiss and Degiusti. Immunization of rodents against malaria by extracts of parasite origin has been reported by several workers. Zuckerman et al. immunized weanling rats with extracts of P. berghei prepared by saponin lysis of infected red cells while D'Antonio et al. immunized mice against P. berghei by use of partially purified extracts also derived from infected erythrocytes. Another approach to protective immunization against rodent malaria has been the use of irradiated parasites. Spitalny and Nussenzweig reported protection in mice through immunization with irradiated sporozoites of P. berghei. Wellde and Sadun reported a significant protection of young rats and mice following treatment with irradiated P. berghei infected erythrocytes; multiple injections are required to induce a sufficient response to protect most of the animals from a fatal outcome on challenge. Briggs and Wellde provided evidence that escape of the parasite from the immune response may result from antigenic variation in the case of P. berghei infections.

The present studies examined protective immunization against two strains of P. berghei yoelii (P. yoelii) with irradiated blood forms of these parasites serving as the immunogen. The goal of this work was to develop an inbred murine model for further studies on the mechanisms involved in the protective immune response and to better characterize the immunogenic features of the antigens.

Animals. Female 6-8 week old BALB/cJ mice from Jackson Laboratories, Bar Harbor, Maine, were utilized throughout these experiments.

The lines of P. yoelii used in these studies were originally designed as 17XL and 17XNL. The 17XL line is a derivative of 17XNL which developed during passage at the NIH in a manner analogous to the development of the lethal line of P. yoelii described by Yoeli and Hargreaves and Hargreaves, et al. During the course of the present investigations it was found that both lines produced lethal infections in unprotected BALB/cJ mice, but

that the course for each strain was markedly different. The strains were therefore redesignated as either rapidly-killing lethal (RAP) or slow-to-kill lethal (SLO) *P. yoelii*. The parasites were maintained by serial blood passage and were prepared for immunization or passage from blood collected into heparin by heart puncture from BALB/cJ mice 3 or 4 days after inoculation with 2×10^7 RAP strain parasitized erythrocytes or 7 days after inoculation with 1×10^6 SLO strain parasitized erythrocytes.

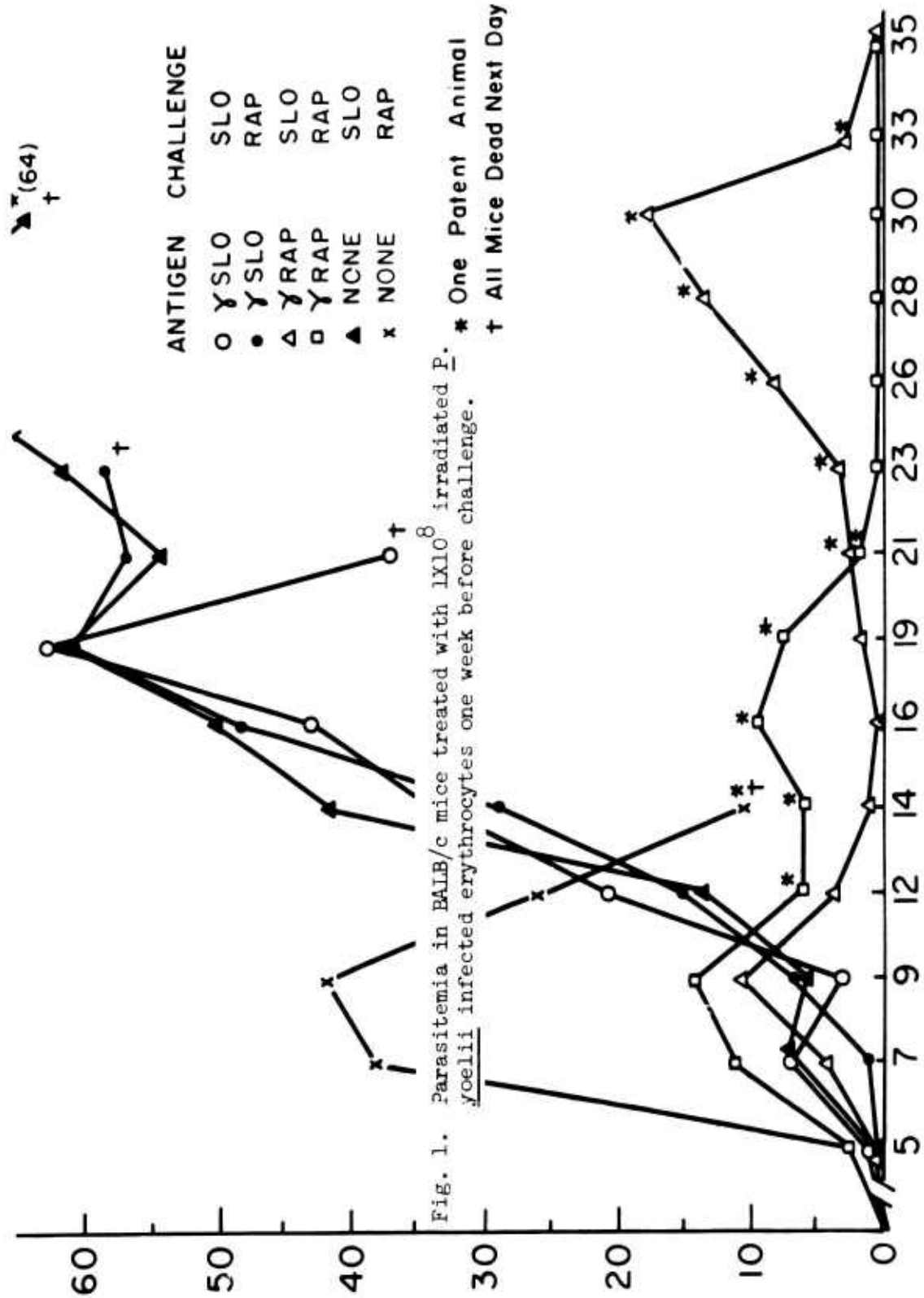
Immunization and Challenge. One volume of infected blood was suspended in two volumes of saline citrate. The blood was then centrifuged at 900g for 10 minutes at 4°C and the supernatant fluid discarded. The packed cells was resuspended in 2 volumes of chilled saline citrate and the number of parasitized erythrocytes per ml determined by the vital staining technique of Hillyer and Diggs. The infected cells were diluted to the desired concentration in chilled saline and used to infect mice or irradiated for use as immunogen. For the latter purpose infected blood was placed in 50 ml plastic tubes and exposed to 20,000 r of gamma irradiation in a 60 Co irradiator described by Rice and Smythe. The inocula were kept in an ice bath until injection into recipient except during the time of irradiation. Mice were given selected immunizing regimens as described for the individual experiments, and challenged at selected time intervals with a standard inoculum of 1×10^5 *P. yoelii* infected BALB/c erythrocytes. Immune status was evaluated by determination of parasitemia as estimated from thin blood films and mortality after challenge. Parasitemia levels were recorded as the number of infected erythrocytes per 500 cells. A slide was considered to be negative when no parasites were seen in 20 microscope fields. Deaths of the challenged animals were recorded at 24 to 72 hour intervals.

Effect of Homologous or Heterologous Challenge Against γ RAP or γ SLO Immunization

Preliminary studies indicated that infection with either RAP or SLO parasites resulted in 100% mortality of BALB/c mice. We therefore studied the effects of γ RAP and γ SLO immunization on these infections. Mice were given 1×10^8 irradiated *P. y.* infected mouse erythrocytes of either the 17X rapidly-killing strain (γ RAP) or the 17X slow-to-kill strain (γ SLO) of the parasite. After 1 week all animals were challenged with 1×10^5 non-irradiated RAP or SLO infected erythrocytes. Figure 1 illustrates the mean parasitemias of mice treated with each regimen. There was no evidence of protection of mice which had been injected with γ SLO parasites, when they were challenged with the homologous parasites; the resulting parasitemia was similar to that of the normal control group. In contrast, this γ SLO immunogen induced resistance against challenged with the RAP strain as compared to the normal RAP challenged mice. Again the parasite levels were nearly identical to those of normal mice challenged with the SLO parasites. On the other hand when γ RAP immunogen was followed by challenge with the homologous parasite there was a markedly lower parasitemia than in nonimmunized RAP controls. On day 9 after challenge the control group showed a mean parasitemia of 42 per cent as contrasted to that of 15 per cent in the immunized groups. In one

TABLE I
Survival of BALB/c Mice Challenged with Plasmodium yoelii One Week After Treatment With Malarial Antigen

Antigen	Challenge Parasite	Proportion of Mice Surviving Day				
		7	14	21	30	35
γSLO	SLO	10/10	5/10	2/10	0/10	—
γSLO	RAP	10/10	6/10	3/10	0/10	—
γRAP	SLO	10/10	8/10	8/10	3/10	2/10
γRAP	RAP	10/10	7/10	7/10	7/10	7/10
NONE	SLO	10/10	10/10	8/10	1/10	0/10
NONE	RAP	10/10	1/10	0/10	—	—



mouse reduction in parasitemia (days 12, 14) was followed by a recrudescence on day 16. All γ RAP immunized, RAP challenged mice remained subpatent after day 23. There was also evidence of protection when the γ RAP immunogen was followed by challenge with the SLO parasite. Here the resultant parasitemia was similar to that of the homologous (γ RAP-RAP) challenge for the first 19 days. After day 19, however, relapses emerged among these mice, some of which were self-limiting and others which were fatal.

Table I summarizes the survival of mice in this experiment. Among the 6 groups studied the most marked protection as shown by survival was in the γ RAP-RAP group where 7 or 10 animals were alive at the end of the experiment. There was an indication of transient protection in the γ RAP-SLO group in that 8 of 10 of the mice were alive on day 21; yet only 2 of 10 survived to the end of the study (day 35). There was no apparent protective immunization in either of the two groups initially treated with γ SLO as compared to nonimmunized controls mice challenged with the SLO parasite.

Effect of Time After Immunization on Immune Status

Mice were treated with 1 dose of 1×10^8 γ RAP with subsequent homologous challenge at selected intervals. The data summarized in Figure 2 is pooled from 2 experiments as results from these studies were indistinguishable from each other. Normal mice challenged with the RAP parasite showed an expected peak parasitemia on day 8 with death shortly thereafter. In those mice challenged 3 weeks after immunization there was an early peak in parasitemia on day 6 with all animals clearing infections by day 13. All animals in these groups remained subpatent until day 29 when a transient low-grade infection (less than 1%) was noted in one mouse. Mice challenged 2 weeks after immunization showed similar patterns of parasitemias as in the 1 week interval group. All mice in the 2 week interval groups were subpatent by day 15, and then showed occasional transient low-grade infections beginning on day 20. Animals challenged 1 week after antigen showed a rebound type pattern of parasitemia between days 8 and 22. All mice in the 1 week groups became subpatent by day 22 and showed occasional low-grade parasitemia starting on day 22. Mice given antigen on the same day as challenged showed high parasitemia levels which peaked on day 15; thereafter the 2 surviving animals became subpatent by day 20 and so remained throughout the study. Table II summarizes the survival of the mice who parasitemias were described above. In brief, a similar degree of protection (survival) was noted where a 1, 2 or 3 week interval was allowed between antigen and challenge. There was little evidence of protection when mice were given antigen on the same day as challenge.

Effect of Dose of Immunogen on Immune Status

Figure 3 shows the parasitemias of mice treated with selected single doses of γ RAP antigen prior to homologous challenge 1 week later. Normal, RAP-challenged mice showed a typical peak parasitemia on day 7 and all mice were dead before day 21. Mice immunized with 10^9 γ RAP showed a marked capacity to suppress their infections. The mice in this group were sub-

TABLE II
Survival of BALB/c Mice Challenged With *Plasmodium yoelii* at Selected Intervals
After Treatment With Malarial Antigen

Interval Between Antigen and Challenge	Proportion of Animals Surviving Day After Challenge					
	8	15	22	29	36	
3 Weeks	15/19	14/19	14/19	14/19	14/19	14/19
2 Weeks	15/20	13/20	13/20	13/20	13/20	13/20
1 Week	20/20	16/20	15/20	15/20	15/20	15/20
0 Weeks	18/20	11/20	2/20	2/20	2/20	2/20
Challenge Control	11/17	0/17	0/17	0/17	0/17	0/17

TABLE III

Survival of BALB/c Mice Challenged With Plasmodium yoelii One Week After Treatment With Selected Single Doses of Malarial Antigen

Antigen Dose	Proportion of Animals Surviving Day After Challenge				
	7	14	21	28	35
1×10^9 γ RAP	4/4	4/4	4/4	4/4	4/4
1×10^8 γ RAP	10/10	5/10	5/10	5/10	5/10
1×10^7 γ RAP	10/10	8/10	5/10	6/10	6/10
1×10^6 γ RAP	10/10	6/10	5/10	5/10	5/10
Challenge Control	10/10	2/10	0/10	0/10	0/10

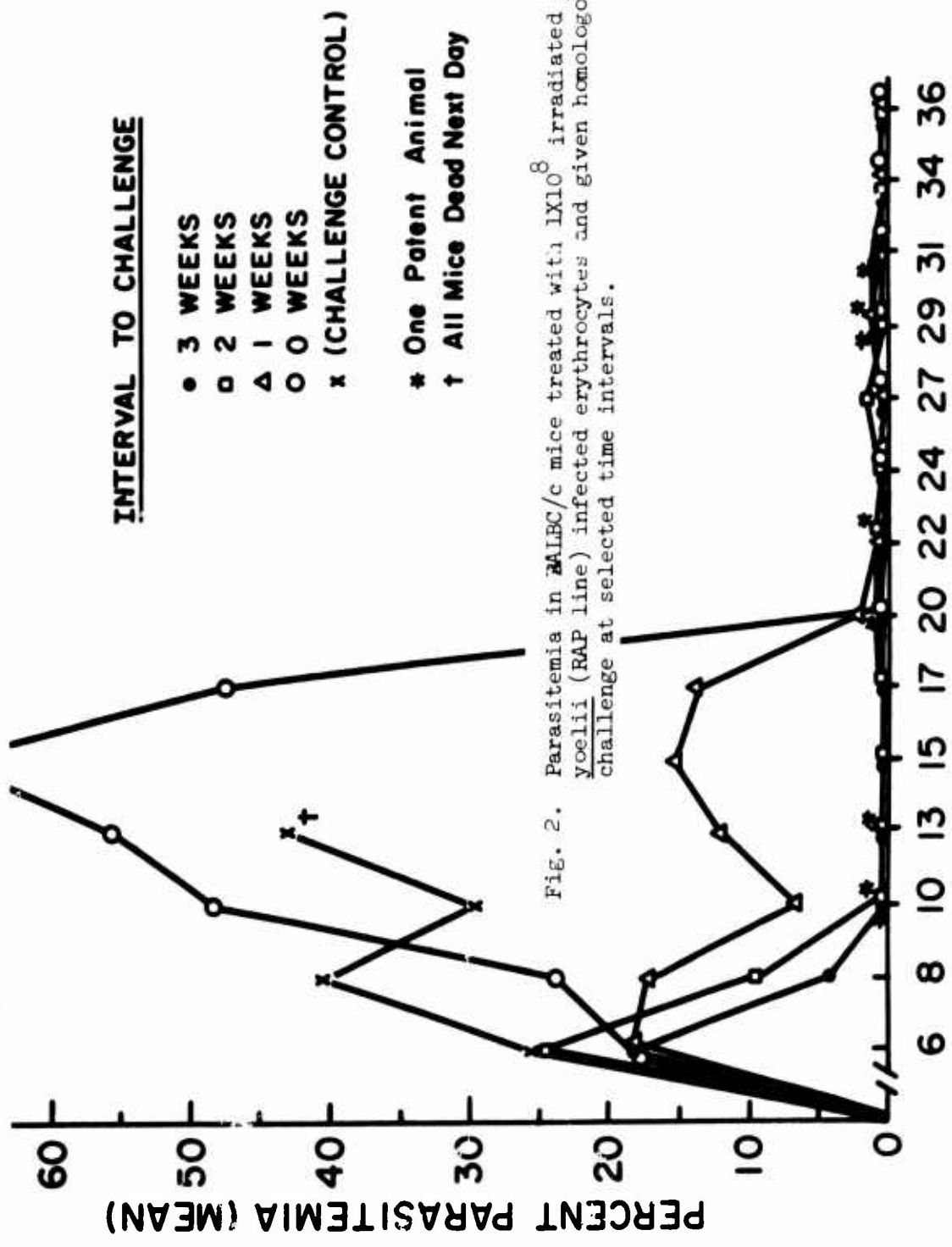


Fig. 2. Parasitemia in BALB/c mice treated with 1×10^8 irradiated P. yoelii (RAP line) infected erythrocytes and given homologous challenge at selected time intervals.

patent by day 9 and remained so for the duration of the experiment. Mice in groups treated with 10^8 , 10^7 or 10^6 γ RAP antigen showed variable levels of parasitemia which conformed to the previously described biphasic pattern over the first weeks with all animals become subpatent approximately 1 week later. No recurrence of patency was observed thereafter.

Table III summarizes the survival of mice whose parasitemias were described above. Those animals immunized with 10^9 γ RAP cells all survived challenge whereas groups which were initially treated with doses of 10^8 , 10^7 or 10^6 γ RAP antigen each showed a final cumulative mortality of 50%. As before, there were no survivors among normal mice challenged with the parasite.

Figure 4 shows the parasitemias of mice initially given the same single dose regimens of antigen as above but with a 2 week interval prior to challenge. The unimmunized, RAP-challenged mice followed a predictable course. Those animals treated with 10^9 γ RAP antigen before RAP challenge showed parasitemias essentially identical to the earlier dose-response experiment. Mice treated with 10^8 , 10^7 or 10^6 irradiated infected red cells were able to suppress their infections in proportion to the dose of antigen initially administered.

As indicated in Table IV, the survival of these animals was reduced with the extended period until challenge in all treated groups except those given 10^9 γ RAP antigen. In this group all but one of the immunized mice were alive at the end of the experiment. An intermediate degree of protection resulted where 10^8 or 10^7 irradiated infected red cells had been given. There was little evidence of protection where 10^6 γ RAP antigen had been administered.

As indicated in Table IV, the survival of these animals was reduced with the extended pre-challenge period until challenge in all treated groups except those given 10^9 γ RAP antigen. In this group all but one of the immunized mice were alive at the end of the experiment. An intermediate degree of protection resulted where 10^8 or 10^7 irradiated infected red cells had been given. There was little evidence of protection where 10^6 γ RAP antigen had been administered.

These studies demonstrate the efficacy of the RAP line of Plasmodium yoelii as an immunogen when administered after inactivation of its proliferative capacity by gamma irradiation. Immunization with γ RAP provided resistance both against challenge with the RAP line and with the SLO line of the parasite to an extent which allowed survival of the majority of the animals under appropriate conditions. In contrast γ SLO was much less effective as an immunogen. Whereas it converted the course of infection after challenge with RAP to a somewhat slower course indistinguishable from that exhibited by SLO, it had no discernible effect on the course of parasitemia after SLO challenge (Figure 1 and Table I). The reasons

TABLE IV

Survival of BALB/c Mice Challenged With *Plasmodium yoelii* Two Weeks After Treatment With Selected Single Doses of Malarial Antigen

Antigen Dose	Proportion of Animals Surviving Day After Challenge				
	7	14	21	28	35
1×10^9 RAP	10/10	9/10	9/10	9/10	9/10
1×10^8 RAP	6/7	3/7	3/7	3/7	3/7
1×10^7 RAP	10/10	6/10	3/10	3/10	3/10
1×10^6 RAP	6/6	3/6	0/6	0/6	0/6
Challenge Control	10/10	1/10	1/10	0/10	0/10

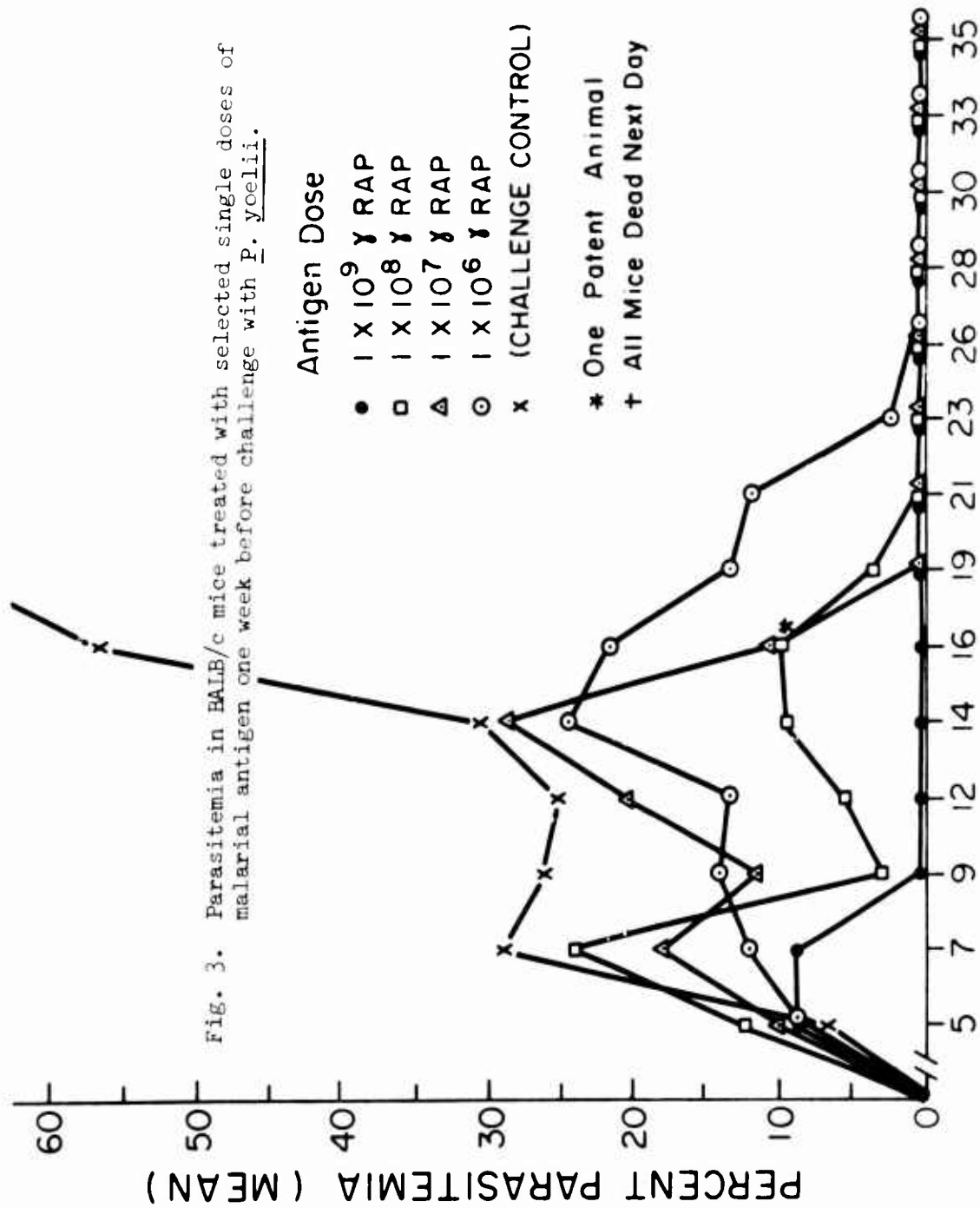


Fig. 3. Parasitemia in BALB/c mice treated with selected single doses of malarial antigen one week before challenge with P. yoelii.

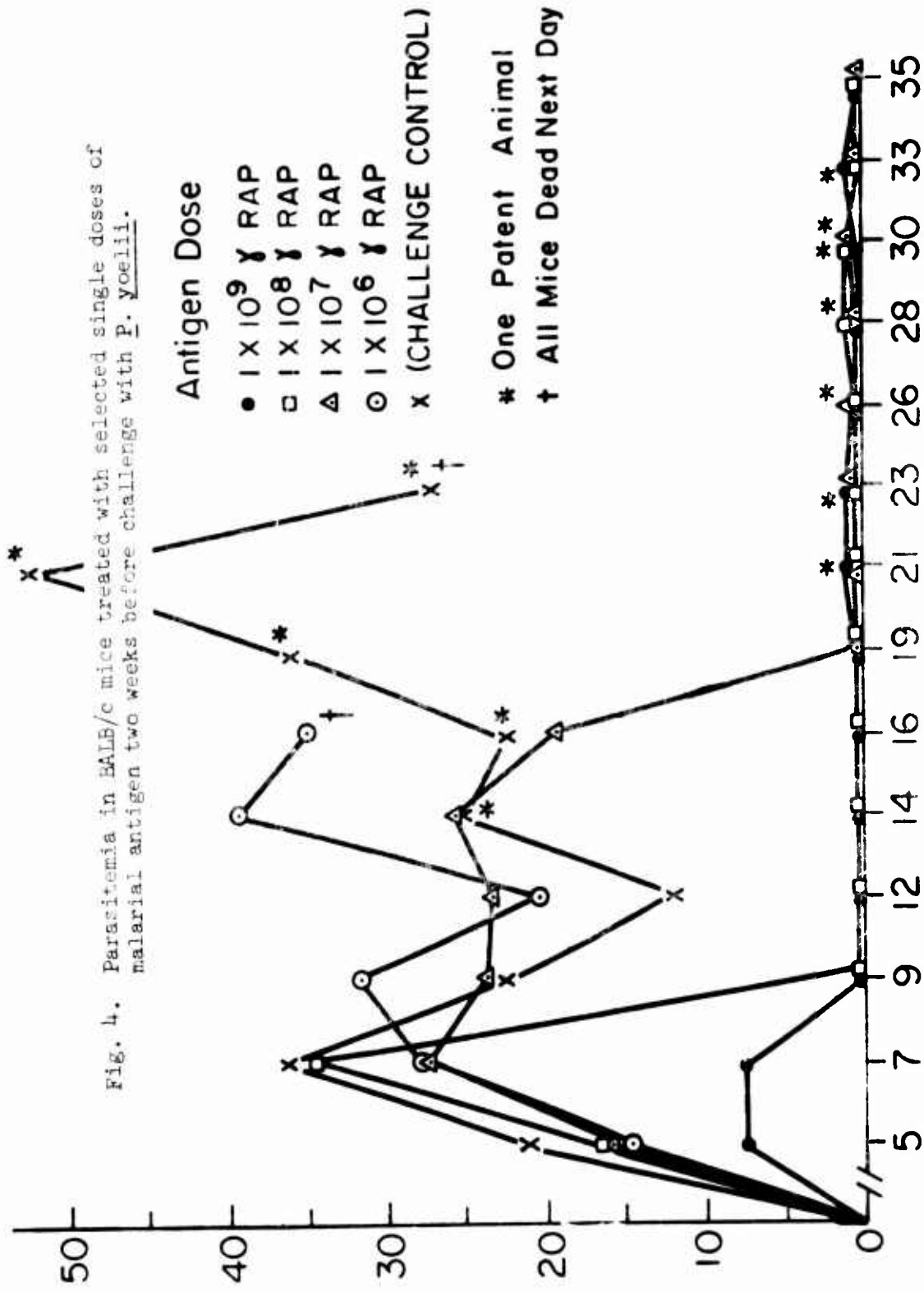


Fig. 4. Parasitemia in BALB/c mice treated with selected single doses of malarial antigen two weeks before challenge with *P. yoelii*.

behind the behavior of these two lines of parasites can only be speculated upon. Within the limits of the counting techniques employed, the same dose of parasites, and presumably of parasite antigen was given in both cases. However, it is possible that the stages of parasites in the two preparations were different, due to a differential predilection of the different blood forms for peripheral blood. This possibility was not examined. In addition the possibility that the RAP line does in fact contain a more immunogenic antigen must be considered. This being the case, the speculation that a connection between immunogenicity and virulence cannot be avoided. No information relevant to these possibilities is, however, presently available.

These experiments were made possible by the observation (Figure 1, Table I) that in our hands, in BALB/c mice, both the RAP and SLO lines of P. yoelii are fatal to all recipients in the absence of immunological or chemotherapeutic intervention. This observation suggests that BALB/c mice are relatively immunologically incompetent with respect to their ability to resist P. yoelii, since the SLO line is not lethal in other animals studied, to our knowledge, the SLO line is similar to the original 17X strain of the parasite. Because of the behavior of the RAP line in BALB/c's, a model is available in where there is 100 per cent mortality in unimmunized animals, and the ability to protect virtually all of the animals with a single dose of artificial immunogen. This model is very convenient for many types of immunological experiments.

Resistance to challenge appears to be influenced only slightly by changes in the dose of γ RAP over a relatively wide range of doses. Thus, most of the protection was obtained at 1×10^6 γ RAP in one experiment whereas little additional protection is obtained even with 1×10^8 γ RAP; 1×10^9 γ RAP are required for full induction of resistance (Figure 3, 4 and Tables III, IV). The requirement for such a large dose of cells suggests that the active fraction may be a very small fraction of the total material injected. It will be important to attempt to identify the fractions of the immunizing inoculum which do, in fact, contain the activity.

The kinetic studies of the induction of immunity to γ RAP indicate that at least a few days are required for induction of the response since simultaneous injection of the immunizing and the challenge dose resulted in suboptimal, although measurable, protection. Similarly, it would appear that once induced the protection lasts for at least three weeks (Figure 2 and Table II). It will be of interest to determine the longevity of the protection to see if it is similar to that observed in the P. berghei model. The model described in this report provides the most complete protection of mice against a lethal challenge with malaria parasite in the absence of previous active infection which has, to our knowledge, been thus far reported. We anticipate that it will be useful for further studies on the mechanism of immunity to rodent malaria parasites.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 324 Host Responses to Malaria

Literature Cited.

Publications:

1. Diggs, C. L., Joseph K., Flemmings, B., Snodgrass, R., and Hines, F.: Protein synthesis in vitro by cryopreserved Plasmodium falciparum. Am. J. Trop. Med. & Hyg. 24: 760-763, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ECT. ^c	6. WORK SECURITY ^d	7. REGARDING ^e	8. DES'N INSTR' ^f	9. SPECIFIC DATA CONTRACTOR ACCESS ^g	10. LEVEL OF SUM. A. WORK UNIT
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO / CODES ^h	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62759A	3A762759A829	00	336			
b. CONTRIBUTING							
X. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ⁱ							
(U) Field Studies on Drug Resistant Malaria							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^j							
003500 Clinical Medicine 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				b. RECEIVING		c. FUNDS (in thousands)	
b. NUMBER ^k				76		4.7	
c. TYPE				77		6	
d. KIND OF AWARD:				f. CUM. AMT.		512	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ^l : Walter Reed Army Institute of Research				NAME ^m : US Army Medical Component, SEATO			
ADDRESS ⁿ : Washington, DC 20012				ADDRESS ^o : Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)			
NAME ^p : JOY, R. J. T., COL				NAME ^q : BEACH, D. J., COL			
TELEPHONE: 202-576-3551				TELEPHONE: 2817776			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: DOBERSTYN, E. B., LTC			
				NAME: PEARLMAN, E. J., LTC			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Malaria; (U) Drug Resistance; (U) Chemotherapy; (U) Chemoprophylaxis; (U) Human; (U) Monkey; (U) Vectors; (U) Chloroquine; (U) Human Volunteer							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede rest of each with Security Classification Code)							
23. (U) To determine the efficacy of conventional and experimental antimalarial drugs in the treatment and prophylaxis of drug resistant falciparum malaria. To define vector bionomics which influence the transmission of drug resistant malaria and to develop rationale for vector control. To evaluate the activity of candidate antimalarial drugs against simian malaria. To characterize the clinical features of malaria in Thailand, and to develop improved methods of hospital management of diseases of military importance.							
24. (U) U.S. Army investigational antimalarial drugs were compared with standard drugs for treatment of drug resistant falciparum malaria in hospitalized human volunteers in Thailand. Chemoprophylactic studies were conducted in rural populations in central Thailand. Chemotherapeutic drugs were studied in rhesus monkeys with <u>P. cynomolgi</u> .							
25. (U) 75 07 - 76 06 WR 142490 (Mefloquine) in a single oral dose was more effective than Fansidar in the treatment of mildly and moderately ill patients with falciparum malaria. In combination with quinine it was 100% curative in patients of all degrees of clinical and parasitological severity. Seventy-one percent of patients treated with Fansidar developed gametocytes infective to vector mosquitoes. In studies of chemosuppressive agents in patients with falciparum malaria, a combination of sulfadoxin-pyrimethamine caused an eight fold reduction in the number of people with a parasitemia. A similar study of Mefloquine (WR 142490) is underway. The malaria drug testing program has used the rhesus monkey - <u>Plasmodium cynomolgi</u> system to test 20 drugs for schizonticidal and/or radical curative effects. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$10,00 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

PII Redacted

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

1112

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 336 Field studies on drug resistant malaria

Investigators

Principals: COL Douglas J. Beach, MSC; COL Anthony P. Hall, MC; LTC Edward B. Doberstyn, MC; LTC Eliot J. Pearlman, MC; MAJ Richard M. Lampe, MC; MAJ John L. Brown, VC; MAJ William T. Watson, VC; MAJ Charles F. Miller, MC; CPT Richard G. Andre, MSC; CPT Gary D. Phillips, MSC; MG Pung Phintuyothin, MC, RTA (Ret); Chul Karnchanachetanee, M.D.; Katchrinnee Pavanand, M.D.; Prayot Tanticharoenyos, DVM, Samran Samransamruajkit, M.D.;

Associate: Douglas J. Gould, Ph.D.; LTC David E. Davidson, Jr., VC; MAJ Garrett S. Dill, VC; MAJ Stephen C. Hembree, MSC; SFC Robert S. Kennedy; Visit Benjapongse, M.D.; Niphon Chuanak; Barnyen Permpanich; Prasit Sookto; Withoon Thiemanun

1. The Suppression of P. falciparum and P. vivax Parasitemias by a Sulfadoxine-Pyrimethamine Combination

OBJECTIVE: To study the effectiveness of the combination of sulfadoxine(s) 500 mg and pyrimethamine (Py) 25 mg given in two dose regimens in suppressing parasitemias in an area with known chloroquine resistant falciparum malaria.

BACKGROUND: The combination of a sulfone or sulfonamide with pyrimethamine in the chemosuppression of chloroquine resistant falciparum malaria has been previously shown to be efficacious. The longer half life of a long acting sulfonamide, such as sulfadoxine ($t_{1/2}$ =150-200 hrs.), should render this, in combination with a matched (in terms of $t_{1/2}$) dihydrofolic acid reductase, a better chemosuppressive agent.

DESCRIPTION: Seven hundred and fifty six semi-immune study subjects from four villages in Prachinburi Province, Northeast Thailand were assigned to one of five drug study groups. Subjects received, under a double blind design, one of the following medications:

- a. Sulfadoxine 1000 mg - pyrimethamine 50 mg biweekly
- b. Sulfadoxine 500 mg - pyrimethamine 25 mg biweekly
- c. Diformyldapsone 200 mg - pyrimethamine 12.5 weekly
- d. Diformyldapsone 400 mg - pyrimethamine 25 mg weekly
- e. Placebo weekly

Each study subject was visited weekly, at which time the medication was given and swallowed under supervision, a capillary blood drawn for a thick-thin malaria smear, and a history of illness since the prior visit noted. For those subjects receiving a biweekly medication regimen, placebo tablets were given on the alternate weeks; thus study subjects received two tablets weekly.

PROGRESS: Six hundred eighty-eight study subjects completed the 26 week trial (92%). Figure 1 shows that the weekly falciparum attack rates (based on a new asexual parasitemia) were lower during the medication phase of the trial in the four treatment groups compared with the placebo group. It can be further seen that an increased number of falciparum parasitemias was detected in the low dose DFD-Py group (weeks 7, 9 and 19); in the high dose DFD-Py group (week 10); and in the low dose S-Py group (week 10). Figure 2 shows the cumulative infection

FIGURE 1.
WEEKLY ATTACK RATE OF SUBJECTS INFECTED WITH *P. FALCIPARUM*
BY STUDY GROUP

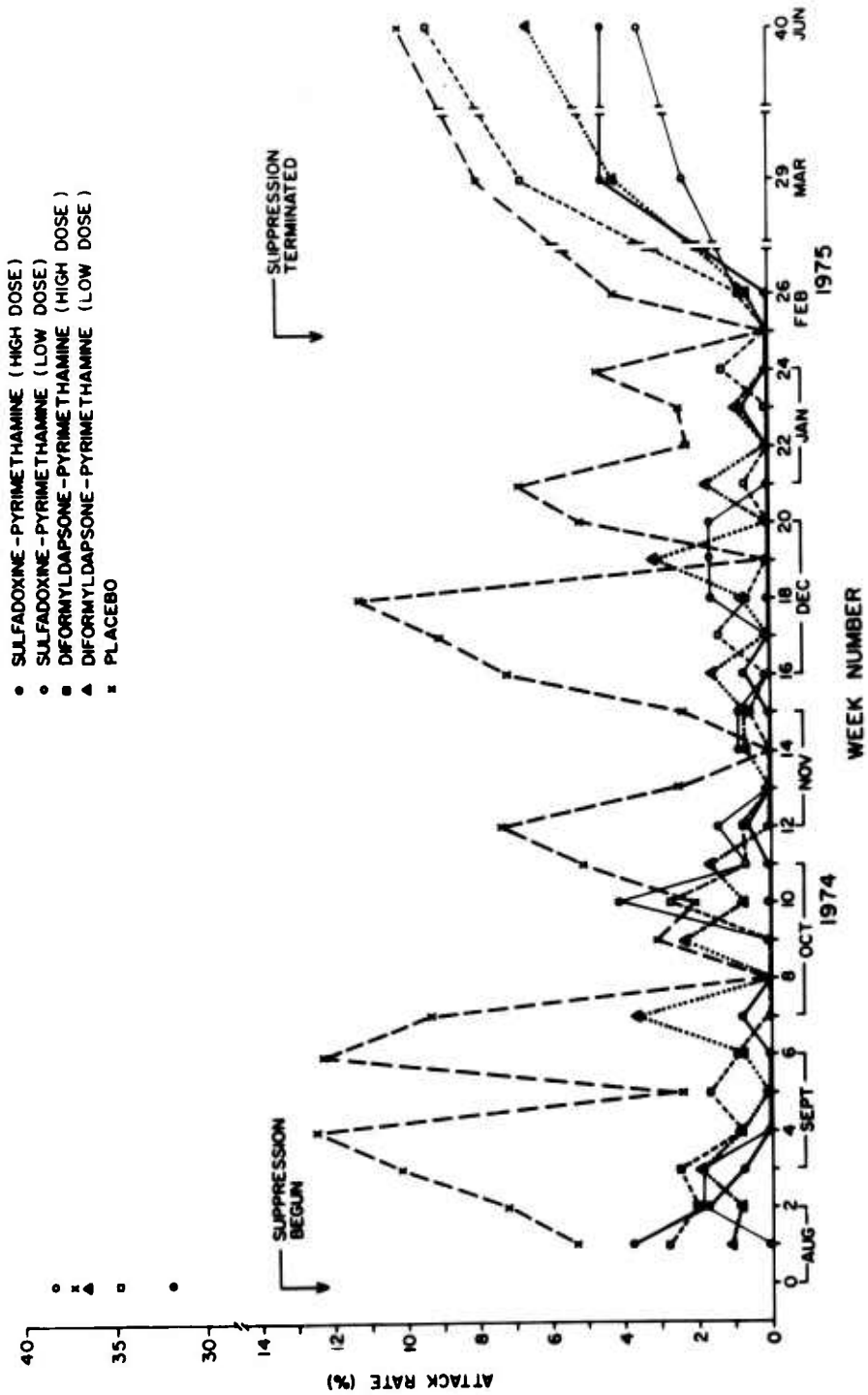
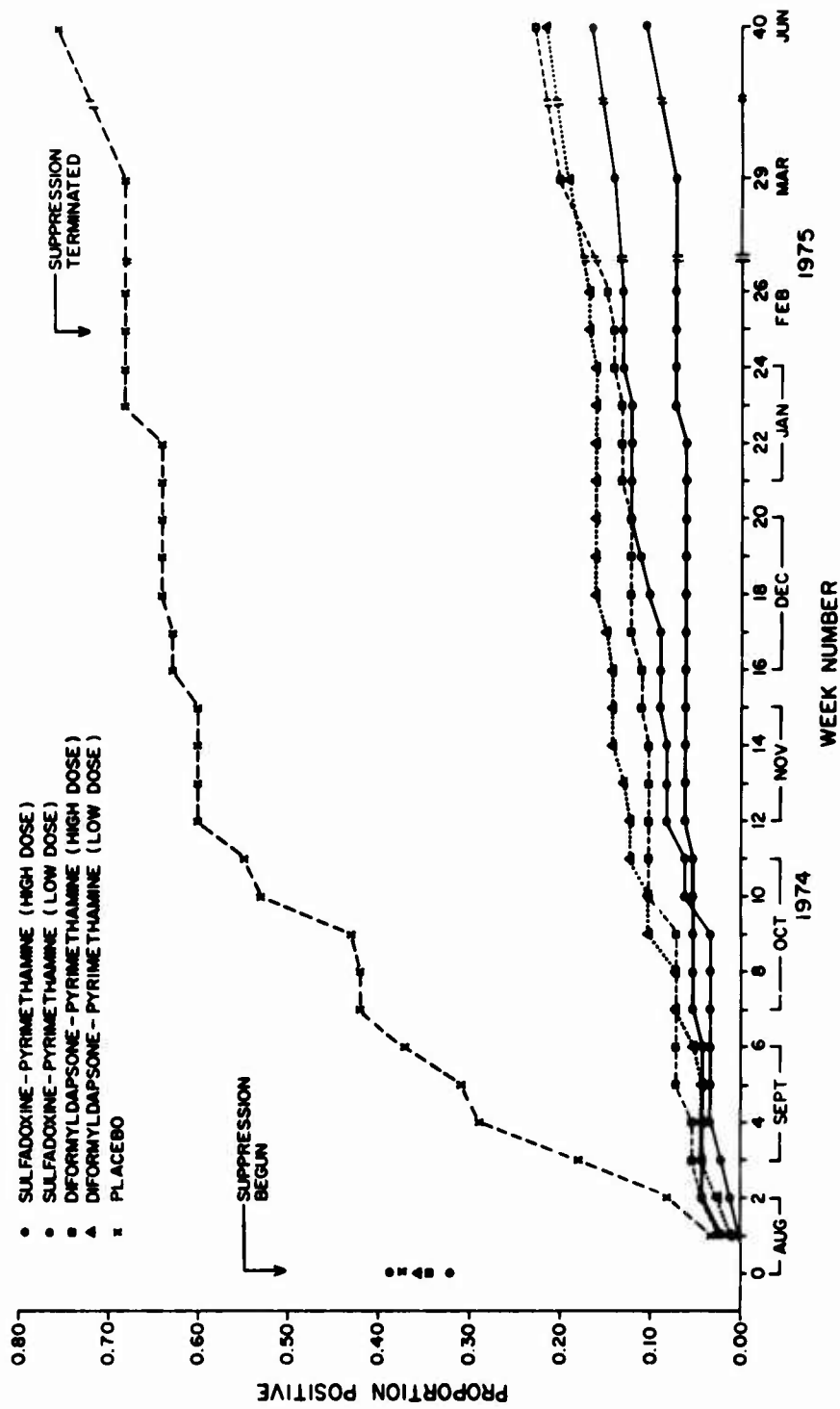


FIGURE 2.
 CUMULATIVE PROPORTION OF SUBJECTS INFECTED WITH *P. FALCIPARUM*
 BY STUDY GROUP



rates of individual study subjects in the course of the 26 week trial and in the subsequent follow-ups. The data indicate an 8-fold reduction in the cumulative parasitemic rate for P. falciparum in the high dose S-Py group, while a 4.4 fold reduction was noted in the high dose DFD-Py group when compared to the placebo group.

Statistical evaluation comparing the various drug regimens was undertaken. Highly significant results were obtained which showed that S(1000 mg)-Py(50 mg) given biweekly; S(500 mg)-Py(25 mg) given biweekly; DFD(400 mg)-Py(25 mg) given weekly; and DFD(200 mg)-Py(12.5 mg) given weekly were effective chemosuppressive agents against P. falciparum when compared to placebo alone ($p < 0.0005$). Furthermore, the higher dose S-Py regimen was more efficacious than the lower dose S-Py group, and more efficacious than either of the DFD-Py regimens in suppressing falciparum parasitemias. A direct comparison between the low dose S-Py group and the two DFD-Py groups failed to reveal any significant differences in efficacy. Table 1 summarizes these statistics.

Table 1. Statistical Evaluation Significance of Difference Between Two Sample Proportions (p value) for P. falciparum Suppression

	S-Py (l)	DFD-Py (h)	DFD-Py (l)	Placebo
S-Py(h)	2.9445 ($p < 0.002$)	3.7240 ($p < 0.0002$)	3.8595 ($p < 0.00015$)	18.2165 ($p < 0.0001$)
S-Py(l)	-	0.6038 ($0.25 < p < 0.30$)	1.1713 ($0.10 < p < 0.15$)	15.9411 ($p < 0.0001$)

(h) high dose

(l) low dose

We were able to identify one hundred seventy study subjects who participated in all three SEATO Medical Research Laboratory chemosuppressive studies. Of these 170, sixty seven individuals were known to have received DDS-Py in 1973; DFD-Py, DFD, or Dapsone-pyrimethamine (DDS-Py) in 1974; and to have received either S-Py or DFD-Py this year. Thus they received a sulfone preparation for a period of six months each year of the past two years, and subsequently received a sulfone or sulfonamide preparation for six months the next year. Analysis of this data was undertaken to discern any indication of increased susceptibility to a falciparum parasitemia among these individuals. When each of the current treatment groups was divided into two parts: those that received the prior sulfone drugs for two years and those that did not; it was found that there was no significant difference of chemosuppression between the groups. Further detailed analysis failed to discern any significant differences when the groups were compared as to which specific drug they received in the past (DDS-Py, DFD-Py, DFD) and which active medication this year.

Individuals (20) who received chloroquine in 1973, Py or Placebo in 1974, and DFD-Py or S-Py for the first time this year did not differ at all in suppression against falciparum parasitemias from those 67 study subjects described above.

With the cessation of chemosuppression the following number of new falciparum parasitemias were seen in the course of the two follow-up visits (weeks 29 and 40): 6 (at week 40) in the high dose S-Py group, 6 in the low dose S-Py group, 18 in the high dose DFD-Py group, 12 in low dose DFD-Py group, and 15 in the placebo group. Of these post-suppression falciparum parasitemias most (37 or 59) occurred at week 40. This would correspond to May, 1975 and the start of a new malaria transmission season.

This year we again had a large number of *P. vivax* parasitemias as shown in Table 2. The placebo group had a 55% cumulative vivax infection rate while the high dose S-Py and high dose DFD-Py had 19%. Both low dose groups were approximately equal with respect to numbers of study subjects infected. Statistical evaluation showed that all four major treatment groups were efficacious vis a vis placebo. However, the high dose S-Py and DFD-Py were both equally effective in preventing vivax parasitemias, and were superior to the lower dose S-Py and DFD-Py regimens. There was no difference in efficacy between the lower dose regimens. Table 3 summarizes this information.

Table 2. P. vivax Parasitemia Experienced by Study Subjects During Chemosuppression

Group	No. Subjects	No. (prop.) Infected
S(1000mg)-Py(50mg)	155	29(0.19)
S(500mg)-Py(25mg)	156	41(0.26)
DFD(400mg)-Py(25mg)	162	31(0.19)
DFD(200mg)-Py(12.5mg)	153	45(0.29)
Placebo	62	34(0.55)

Table 3. Statistical Evaluation Significance of Difference Between Two Sample Proportions (p value) for P. vivax Suppression

	S-Py (l)	DFD-Py (h)	DFD-Py (l)	Placebo
S-Py (h)	2.9445 (0.0016<p<.002)	0.4763 (0.30<p<0.35)	4.6588 (p<0.0005)	13.1462 (p<0.0005)
S-Py (l)	-	2.5383* (0.005<p<0.01)	1.5935 (0.05<p<0.10)	10.3316 (p<0.0005)

* In favor of DFD-Py (h).

(h) high dose

(l) low dose

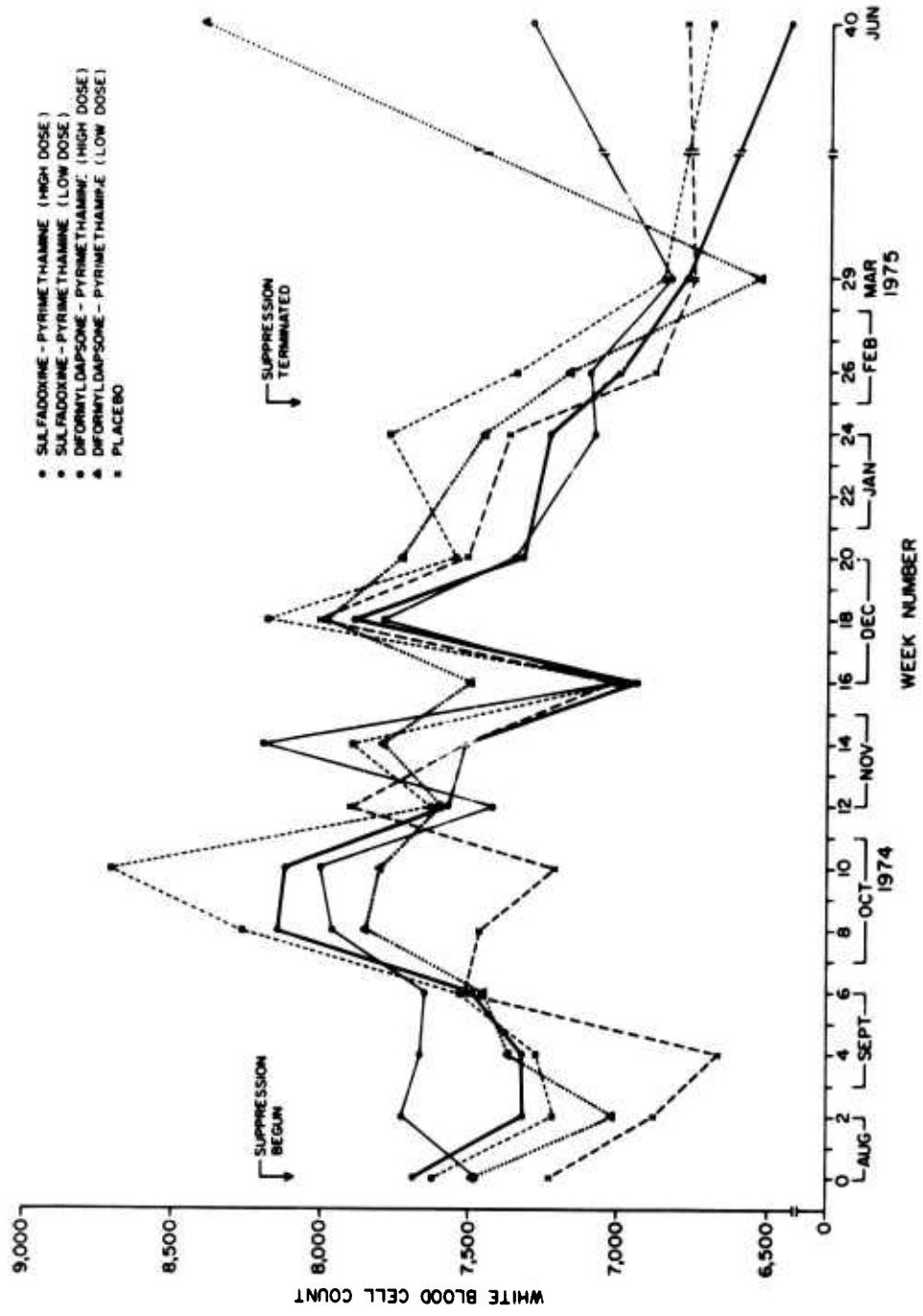
Following completion of the chemosuppressive phase of the study there was an increase in the number of primary vivax parasitemias. These numbered as follows: S-Py (high dose) group - 27; S-Py (low dose) group - 19; DFD-Py (high dose) group - 34; DFD-Py (low dose) group - 18; and placebo group - none. Thus the increase in the cumulative values for vivax parasitemias are for the S-Py (high dose) group from 0.19 to 0.36; for the S-Py (low dose) group from 0.26 to 0.38; for the DFD-Py (high dose) group from 0.19 to 0.40; for the DFD-Py (low dose) group from 0.29 to 0.41; and there was no change in the placebo group.

There were no reported episodes of drug related disease from the 747 study participants who started the study. All study subjects who finished the study were also seen during the follow-up period. Those with parasitemias were treated. Study subjects with a falciparum parasitemia were given a therapeutic dose of sulfadoxine-pyrimethamine, while those with a vivax parasitemia were treated with the standard regimen using chloroquine. Primaquine was given only to those study subjects known to be G-6-PD normal.

Hematocrits were monitored biweekly for evidence of hemolysis secondary to a G-6-PD deficiency. Of major concern were the two DFD-Py groups. There were 26 instances where individuals experienced falling hematocrits. These episodes were evenly distributed among the five treatment groups. In most instances repeat hematocrits a week later pointed to a probable laboratory error. Paired information using hematocrits at week 0 and week 26 were statistically analyzed. The study subjects as a whole in each of the four major treatment groups showed a significant increase in the value of their hematocrit over the twenty-six week trial period. For the DFD-Py high and low dose groups the rises (0.7% and 1.0%) were significant at the 5% level, while for the S-Py high and low dose groups the rises (1.46% and 2.98%) were significant at the 1% level. The result for the placebo group showed a slight increase in the average hematocrit value (0.52%), which was not significant. Evaluation of hematocrits of males with G-6-PD deficiency in the high dose DFD-Py regimen (18 out of 133 males) showed that they too experienced an increase in their hematocrits (2.2%) which was significant at the 2% level.

Likewise all study subjects were monitored for leucopenia. At the start and during the trial there were 240 instances of a leucopenia (defined as < 4000 cells/ml). These episodes of leucopenia were distributed evenly among all five treatment groups, and there was no bias detected ($\chi^2=3.5103$; $0.40 < p < 0.50$). Statistical evaluation

FIGURE 3
EFFECT OF TREATMENT ON WHITE BLOOD CELL COUNTS



was undertaken to detect any significant differences in WBC determinations following a six month ingestion of sulfonamides or sulfones using paired data from weeks 0 and 26. While all groups experienced a decrease in WBC counts, only the S-Py (high dose) group showed a significant decrease (See Figure 3). This amounted to an average decrease of from 7645 WBC/ml at the start to 6850 WBC/ml at the end of the trial ($p < 0.05$).

Follow-up visits to the local health center revealed only four study subjects who received antimalarial therapy (3 received sulfadoxine-pyrimethamine, one received oxytetracycline) from other sources. Weekly smears taken from these four study subjects while under outside therapy were not included in the results.

From the point of view of drug tolerance there seemed to be no instances of untoward reactions, and this agrees with the experience of others. There was no evidence of marked leucopenia except for a lowering of the WBC in the high dose S-Py group. Values for hematocrits in all major treatment groups were increased possibly due to supplementary hematinics given (FeSO_4 , vitamins) to all study subjects at the time of tablet ingestion and blood smearing.

SUMMARY: Sulfadoxine (1000 mg)-pyrimethamine (50 mg) given bi-weekly was shown to be an effective chemosuppressive against both falciparum and vivax parasitemias, causing an eight fold reduction in falciparum parasitemias, and an approximately three fold reduction in vivax parasitemias. While the low dose S-Py group and the two DFD-Py groups were less effective than the high dose S-Py group in suppressing falciparum parasitemias, the high dose DFD-Py combination was as effective as the high dose S-Py combination in suppressing vivax parasitemias.

2. Evaluation of the Sporonticidal Effect of Pyrimethamine-Sulfadoxine, Quinine, and Quinine-Pyrimethamine against P. falciparum

OBJECTIVE: To determine the effect of three therapeutic regimens for malaria upon the subsequent development of sporogonic forms of P. falciparum in mosquitoes fed on infected patients.

BACKGROUND: Fansidar, the fixed combination of pyrimethamine and sulfadoxine, has been shown to be effective against the asexual

forms of chloroquine resistant and chloroquine sensitive strains of P. falciparum as well as P. vivax malaria in many parts of the world. It is currently used in Thailand as an alternative regimen by the clinics of the National Malaria Eradication Project and is widely used in hospitals and clinics throughout the country. A number of studies have proven its effectiveness as a schizonticide, but it has not been thoroughly evaluated as a gametocytocide and sporonticide. Chin and Rattanaarithikul (1) reported that 47% of A. balabacensis fed on gametocytemic patients following single-dose therapy developed sporozoites. Serum levels of the drug in the donor patients at the time of the mosquito feedings were not reported by these authors.

Quinine has always been considered to have no effect on gametocytes or upon the development of P. falciparum in the mosquito host. In the past, pyrimethamine was thought to effectively interrupt the sporogonic cycle, however, since resistance of asexual forms of the parasite to pyrimethamine is common in the region, it is reasonable to expect that the drug's effect as a sporonticide has diminished.

In view of the widespread use of Fansidar in the therapy of malaria in Southeast Asia and its emergence as a drug of choice for chloroquine-resistant malaria in many parts of the world, the effect of this drug upon the infectivity of gametocytes needs clarification. Epidemiologically, it is essential to be aware of the need for the additional use of a sporonticidal drug such as primaquine in combination therapy.

DESCRIPTION: Patients presenting to the outpatient clinics of the National Malaria Eradication Project and the district hospital in Phra Phutthabat, who were at least 15 years of age and had blood smears positive for P. falciparum, were considered eligible for the study. Eligible patients who volunteered were assigned to one of three treatment groups; Group A: Patients were treated with a single dose of Fansidar, two tablets (total 50 mg. pyrimethamine and 1.0 mg. sulfadoxine); Group B: Patients were treated with quinine, 650 mg. every eight hours for six days; Group C: Patients received quinine as in Group B, above, but in addition, were given pyrimethamine 50 mg. daily for the first three days. Fansidar, either alone or in combination with quinine, is a standard therapy for P. falciparum used in the outpatient clinic and on the wards in Phra Phutthabat hospital and in the clinic operated by the National Malaria Eradication Project.

Medications were administered by the nursing staff under the supervision of the study physicians. At the conclusion of the 21-day study period patients from Group B and C were given two tablets of Fansidar.

Parasite counts were performed and blood drawn for pyrimethamine and sulfadoxine levels before treatment was begun on day 0, daily in hospital, and on days 5, 10, 15 and 20 after treatment. Mosquito feeds were performed on days 0, 5, 10, 15 and 20, using previously unfed A. balabacensis from the colony of the SEATO Medical Research Laboratory Phra Phuttabat insectary. Patients were asked to return to the laboratory for follow-up. If necessary, they were followed at home. Ten percent of the mosquitoes fed on the patients were dissected six to ten days after feeding, and all surviving mosquitoes were dissected on day 15, regardless of the results of the previous dissection.

Serum levels of the antimalarial drugs are being determined at the biochemistry laboratory of the SEATO Medical Research Laboratory.

PROGRESS: One hundred and three patients were initially admitted to the study, but 23 of these were not included in the final tabulation of the data. Patients were eliminated for the following reasons:

1. No gametocytes at any time during their course (11 patients)
2. No gametocytes after day 0 (3 patients)
3. Change in therapeutic regimen (1 patient)
4. No follow-up after day 0 (13 patients)

A total of seventy-five patients was included in analysis of the data. The Fansidar treatment group comprised 35 patients, the quinine group 28, and the quinine-pyrimethamine group 12. Choice of therapy was not random, but was often dictated by the clinical condition of the patient. Patients with severe infections were not considered eligible for single-dose oral therapy, and were treated with quinine instead. This fact is reflected in the higher average asexual parasite count in the quinine and quinine-pyrimethamine groups (See Table 1).

Gametocytemia on admission was similar for the three groups, however, the Fansidar-treated group subsequently developed much higher levels. Whether this phenomenon was due to stimulation by Fansidar or was the result of suppression by quinine is not clear. (Figure 1).

Table 1. Gametocytemia

Drug	Day	No. Pts.	Parasite Counts Mean (S.D.)	
			Asexual	Sexual
FANSIDAR	0	35	12622(17562)	234 (365)
	5	35		644 (1067)
	10	31		802 (981)
	15	28		326 (387)
	20	22		120 (156)
QUININE	0	28	78387(125932)	225 (451)
	5	28		211 (252)
	10	25		72 (117)
	15	18		24 (100)
	20	15		6 (8)
QUININE-PYRIMETHAMINE	0	12	58426(104365)	440 (663)
	5	12		526 (896)
	10	12		227 (385)
	15	11		52 (100)
	20	6		13 (23)

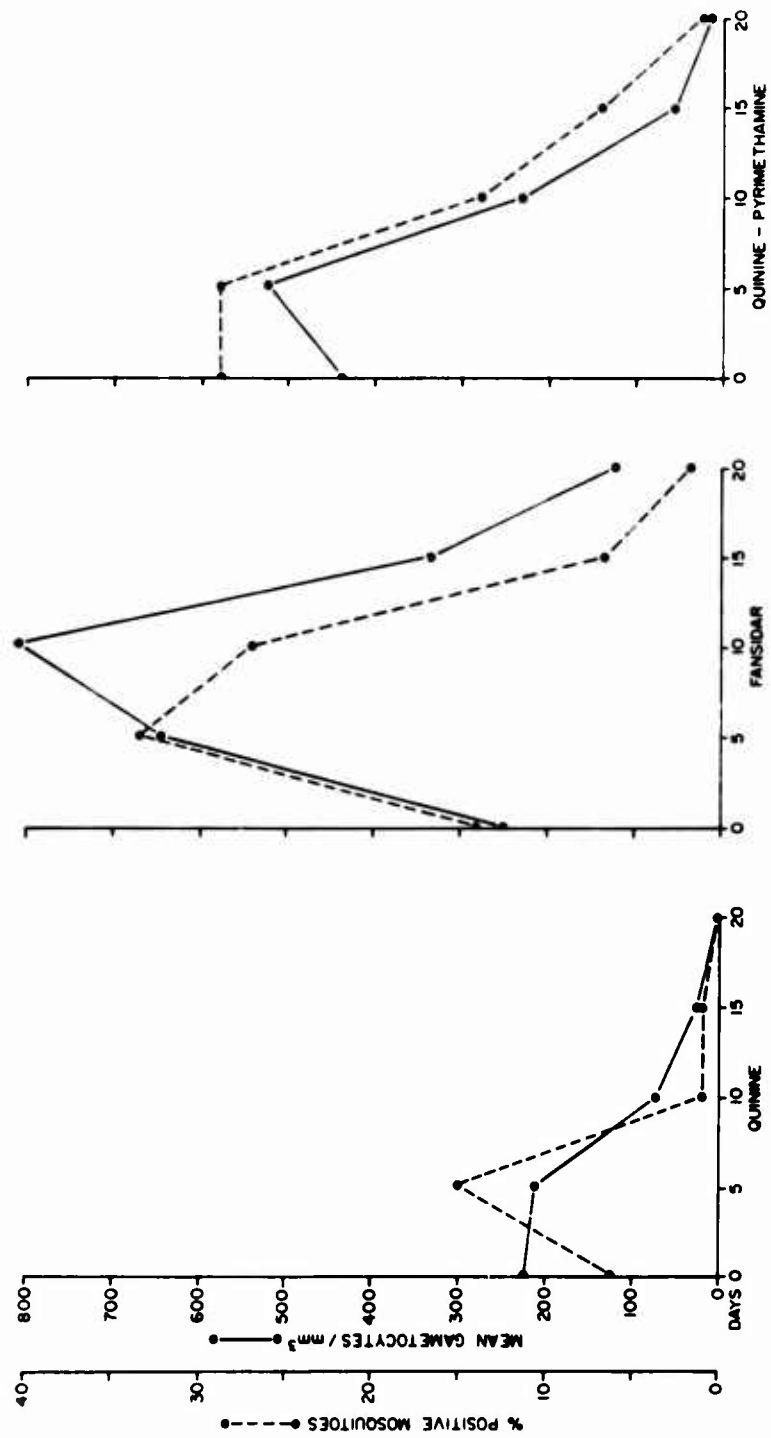


FIGURE 1. GAMETOCYTEMIA AND POSITIVE MOSQUITO FEEDS

Table 2. Mosquito Feeding and Dissection Data

Day	Engorgement (%)	Survival (%)	Positive Mosquito Feeds (%)	
			Lots	Individuals
Fansidar				
0	74	57	39	14
5	61	50	55	33
10	63	55	53	27
15	57	60	23	7
20	<u>55</u>	<u>64</u>	<u>10</u>	<u>2</u>
Mean(S.D.)	62 (7.4)	57 (5.3)	36(19.4)	17(13.1)
Quinine				
0	68	68	15	6
5	50	63	32	15
10	62	67	5	1
15	56	69	22	1
20	<u>62</u>	<u>70</u>	<u>0</u>	<u>0</u>
Mean(S.D.)	52(29.7)	67 (2.7)	15(12.9)	5 (6.3)
Quinine-Pyrimethamine				
0	62	80	43	29
5	47	75	45	29
10	56	71	27	14
15	83	79	17	7
20	<u>94</u>	<u>71</u>	<u>33</u>	<u>1</u>
Mean(S.D.)	68(19.5)	75 (4.3)	33(11.6)	16(12.7)

Table 3. Fansidar Sporonticidal Study Mean Oocyst Diameters
Seven Days Post-Feeding

Drug	Day Post-Treatment	Mean Diameter in Microns	
		Minimum (S.D.)	Maximum (S.D.)
None	0	26 (5.1)	37 (4.6)
Fansidar	5	29 (10.0)	39 (11.6)
Fansidar	10	26 (2.7)	37 (6.4)
Fansidar	15	27 (0)	35 (2.3)
Quinine	5	18 (0)	29 (2.7)
Quinine	10	-	38 (0)

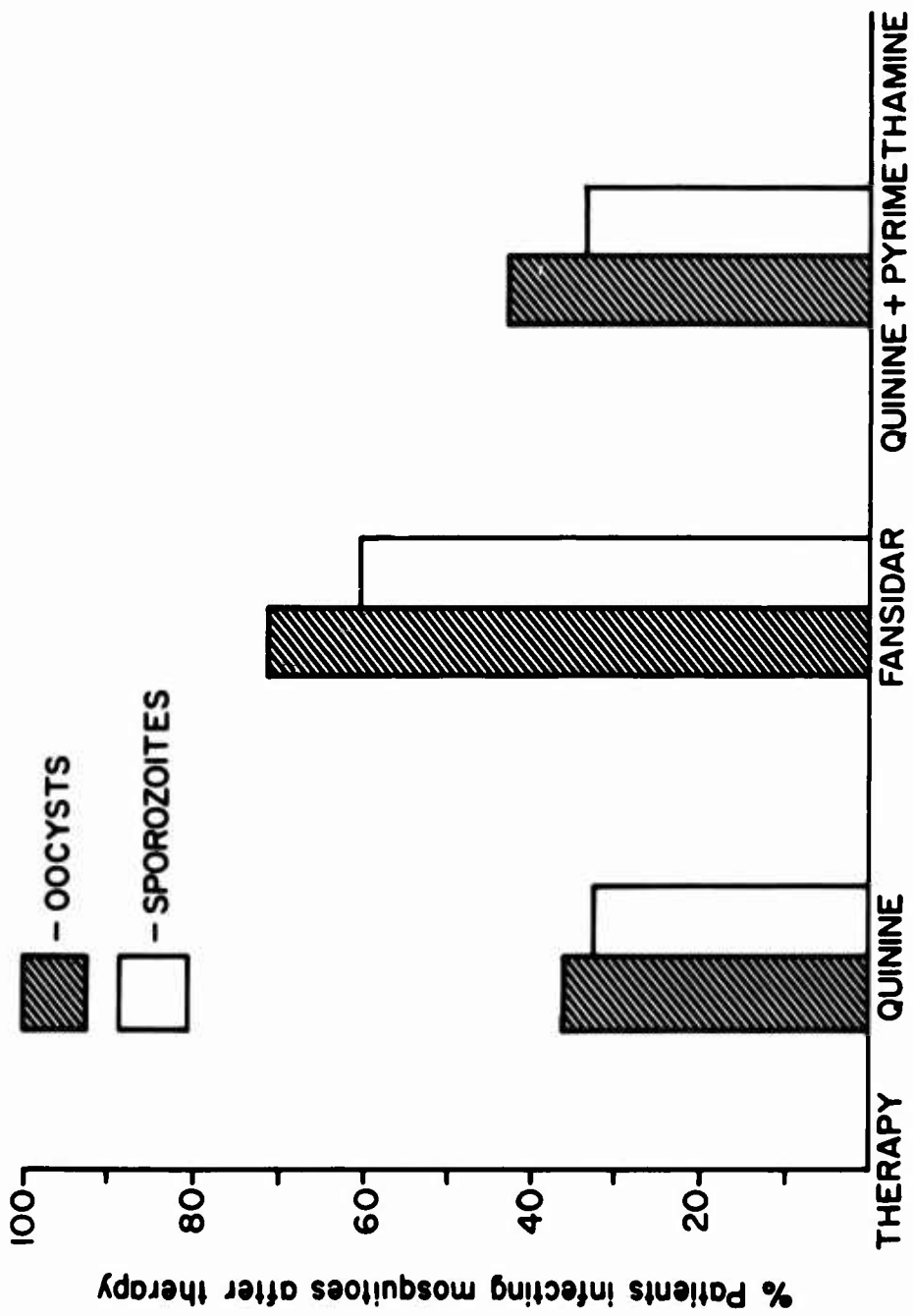


FIGURE 2. MOSQUITOES INFECTED AFTER FEEDING ON TREATED PATIENTS

Positivity of mosquito dissections seems to have been related to gametocyte levels, and when the average gametocyte counts were compared with percentages of mosquitoes showing development of parasites, the relationships were similar for all three groups (Figure 1).

Over 12,800 mosquitoes were allowed to feed upon the 75 study patients. Of the 60% of A. balabacensis that engorged on these patients, 4,880 survived and were dissected and examined for malaria parasites (Table 2). Fourteen percent of the dissected mosquitoes were found to be positive. Measurements of oocyst diameters were made to determine if normal parasite development took place. Guts of mosquitoes fed on patients before treatment or on days 5, 10, 15 or 20 following treatment were examined. Mosquitoes that fed on Day 5 on Fansidar-treated patients had oocysts that were larger than those seen in mosquitoes that fed on quinine-treated patients on the same day (Table 3). Also of interest is the fact that these oocyst diameters are larger than those reported by Coatney et al. (1971) in A. freeborni and A. quadrimaculatus mosquitoes. More measurements are required to determine if this apparent difference of growth rate is significant or the result of inadequate sampling. Sporozoites were not found in all lots of mosquitoes positive for oocysts. Thus, development to the sporozoite stage occurred in 21/25 lots fed on Fansidar-treated patients as compared with 9/10 lots fed on quinine-treated patients and 4/5 lots fed on quinine-pyrimethamine treated patients (Figure 2). Sporozoites, when seen, appeared to be normal in morphology; however, it is not known whether they were infective.

Determinations of serum levels of quinine, pyrimethamine, and sulfadoxine are not complete, but correlation of these data will be included in the final analysis of these results.

3. Mefloquine and Fansidar Alone and in Combination with Quinine for the Treatment of Falciparum Malaria

OBJECTIVE: To determine the efficacy of mefloquine in naturally-acquired falciparum malaria.

BACKGROUND: Mefloquine hydrochloride is an analogue of quinine and of WR 30090, an antimalarial which was developed during the Viet Nam war and was used effectively in the treatment of military personnel with falciparum malaria unresponsive to other drugs. It is a 4-quinolinemethanol containing a 2-trifluoromethyl group which blocks ring oxidation at that point, a major site of enzymatic inactivation of quinine.

Fansidar, a 20:1 combination of sulfadoxine and pyrimethamine has been used widely for the single-dose therapy of falciparum malaria, and has been shown to be 85% and 82% successful in SEATO Medical Research Laboratory studies in Trat and Prachinburi provinces respectively.

Recent SEATO Medical Research Laboratory studies have shown the most effective therapeutic regimen for drug resistant malaria in Thailand to consist of a short course of quinine followed by a single oral dose of Fansidar. Four doses of quinine, 10 grains every eight hours, followed by a single dose of Fansidar produced a cure rate of 96% (2).

DESCRIPTION: The study was opened at the Chao Phya Abhai Phu Bejr Hospital (Prachinburi Provincial Hospital). In the early phase of the study, only patients with clinically mild and moderate infections were considered eligible. Male volunteers over 15 years of age with P. falciparum asexual parasitemias of more than 1,000/cu.mm., were admitted to the study and assigned randomly to single dose oral therapy with either Fansidar, three tablets (total 75 mg. pyrimethamine and 1.5 gm. sulfadoxine) or mefloquine, six tablets (1.5 gm).

Parasite counts were performed twice daily in the hospital and on days 14, 21 and 28 following therapy, at follow-up visits. Hematocrit, WBC count, urinalysis and liver function studies were also performed.

In the later phase of the study, patients of all degrees of severity were admitted to the study; they were treated with quinine, administered either orally or intravenously, 10 grains every eight or twelve hours, followed by a single dose of either mefloquine or Fansidar when their clinical condition had improved.

Therapeutic response was judged by a modification of the WHO criteria (3). An RIII response was diagnosed if the patient's clinical condition and/or parasitemia failed to improve or worsened within a few hours after administration of the test medication. An RII response indicated reduction but not elimination of asexual parasites following therapy, and an RI response was arbitrarily diagnosed if asexual parasites reappeared on or before the 28th day following the initiation of therapy, whether or not the patient had returned to an endemic area.

In some patients, it was later decided that an RIII response was diagnosed prematurely, and that the test regimen may have proven

Table 1. Therapy of P. falciparum Malaria, Prachinburi 1975

Regimen	Total Patients Studied	Initial Parasite Count (Average/Range)	Response*					Total Patients Followed-up	Cure-Rate
			RIII	RII	RI	S			
Quinine: Mefloquine	40	58,000 (1,700-750,000)	0	0	0	35	35	100% (35/35)	
Mefloquine	32	47,000 (1,440-177,000)	0	0	1	29	30	97% (29/30)	
Quinine: Fansidar	44	56,000 (1,100-656,000)	0	0	3	36	39	92% (36/39)	
Fansidar	36	42,000 (1,200-134,000)	1	3	2	24	30	80% (24/30)	

* RIII, no marked reduction of asexual parasitemia;
 RII, marked reduction of asexual parasitemia, but no clearance;
 RI, clearance of asexual parasitemia, followed by recrudescence;
 S, clearance of asexual parasitemia, without recrudescence (radical cure).

Table 2

Average Parasite and Fever Clearance Times
for Mefloquine and Fansidar^R

	Number Patients	Average Parasite Count	Parasite Clearance Time in Hours (Average/Range)	Average Initial Temperature (°C)	Fever Clearance Time in Hours (Average/Range)
Mefloquine	32	47,000	66* (19-116)	39.4	48** (10-104)
Fansidar	36	42,000	76* (42-135)	39.5	61** (9-114)

* $t = 1.06, p > 0.1$

** $t = 1.11, p > 0.1$

effective on its own. These cases were classified as undetermined results.

An S response (radical cure) was diagnosed in patients in whom asexual parasitemia cleared promptly and did not reappear during the 28-day follow-up period.

PROGRESS: Fansidar - Thirty-six patients were treated with Fansidar (Table 1). All patients received three tablets except one boy weighing 31 kg., who received two. In four patients, RII or RIII early failures were diagnosed, and quinine was added. In two additional patients, increases in parasitemia occurred, but by the time quinine was added, improvement in the clinical picture and fever had already occurred. It was considered that quinine was given prematurely, and treatment failures were therefore not diagnosed. Two patients responded in hospital, but recrudesced before the end of the 28-day follow-up period, and were considered RI failures. Six patients did not complete follow-up. The overall cure rate was 80%. Parasite and fever clearances were 76 and 61 hours respectively (Table 2).

Mefloquine - Thirty-two patients received a single oral dose of mefloquine. Four patients weighing between 30 and 40 kg. received 1.25 gm.; the others were treated with 1.5 gm. One patient did not complete follow-up; one patient had a recrudescence on the 24th day following treatment, after having returned to an endemic area; and one patient was given quinine seven hours after the oral dose of mefloquine because of an increasing asexual parasite count. In retrospect, this last patient probably should not have been a candidate for oral therapy on admission because of the symptom combination of borderline hypotension, lack of fever, and prostration, the complex known as "algid malaria." Drug absorption in such a patient would be expected to be slow, and effective blood levels of the drug difficult to obtain. It is likely that the drug was eventually absorbed since the patient went on to complete recovery following only three doses of quinine. Eliminating this last patient from consideration, mefloquine produced a 97% cure rate. Parasite and fever clearance times were 66 and 48 hours respectively.

Quinine-Fansidar - Forty-four patients were treated with from one to eleven doses of quinine followed by a single dose of Fansidar (average 4.7 doses of quinine). Three patients developed RI recrudescences, making the overall cure rate 92% (36 of 39 patients followed up).

Quinine-Mefloquine - Forty patients were treated with quinine, one to eleven doses (average 4.3) followed by a single oral dose of mefloquine. Complete follow-up was achieved in 35 patients, all of whom were radically cured.

Gastrointestinal side-effects were most often seen in patients receiving the quinine-mefloquine regimen. This is not surprising, since the drugs are similar and would be expected to produce additive toxicity. Mefloquine alone was occasionally responsible for protracted vomiting and diarrhea. These side-effects were controlled with symptomatic medication and none considered severe. Fansidar alone was not associated with any drug-related side effects. Quinine-Fansidar patients showed only side effects related to quinine therapy; tinnitus, dizziness and nausea. (There were no side effects attributable to Fansidar).

A method of determination of serum levels of mefloquine has recently been developed and specimens from this study are currently being analyzed. In view of the relatively small numbers of patients who received single oral doses of mefloquine and Fansidar, the study is being continued at the Phra Phuttabat Hospital, Saraburi Province.

4. Evaluation of Experimental Antimalaria Drugs in Rhesus Monkeys Infected with *P. cynomolgi*

OBJECTIVE: To evaluate the effectiveness of selected experimental drugs against *P. cynomolgi* malaria in rhesus monkeys. These studies are coordinated by the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, and the results are used to aid in selection of more effective antimalarial drugs for human use.

BACKGROUND: This is a continuation of studies initiated in 1971. A chronological report of methodology and results are available in SEATO Medical Research Laboratory Annual Reports for 1971 through 1975. During 1975-76 work was oriented toward perfection of a sporozoite induced test system for evaluation of radical curative drugs.

DESCRIPTION: Blood Schizonticidal Tests: Experimental drugs are evaluated in rhesus monkeys (*Macaca mulatta*), which are infected by intravenous administration of 5×10^8 parasitized erythrocytes obtained from donor monkeys infected with *P. cynomolgi* strain B. Post-inoculation day 4, test drugs were administered orally (by gastric intubation) for seven days.

Table 1. Summary of Blood Schizonticidal Tests in Rhesus Monkeys

Type of Compound	WRAIR Drug Number	Minimum Curative Dose (mg/kg/day)
Pyridine methanol	180409 AC	10.0
	180409 AD	10.0
	180117	10.0
Phenanthrene methanol	33063	* NC (13.6)
2,4-Diamino quinazoline	150012	3.16
Quinoline methanols	142490	31.6
	199426	10.0
Miscellaneous	151136	* NC (100.0)
	38839	3.16
	194905	* NC (31.6)
	99210	31.6

* Not curative. The compound had suppressive activity but did not cure at the maximum dose tested. The maximum tested dose is indicated in parentheses.

Table 2. Blood Induced Schizonticidal Tests in Rhesus Monkeys
Summary of Combination Studies

WRAIR Drug Number	Combination Ratio	Minimum Curative Dose (mg/kg/day)	Number of Monkeys (total cured/total treated)
158122+180872	1:3.2	0.33:1.05	2/2
2978+6798	1:16	0.005:0.08	1/2
4629+5949	1:1.5	10:15	1/2
2978+448	1:8	0.04:0.32	2/2

Table 3. Summary of Radical Curative Tests in Rhesus Monkeys
Sporozoite Induced Tests

Type of Compound	WRAIR Drug Number	Minimum Curative Dose** (with 4.0 mg/kg/day of chloroquine phosphate (mg/kg/day))
8-Aminoquinoline	4234	* NC (10.0)
	106147	3.16
	182232	3.16
	183489	3.16
	211820	10.0
Miscellaneous	14262	* NC (10.0)
	222249	* NC (10.0)
	223660	* NC (10.0)

* Not curative. The compound did not cure at the maximum dose tested. The maximum tested dose is indicated in parentheses.

** Administered with 4.0 mg/kg/day of chloroquine phosphate

Suppression of parasitemia was indicative of blood schizonticidal activity, and post-treatment day 20, splenectomy was performed on monkeys in which there was no evidence of parasitemia. Splenectomized monkeys which were continuously negative for malarial parasites through post-treatment day 50 were considered cured.

Sporozoite Induced Radical Curative Tests: A. balabacensis mosquitoes were used for production of sporozoites. A rhesus monkey, inoculated with P. cynomolgi strain B. approximately one month prior to the anticipated mosquito feed, was used as a donor. Splenectomy was performed to assure a rising parasitemia at the time of mosquito feed. Once the parasitemia reached 10^4 parasitized erythrocytes per cmm, but prior to the period of descending parasitemia, three separate batches of mosquitoes were fed on consecutive days.

Post-feed day 6, mosquitoes were examined for gut oocysts. Twenty to 80 oocysts per gut was considered optimum for sporozoite development. Absence of oocysts was indicative of an unsuccessful mosquito feed. Post-feed day 14, sporozoites were harvested and diluted to contain $5-20 \times 10^5$ sporozoites per ml, (approximately 15 mosquito salivary gland pairs per ml). Each test monkey was inoculated intravenously with one ml of the sporozoite suspension.

Parasitemia normally developed in eight days, and once there were $5-25 \times 10^3$ parasites per cmm, drugs were administered orally for seven days. Chloroquine phosphate (WR1544) at 4.0 mg/kg/day was given simultaneously with all test drugs by the same route. Blood was examined for malarial parasites daily for 12 days, then every two days thereafter. Monkeys which remained negative through post-treatment day 20 were splenectomized and observed for an additional 33 days. If parasites were absent through post-treatment day 53 the drug was considered curative.

RESULTS: Blood Schizonticidal Tests: A total of 12 experimental drugs were evaluated for blood schizonticidal activity. Minimum curative doses are indicated in Table 1. Combination studies were conducted to meet special drug development requirements. Four sets of drug combinations were tested and the initial results shown in Table 2 suggest that there is synergistic action.

Sporozoite Induced Radical Curative Tests: Development of sporozoites following engorgement of mosquitoes on splenectomized donor monkeys was not consistent. In order to obtain a more reliable system for mass sporozoite production, plans were initiated to utilize intact donor monkeys for future experiments. A total of eight experimental drugs were evaluated for radical curative properties. A minimum curative dose for each is indicated in Table 3.

SUMMARY: Rhesus monkeys infected with trophozoite induced P. cynomolgi strain B. were used to evaluate twelve single drugs and four drug combinations for blood schizonticidal activity. A sporozoite induced test system was used to evaluate eight drugs for radical curative activity.

5. In vitro Gametogony of P. falciparum

OBJECTIVE: To establish an in vitro technique for the production and maintenance of sexual erythrocytic forms of P. falciparum.

BACKGROUND: Despite extensive studies of gametocytes the gametogony of plasmodial species is still not fully known. To date no conclusion has been made on the origin of gametocytes regarding whether they arise from special merozoites or if a particular stimuli is required to trigger their development.

In human infections mature gametocytes appear in peripheral circulation, with the developing stages being confined to the bone marrow. Only during severe infections do immature forms appear in peripheral smears. Although these sexual parasites do not cause clinical symptoms, their existence could prevent the eradication of malaria in a particular area. Some anti-malarials utilized in treatment affect the crescent wave, where gametocyte numbers are not suppressed during treatment but are increased (4). Gametocidal effects of various drugs therefore need to be tested. An in vitro test system would greatly facilitate determination of these effects.

PROGRESS: In previous studies a technique for the in vitro culture of erythrocytic asexual P. falciparum parasites was developed (5). The culture technique proved to be well suited for studies of plasmodial response to different antimalarials both morphologically and radiochemically (6). Studies of sexual parasites in vitro are now being performed utilizing a modification of this technique. During the culture period,

nutrients for the growing parasites are replenished and their metabolites are removed at various intervals. Parasites have been maintained in culture up to 228 hours using this procedure. Bacterial contamination has prevented a longer culture period. Infected blood specimens for culture were obtained from patients attending the Somdej Sri Racha Hospital, Cholburi, Thailand. Pretreatment samples were collected randomly. In all cases, a control culture was initiated within a few hours and aliquots of blood were frozen for subsequent studies (7).

The development of gametocytes in vitro was successful in cultures of blood from four different patients. None of these infected blood specimens exhibited the sexual form of the parasites upon initial examination. Gametocytes were detected after 48 hours incubation in one patient, while the remainder were observed at 65, 132 and 138 hours incubation respectively. The gametocytes progressed to more mature stages during the culture period. The development of gametocytes in vitro was confirmed by the observation of two immature gametocytes in one erythrocyte. This form of parasitized red cell normally occurs in the bone marrow or spleen. In the same smear, a number of erythrocytes with multiple infection of trophozoites were seen indicating an active production of new broods in the culture.

Intact gametocytes varied from 1.4% to 5.4% of asexual parasites (counted against 10,000 r.b.c.). A small number of mature gametocytes were detected after 204 hours incubation. Mature female gametocytes were long and thin, but not typically curved. The nuclear chromatin appeared as a dense, deeply stained mass located in the center, but not obscured by the pigment rodlets. Mature males were short with round ends, and pink cytoplasm. The chromatin was diffused, pale staining and somewhat obscured by the pigment granules.

Late gametocytes were numerous with the females appearing in various shapes. Those most often seen were spindle shaped and pointed while a small number were elliptical. The chromatin appeared as a deeply stained mass, centrally located or displaced to one side. The pigment was rod shaped and was seen clumped near the chromatin mass.

The number of late male gametocytes was very small when compared to the female population. The morphology was similar to that described in the fully matured males except that they were more rounded than spindle shaped. In both populations the female/male gametocyte ratio was approximately 3:1.

The morphology of both mature gametocytes varied from the typical crescent shapes found in peripheral blood smears. These alterations may be the result of the unnatural conditions produced in the in vitro culture system. On only one occasion did it appear that the gametocytes were in the exflagellation stage. The appearance of these gametocytes were similar in that there was only one long flagellum extending from a single pole of the gametocyte. It has been confirmed that under unnatural conditions the exflagellation process results in such appearance (8).

In addition, a number of gametocytes with a pink staining mass extruding from the body of the gametocytes were seen. A similar observation of this form of gametocyte was first described in 1960. It was confirmed that this appearance was confined to P. falciparum only (9).

These observations prompted efforts to study the infectivity of these gametocytes. A number of clean Anopheles balabacensis mosquitoes were fed on cultures of P. falciparum at varying intervals. Satisfactory numbers of mosquitoes were fully engorged after each feeding, but no oocyst development was detected seven days later.

Gametocytes also developed in subsequent cultures made utilizing frozen blood specimens from two patients. This compared favorably with cultures of freshly drawn blood from the same patients. No oocysts developed in mosquitoes fed on these cultures.

It is significant that the sexual cycle can be initiated in vitro with blood containing asexual parasites. The observation of gametocyte production and exflagellation indicates that this culture system possibly provides the proper conditions for the generation, growth, and maintenance through maturity, of the sexual forms of the parasite. Failure to produce oocysts in mosquitoes may be due to various factors. Gametocyte population may be too low, or they may not be infective at the time of feeding. The in vitro environment may not provide the conditions necessary to produce gametocytes which are infective. Efforts to improve the existing culture technique are currently being pursued in this laboratory.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 336 Field studies on drug resistant malaria

Literature cited:

References:

1. Chin, W., and Rattanarithkul, M.: The Evaluation of the Presumptive and Radical Treatments Against Falciparum Malaria in Thailand. Southeast Asian J. Trop. Med. Pub. Hlth. 4:400, 1973.
2. Hall, A.P., Doberstyn, E.B., and Mettaprakong, V., Sonkom, P.: Falciparum Malaria Cured by Quinine Followed by Sulfadoxine-pyrimethamine. Brit. Med. J., 2:15-17, 1975.
3. Doberstyn, E.B., and Hall, A.P., Vetvutanapibul, K., and Sonkom, P.: Single Dose Therapy of Falciparum Malaria Using Pyrimethamine. Am. J. Trop. Med. Hyg. 25:14-19, 1976.
4. Field, J.W., Shute, P.G., and Fong, Y.L.: The Microscopic Diagnosis of Human Malaria part II. Institute for Medical Research, Federation of Malaya No. 24: 36, 1956.
5. Diggs, C., Pavanand, K., et al: Penetration of Human Fetal Erythrocytes by Plasmodium falciparum In vitro. J. Parasit. 57:187-188, 1971.
6. Iber, P., Pavanand, K., et al: Evaluation of In vitro Drug Sensitivity of Human Plasmodium falciparum by Incorporation of Radioactive Isoleucine. J. Med. Asso. Thailand. 58:559-566, 1975.
7. Pavanand, K., Permpnich, B., et al: Preservation of Plasmodium falciparum Infected Erythrocytes for In vitro Culture. J. Parasit. 60:537-539, 1974.
8. Garnham, P.C.C.: Malaria Parasites and Other Haemosporidia. Blackwell Scientific Publications. p. 21, 1966.
9. Field, J.W., Sandosham, A.A., and Fong, Y.L.: The Microscopical Diagnosis of Human Malaria part I. Institute for Medical Research Federation of Malaya No. 30:136, 158, 1963.

Publications:

1. Davidson, D.E., Johnsen, D.O., Tanticharoenyos, P., Hickman, R.L., and Kinnamon, D.E.: Evaluating New Antimalarial Drugs Against Trophozoite Induced Plasmodium cynomolgi Malaria in Rhesus Monkeys. *Amer. J. Trop. Med. & Hyg.* 25:26-33, 1976.
2. Doberstyn, E.B., Hall, A.P., Vetvutanapibul, K., Sonkom, P.: Single-Dose Therapy of Falciparum Malaria Using Pyrimethamine in Combination with Diformyl-dapsone or Sulfadoxine. *Amer. J. Trop. Med. & Hyg.* 25:14-19, 1976.
3. Hall, A.P., Segal, H.E., Pearlman, E.J., Phintuyothin, P., Kosakal, S.: Comparison of a 9-Phenanthrene Methanol (WR 33063) a 4-Quinoline Methanol (WR 30090), and Quinine for Treatment of Falciparum Malaria in Thailand. *Trans. Roy. Soc. Trop. Med. & Hyg.* 69:342-349, 1975.
4. Hall, A.P., Doberstyn, E.B., Nanakorn, A., Sonkom, P.: Falciparum Malaria Semi-Resistant to Clindamycin. *Brit. Med. J.* 2:12-14, 1975.
5. Hall, A.P., Segal, H.E., Pearlman, E.J., Phintuyothin, P., Kosakal, S.: Amodiaquine Resistant Falciparum Malaria in Thailand. *Amer. J. Trop. Med. & Hyg.* 24:575-580, 1975.
6. Hall, A.P., Doberstyn, E.B., Mettaprakong, V., Sonkom, P.: Falciparum Malaria Cured by Quinine Followed by Sulfadoxine-Pyrimethamine. *Brit. Med. J.* 2:15-17, 1975.
7. Hall, A.P.; The Treatment of Chloroquine-Resistant Falciparum Malaria. Ninth International Congress on Chemotherapy, London, July 1975.
8. Hall, A.P.: The Treatment of Malaria. *Brit. Med. J.* 1: 323-328, 1976.
9. Pearlman, E.J., Thiemanun, W. and Castaneda, B.F.: The Suppression of P. falciparum and P. vivax Parasitemias by a Diformyl-dapsone-Pyrimethamine Combination. *Am. J. Trop. Med. & Hyg.* 24:901-999, 1975.
10. Pearlman, E.J., Hall, A.P.; Prevention of Chloroquine-Resistant Falciparum Malaria. *Ann. Intern. Med.* 82:590-591, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
					DA OB 6495	76 07 01	DD-DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DOD'S INSTR ^a	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 ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62759A	3A762759A829		00	337			
B. CONTRIBUTING								
C. CONTRIBUTING	CARDS 114F							
11. TITLE (Precede with Security Classification Code) ^a								
(U) Synthesis of Antimalarial Drugs								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a								
012100 Organic Chemistry								
13. START DATE			14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07			CONT		DA		C. In-House	
17. CONTRACT, GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS		B. FUNDS (in thousands)
A. DATES/EFFECTIVE:		EXPIRATION:		RECEIVING				
B. NUMBER:				FISCAL YEAR		7.2		375
C. TYPE:		4. AMOUNT:		CURRENT YEAR		7.0		498
D. KIND OF AWARD:		I. CUM. AMT.						
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research				
ADDRESS: Washington, DC 20012				Div Medicinal Chemistry				
				ADDRESS: Washington, DC 20012				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				
NAME: Joy, COL R.J.T.				NAME: Sweeney, T.R., PhD				
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3731				
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]				
21. GENERAL USE				ASSOCIATE INVESTIGATORS				
Foreign intelligence not considered				NAME: Canfield, C.J., COL				
				NAME:				
22. KEYWORDS (Precede EACH with Security Classification Code)								
(U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Chemical Syntheses								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23. (U) The objective is to manage, integrate, and provide technical direction for both a contract and in-house program to obtain potentially active antimalarial compounds for military use through rational organic syntheses.								
24. (U) Necessary research areas are defined, proposed research evaluated, ongoing research guided, evaluated, and integrated with the other program elements. Technical advice is obtained through an Ad Hoc Study Group on Medicinal Chemistry. Contract exchange by contractors through technical meetings.								
25. (U) 75 07 - 76 06 The area of potential curative and prophylactic agents has received the greatest attention with the 8-aminoquinolines receiving the most attention synthetically. Structural requirements for greatest activity in the <u>P. cynomolgi</u> /Rhesus system have been established but these may be modified as the result of testing yet to be conducted on a number of compounds still in the pipeline. The toxicity inherent in this class of compounds necessitates a cautious development. Other active synthesis areas are of a probing nature. Two compounds that emerged from the large scale synthesis program this year, viz. WR-177 602 and WR-194 965 are targeted for INDs in FY-77 as is one earlier compound, WR-172 435. An IND on WR-180 409 was also prepared this year. During the year 498 compounds were received from the rational synthesis program of which about 250 were target compounds. An additional 5155 were submitted under the no-dollar agreement, 216 as gifts and 168 from purchases. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$99,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.								

PII Redacted

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

1144

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 337 Synthesis of Antimalarial Drugs

Investigators:

Principal: Thomas R. Sweeney, Ph.D.

Associate: COL Craig J. Canfield, MC; Richard E. Strube, Ph.D.;
Edgar A. Steck, Ph.D.; Bing T. Poon, Ph.D.; June A. Page;
Daniel L. Klayman, Ph.D.; LTC Peter S. Loizeaux, VC;
CPT John P. Scovill, Ph.D.; Ruby N. Boyd; SP5 Joseph F.
Bartosevich; SP5 Carl J. Mason; SP4 Larry J. Hooks.

The Research Contract Chemical Synthesis Program

During FY-76 there were 13 contracts active in the rational synthesis program; 8 of these will be carried over into FY-77. Three proposals in new areas, approved by the Medicinal Chemistry Study Group, are awaiting funding. During the year five papers were published, two patents issued and one applied for as the result of work done on the contract synthesis program.

Research areas that will be discontinued at the end of FY-76 because of contract terminations include the oxazinediones, distamycin and its analogs, and the 3'-alkyl cordycepin analogs. No activity was observed with the oxazinediones or the Cordycepin analogs. Distamycin and analogs have not yet been submitted; they will be tested as soon as possible.

The largest concerted synthetic effort during FY-76 was in the synthesis of 8-aminoquinolines as potential prophylactic and curative agents. Several compounds more active than primaquin were obtained but the toxicity associated with them will necessitate a cautious development. The difficulties associated with the termination of the P. cynomolgi/rhesus curative test system at the Southern Research Institute and its reinstatement at SEATO have resulted in the accumulation of a number of untested 8-aminoquinolines. The synthesis of additional compounds in this class will be curtailed until the biological feedback can be brought up to date.

Because of the consistent, but moderate, activity uncovered in the 3-amino-6-aryl-1,2,4,5-tetrazines, a new class of compounds to show antimalarial activity, a number of these were synthesized in order to investigate the structure-activity relationships. One of the better ones, WR-216 693, was selected for monkey studies.

Probing synthetic work has been carried on in a number of areas. These include analogs of the above mentioned tetrazines; basically

substituted (trichloromethyl)heterocycles and certain of their N-oxides; amodiaquin analogs; 1,3-bis-[[4-(aminoalkylamino)benzylidene]amino]-quinadines; acridones; carboxylic acid derivatives of known structural types; 3,5-diamino-1,6-dihydro-1,2,4-triazines. Should any of these classes prove to have significant and interesting activity, the class would be expanded.

Work on the synthesis of potential hypoxanthine-guaninephosphoribosyl transferase inhibitors was continued because of the compelling biochemical rationale. Emphasis was directed to the 8-substituted hypoxanthines and guanines when no activity was uncovered in the 7-substituted series. Some encouragement was received from the former groups when enzyme inhibitory activity was demonstrated in an *in vitro* system; this activity notwithstanding, the same compounds were inactive as antimalarials. In addition to the 8-substituted compounds some ring-modified compounds analogs of hypoxanthine are being synthesized as potential enzyme inhibitors.

The Preparations Laboratories

The preparations laboratories, used chiefly to resynthesize on a larger scale, selected compounds that are needed for testing in large animals, or for toxicological or clinical studies, have continued to support the program. Only two of the three contracts active in FY-76 will be carried into FY-77.

The work of the preparations laboratories is summarized in the following table. It should be realized that some of the compounds received in FY-76 were requested in FY-75.

FY-76	Target Compounds			Intermediates	Total
	>1000 g	100-1000 g	<100 g		
Requested	1	15	20		36
Received	6	12	23	61	102

The compounds that were received in quantities of greater than 1 kg include the following IND compounds: WR-142 490 (2 batches), WR-142 490/WR-177 602 mixture in the ratio 43/57, WR-177 602, and WR-194 965.

The one contract dedicated to the synthesis of radioactively tagged compounds for use in pharmacological studies was also active during the year.

The Analytical Laboratory

The analytical laboratory is used to check the identity and purity of bulk and formulated drugs and to determine the stability of formulations. The following table summarizes the reports received from this laboratory.

Analyses

	Bulk	Formulations	Stability Determinations
Requested	9	9	9
Received	12	5	4

Acquisition of Compounds

The following table summarizes the number of various classes of compounds received during FY-76.

	<u>Original</u>	<u>Not Original</u>
Purchased	168	8
Gifts	216	114
Synthesized	498	67
Discreet	5155	662
Total	6037	851

The following table shows the evolution in the preparations laboratory cost of Mefloquine. The reduced costs are the result of experience and improved synthetic approaches. Because of the growing importance of Mefloquine these figures may have important commercial significance.

<u>Date</u>	<u>Amount Made (g)</u>	<u>Cost (\$/kg)</u>
Sept. 71	2,541	20,000-25,000
Oct. 74	4,064	8,351
Sept. 75	8,414*	7,563
June 76	19,000	2,604

*erythro-threo mixture

Organic Synthesis Section

An investigation of the synthesis of a new class of potential causal prophylactic agents, the 4-amino-2-methoxyacridines was started with a series of model experiments using 8-amino-4-methyl-6-methoxyquinoline to evaluate various methods for the introduction of sidechains onto the amino group. Upon arrival of the 4-amino-2-methoxy acridine from the prep laboratory in modest quantity, the compound has been successfully alkylated with 6-bromohexylphthalimide and 4-bromopentylphthalimide. The resultant products have been converted by hydrazinolysis to the desired primary amino compounds.

Alkylations of 4-amino-2-methoxyacridine with other secondary alkyl halides, however, have proceeded poorly yielding either unchanged starting materials under mild conditions or complex mixtures under drastic conditions. These difficulties can probably be ascribed to the fact that the least basic amino group located on the acridine nucleus is the one located in the 4-position.

Since October 1, 1975, a total of 64 new thiosemicarbazones have been submitted for screening. Most of these have been synthesized via the novel route developed by us, namely the reaction of an amine with an arylidene derivative of S-methyl-dithiocarbamate.

The following structure/activity relationships have been observed for the thiosemicarbazones: (1) the aldehyde or ketone precursor leading to the most active thiosemicarbazone is 2-acetylpyridine; 2-propionylpyridine imparts diminished activity. The placement of the acetyl group elsewhere on the pyridine ring or on another nucleus tends to eliminate activity; (2) substitution in the 4-position of the thiosemicarbazone with antimalarial sidechain diamines such as novoldiamine, as well as other aliphatic groups, destroys activity. Activity is imparted, however, by aromatic and benzyl-type substitution. What is unknown, as yet, is what substituent on the aromatic or benzyl ring systems optimizes antimalarial activity.

Data Processing

During the past year activity has continued to center around efforts to bring all data systems in-house and up grade existing capabilities.

1. Inventory

Work on the new inventory system was temporarily suspended pending decisions on the direction of the chemical system. Work has resumed and the system is scheduled to begin parallel processing in January 77. Certain improvements planned for the new system, such as special codes for open and discreet return samples, combination studies, and blind samples, extension of 30-day edit criteria to 60 days and the retention of the original bottle number in the return sample record, were

implemented in the existing system to facilitate operations during the delay. The computer file was verified by checking against the physical inventory.

2. Chemistry

Personnel and administrative changes necessitated a review of the status of the proposed chemical system conversion. Final plans have been submitted and approved. The initial conversion of the data base was accomplished and correction of structures is currently being conducted on line through the IMLAC terminal. Work is proceeding according to schedule with the first series of programs due to be operational early in 1977.

3. Biology

The biology computer system continues to expand as new testing programs are developed to cover a broader spectrum of parasitic diseases. Programming of the biology system for the CDC 3500 and conversion of current master files was completed. System and program documentation is 80% completed. Parallel processing has been delayed until the chemistry indexing method for interfacing biology, inventory, and chemistry files is completed.

An additional 52,000 records have been added to the biology data base during FY-76. Two new test systems were incorporated into both old (IBM 7090/7094) and new (CDC 3500) biology systems and several programs were modified to accept additional test information. In order to interface biology with chemistry, the old system must be kept operational. Completion of the chemistry system conversion to the CDC 3500 will eliminate the necessity of maintaining the old biology system. Additional modifications will be necessary to accommodate files generated under Data Management Routines, a new software package obtained by the Division of Biometrics and Medical Information Processing.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	3. REPORT CONTROL SYMBOL DD-DR&E(AR)636	
4. DATE PREV. SUMMARY	5. KIND OF SUMMARY	6. SUMMARY SCTY ³	7. WORK SECURITY ⁴	8. REGRADING ⁵	9. DISB'N INST'N	10. SPECIFIC DATA - CONTRACTOR ACCESS	11. LEVEL OF SUM ⁶
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
12. NO / CODES ⁷	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	62759A	3A762759A829	00	338			
13. CONTRIBUTING							
14. COMMUNICATING		CARDS 114F					
15. TITLE (Precede with Security Classification Code) ⁸							
(U) Protective Immunity in Malaria							
16. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹							
002600 Biology 010100 Microbiology							
17. START DATE		18. ESTIMATED COMPLETION DATE		19. FUNDING AGENCY		20. PERFORMANCE METHOD	
74 07		CONT		DA		C. In-House	
21. CONTRACT/GRANT				22. RESOURCES ESTIMATE		23. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER ¹⁰				FISCAL YEAR		76	
C. TYPE				CURRENCY		1.0	
D. KIND OF AWARD:				E. CUM. AMT.		217	
77				2.0		218	
24. RESPONSIBLE DOD ORGANIZATION				25. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Div of CD&I			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Robert J.T. Joy, COL				NAME: Carter L. Diggs			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3544			
26. GENERAL USE				27. ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME:			
				NAME:			
28. KEYWORDS (Precede EACH with Security Classification Code) ¹¹							
(U) Immunity; (U) Antigens; (U) Protozoa;							
(U) Tropical Medicine; (U) Antibodies							
29. TECHNICAL OBJECTIVE, 30. APPROACH, 31. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) The objective of this work unit is to elucidate the protective mechanisms involved in immunity to malaria, a disease which has repeatedly impeded military operations, and to investigate the feasibility of immunoprophylaxis against this disease.							
24 (U) The approach used in these studies is to study both in animal models and through the use of in vitro techniques the response elicited by the malaria parasite on the immune system, and to determine the relative roles of cellular and molecular mediators in these processes.							
25 (U) 75 07-7606 Marked improvement in culture of Plasmodium falciparum has been achieved with maintenance of cultures for three weeks, starting with cryopreserved inocula. Insight into the characteristics of the receptors on the erythrocyte for P. falciparum has been gained through use of this technique. Studies in murine models have confirmed and extended previous work indicating the efficacy of irradiated blood forms as experimental immunogen and of antibody as effector of immunity. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1975 - 30 June 76.							
Support in the amount of \$38,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

PII Redacted

DD FORM 1498
1 MAR 66

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

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 338 Protective immunity in malaria

Investigators: COL Carter L. Diggs, M.D., MAJ David Haynes, M.D.

Assistants : Barbara Flemmings, B.S.; Andre Toussaint, B.S., M.S.;
James Dillon, B.S.; SP5 Karen Czarnecky, B.S.

I. Inhibition of Plasmodium berghei parasitemia by Purified IgG from Immune Rat Serum.

Description. The protective efficacy of immune serum against P. berghei is well established. Recent studies involving experimental immunization against murine malaria with BCG have raised the possibility that protection may not be mediated through immunoglobulin, but by other serum factors in immunized animals. The present studies were designed to test the activity of highly purified IgG from rats immunized against P. berghei by repeated infection and spontaneous cure. Immunoglobulin G was isolated by protein A sepharose affinity chromatography. It was assayed by intravenous administration into young syngeneic rats (CDF, of the Charles River Breeding Laboratory) at the same time the animals were challenged with viable parasites.

Progress. The results are shown in Table I in which it can be seen that immunoglobulin G from immune animals was comparable in its antiparasitic efficacy with the serum from which it was derived. In contrast, control animals which received normal serum or the IgG fraction of normal serum exhibited parasitemia which was indistinguishable from animals receiving saline. Immunoelectrophoretic analysis of the IgG preparations revealed no non-IgG contaminants.

Discussion. This study demonstrates that highly immunochemically purified IgG antibody is active against Plasmodium berghei. The studies do not rule out, of course, the presence of other active serum factors in this serum or in serum from other sources.

II. Cryopreserved Plasmodium falciparum: Long term in vitro culture and host erythrocyte specificity.

Description. The objectives of this research are to develop a reliable method of culturing P. falciparum in vitro which allows (1) the use of a cryopreserved inoculum which may be thawed at the convenience of the experimenter, (2) continuous growth and production of merozoites in large batches for biochemical and immunological studies, (3) the biochemical and genetic analysis of the human erythrocyte - malaria merozoite surface interaction, and (4) the eventual production of an effective malaria merozoite component vaccine.

This project has utilized the cryopreservation method of Diggs, et. al.

TABLE I

Effect of Normal and Immune Serum and Staphylococcal Protein A Purified IgG on Parasitemia Due to Plasmodium berghei

Treatment	Median % Erythrocytes Parasitized on Day										
	3	4	5	6	7	9	12	14	16	19	21
Immune Serum	0	0	0	0	0.5	3	2.5	0.5	1	0.5	0
Normal Serum	0	0	2	2	3	7	10	10	11	1	0
Immune IgG	0	0	0	0	1	5.5	4	0.5	0	0	0
Normal IgG	0	0	2	3.5	3	5	9	6	13	1	0.5
Buffer Control	0	0	0.5	2.5	6.5	4	6	11	20	2	0

(1975 Am. J. Trop. Med. & Hyg. 24:760) combined with a tissue culture technique. Briefly, the parasitized inoculum is mixed with test erythrocytes and incubated at 37° C in Medium 199 (containing 1.25 g/l of NaHCO₃) with added glucose, glutamine, gentamycin, TES buffer, vitamin E, 2-mercaptoethanol, and heat inactivated fetal bovine serum. All leucocytes have been removed from the culture by passing the blood cells through a powdered cellulose column. The culture is incubated in equilibrium with 3% CO₂, 6.6% O₂, and 90.4% N₂. The medium and erythrocytes were changed every 2 days in the long term culture by centrifuging out the schizont layer and resuspending with fresh erythrocytes and medium. The short term cultures were incubated for three days and sampled at 24, 48, and 72 hours. Erythrocytes were obtained from various species, from humans with rare blood types, and in some cases human erythrocyte were treated with various enzymes before culture. Merozoites were obtained by differential centrifugation of 48 hour cultures.

Progress. Cryopreserved blood has been cultured successfully for as long as 22 days (11 generations) of continuous culture, and on numerous instances for three day periods. Parasite forms seen after 22 days of culture include schizonts, trophozoites, ring forms, invading merozoites, and a few immature gametocytes. In the three day culture erythrocytes from humans (including Duffy negative individuals and others with rare blood types), chimpanzee, and Aotus monkeys were invaded and supported parasite growth. Erythrocytes from rhesus monkeys and guinea pigs were not invaded (see tables II and III). Analysis the invasion of enzyme treated erythrocytes is in progress in collaboration with Dr. Lou Miller of the NIH. The yields of merozoites have been small, and we hope to

increase production by using a continuous flow culture system.

Discussion. This culture system is being used to study the nature of P. falciparum interactions with erythrocytes (Miller, et. al., manuscript in preparation), as has already been done for P. knowlesi using enzyme treated and rare blood type human erythrocytes. We expect that the culture of P. falciparum will allow further biochemical and immunological studies, which, if accompanied by the production of large numbers of merozoites, may lead to the development of an effective vaccine for malaria.

TABLE II

Malaria Grown in Four Species of Erythrocytes

Test Erythrocytes	Trophozoites* at 24 hours	Trophozoites* at 72 hours	Ratio of 72 to 24 hours
Human	3.7 \pm 1.5	23.0 \pm 3.1	6.2
Chimpanzee	5.3 \pm 0.9	33.3 \pm 1.7	6.3
Rhesus	3.0 \pm 1.2	1.3 \pm 0.3	0.4
Guinea pig	5.5 \pm 2.5	1.8 \pm 0.5	0.3

*Numbers of trophozoite forms per 1,000 erythrocytes (mean \pm S.E. of triplicate samples).

TABLE III

Erythrocytes	I and R	T	S	Ab
		<u>24 hours</u>		
Human	0	11	0	6
Rhesus	5	12	0	10
		<u>48 hours</u>		
Human	23	5	1	11
Rhesus	3	5	2	8
		<u>72 hours</u>		
Human	4	39	0	6
Rhesus	0	1	0	7

Numbers of parasite forms per 1,000 human or rhesus erythrocytes at 24, 48, and 72 hours after the beginning of culture. I and R = invading merozoites and ring forms, T = trophozoites, S = schizonts, and Ab = abnormal degenerating forms.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 338 Protective immunity in malaria

Publications:

1. Protein Synthesis in vitro by Cryopreserved Plasmodium falciparum
Carter Diggs, Kenneth Joseph, Barbara Flemmings, Ralph Snodgrass, and
Fred Hines. Am. J. Trop. Med. Hyg. 24 760-763, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E (AR) 636	
				DA OC 6437	76 07 01		
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^b	6 WORK SECURITY ^b	7 RESRADING ^c	8A DR&E INSTR ^d	8B SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
75 07 01	D. Change	U	U	NA	NL		
10 NO / CODES ^e		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
A. PRIMARY		62759A		3A762759A829		00	
B. CONTRIBUTING						339	
C. XXXXXXXX		CARDS 114F					
11 TITLE (Precede with Security Classification Code) ^g							
(U) Studies of Malaria Parasitic Metabolism and Drug Action							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^h							
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		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE NA				B. PRECEDING		C. FUNDS (in thousands)	
B. NUMBER ⁱ				76		2	
C. TYPE				77		1	
D. KIND OF AWARD				1		115	
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME ^j Walter Reed Army Institute of Research				NAME ^k Walter Reed Army Institute of Research			
ADDRESS ^j Washington, DC 20012				ADDRESS ^k Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish OASD if U.S. academic institution)			
NAME JOY, R. J. T., COL				NAME ^l DAVIDSON, David E., LTC			
TELEPHONE: 202-576-3551				TELEPHONE 202-576-2292			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
22 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: McCORMICK, G. J., Ph.D.			
23 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cells							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRAM (Furnish individual paragraphs identified by number precede text of each with Security Classification Code)							
<p>23. (U) Document metabolic alterations of human and animal red blood cells when infected with malaria parasites and to assess the effect of antimalarial drugs on these alterations in order to guide the development of new drugs effective against resistant falciparum malaria, a disease of continuing military importance.</p> <p>24. (U) Measure the effects of antimalarial drugs on morphologic growth and radio-labeled precursor incorporation into protein and nucleic acids during in vitro schizogony. Establish presence of metabolic pathways in the malaria parasite. Assess anti-malarial activity in vivo of selected drug preparations.</p> <p>25. (U) 75 07 - 76 06 In vitro culture of P. knowlesi-infected rhesus blood has continued for screening for antimalarial activity, and for study of metabolic activities. A number of candidate compounds demonstrated antimalarial activity in this system. Enhancement of growth was observed when certain fatty acids were added to the culture. Using inhibition of incorporation of 14-C orotic acid into DNA as the parameter in vitro culture of P. falciparum in aotus monkey blood and of P. berghei in mouse blood demonstrated differences in effective dose levels of pyrimethamine between pyrimethamine sensitive and resistant strains. Sustained release preparations containing WR 158122 or sulfadiazine were implanted in mice and tested for efficacy against repetitive weekly challenges with P. berghei. In some experiments, protection was observed for many weeks (5-15). For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$25,000 from FY 77 funds is programmed for the period 1 Jul - 30 Sep 76.</p>							

PII Redacted

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 339 Studies of malaria parasitic metabolism and drug action

Investigators.

Principal; Gerald J. McCormick, Ph.D.

Associate: COL Craig J. Canfield, MC; Gloria P. Willet

1. Description

The objectives of these studies are to define metabolic alterations of human and animal red blood cells when infected with malaria parasites, to elucidate the metabolism of the parasite, and to assess the effect of antimalarial drugs on the erythrocytic alterations, parasite metabolic activity, and the course of infection, in order to guide the development of new drugs and preparations effective against resistant falciparum malaria, a disease of continuing military importance. Studies are conducted both in vivo and in vitro.

2. Progress

In vitro cultivation studies with P. knowlesi-infected rhesus monkey red blood cells were continued. Screening studies were completed on 90 candidate drugs, using as the parameter the inhibition of incorporation of radioactivity from ¹⁴C-orotic acid into DNA and RNA. Of these drugs, 34 were considered to be effective (greater than 80% inhibition); nine were less effective (50-80% inhibition); and 47 were ineffective at a concentration of 10 mg/L. The results for the effective compounds are summarized in Table 1.

3. A previously completed study which described the presence of a new metabolic pathway in P. knowlesi was accepted for publication (1). This study showed that the parasite biosynthesized methionine, a capability previously considered to be lacking.

Serine was a source of the methyl groups of methionine and thymidylic acid.

4. In vivo studies of implanted drug preparations have continued in collaboration with Dynatech R/D Company, under the protocol entitled "Development of an implantable sustained release system for the prevention of malaria." Several studies were completed and one is underway. Two preparations with WR 158122 as the antimalarial drug were received. One was protective in mice against repetitive challenges with P. berghei for more than three weeks at 20 to 160 mg/kg.

Evaluation of the second preparation is in progress. Three preparations with sulfadiazine were evaluated. One, in bead form, was protective for three weeks with eight beads implanted (equivalent to 132 mg/kg). A second preparation in bead form was protective at one to eight beads per mouse (equivalent to 57-456 mg/kg) through a 15-week period of repetitive challenges. With the third preparation, in powder form, protection was observed for two weeks at 160 mg/kg.

5. Studies of drug resistant malaria have continued. Through the courtesy of Dr. Leon H. Schmidt of Southern Research Institute several Aotus trivirgatus monkeys infected with pyrimethamine-sensitive or resistant P. falciparum strains were provided, and in vitro studies were done using samples of their blood. A difference of effective level of pyrimethamine in vitro was found between the strains. Due to the shortage of Aotus monkeys, these studies with P. falciparum have been suspended. Pyrimethamine resistance in P. berghei in mice has been induced and a change in effective level of pyrimethamine in vitro was observed during the in vivo induction.

6. A previously completed study of levels of phospholipids in normal and parasitized (P. knowlesi) blood of rhesus monkeys, done in collaboration with the University of Maryland, was published (1).

Table 1. Antimalarial Activity of Candidate Compounds at 10 mg/L vs. P. knowlesi in vitro.

Compound	Incorporation ²		Compound	Incorporation	
	DNA	RNA		DNA	RNA
<u>"Effective"</u>					
WR 2712	3	6	199881	11	38
4931	1	4	202147	1	0
124949	0	4	202148	1	1
139008	3	4	202930	3	87
159412	2	6	202931	2	66
180872	13	61	206274	5	1
182067	2	37	206275	11	155
182713	7	85	206276	5	14
182829	4	80	206290	2	89
183345	2	4	208465	8	109
184363	8	29	212217	0	133
186937	2	92	212829	19	135
191031	2	2	214090	15	55
193575	6	11	217277	1	3
197629	12	105	217384	0	4
198279	13	83	219070	2	6
199387	10	108	219381	2	126
<u>"Less Effective"</u>					
106911	43	85	214081	49	94
109018	39	102	214740	38	78
199071	38	94	219255	33	154
209363	50	89	219391	32	120
209847	33	51			

²Incorporation of radioactivity from (6-¹⁴C)-orotic acid as percentage of that incorporated in absence of any drug.

Project 3A762759A829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 339 Studies of malaria parasitic metabolism and drug action

Literature Cited.

References:

1. Smith, D. C., McCormick, G. J., and Canfield, C. J.:
Plasmodium knowlesi: In vitro Biosynthesis of Methionine. Experimental Parasitology, in press.

Publications:

1. McLean, S., Purdy, W. C., Kabat, A., Sampugna, S., DeZeeua, R.,
and McCormick, G.: Analysis of the phospholipid composition of
Plasmodium knowlesi and rhesus erythrocyte membranes. Analytica
Chimica Acta, 82, 175-185, (1976).

Project 3A762759A831
TROPICAL MEDICINE

Task 00
Tropical Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OB 6525	76 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ³	6. WORK SECURITY ⁴	7. REGRADING ⁵	8. DES'N INST'N ⁶	9. SPECIFIC DATA - CONTRACTOR ACCESS	
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO / CODES ⁷	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62759A	3A76, 759A63	00	070			
b. CONTRIBUTING							
9. XXXXXXXX CARDS 114F							
11. TITLE (Precede with Security Classification Code) (U) Anti-schistosomal Drug Development							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁸ 012600 Pharmacology 002600 Biology 010100 Microbiology							
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				b. FISCAL YEAR		c. FUNDS (in thousands)	
b. NUMBER ⁹				76		1.1	
c. TYPE				d. CUM. AMT.		31	
e. KIND OF AWARD:				1.1		29	
10. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ¹⁰ Walter Reed Army Institute of Research				NAME ¹¹ US Army Medical Research Unit			
ADDRESS ¹⁰ Washington, DC 20012				ADDRESS ¹¹ Brasilia, Brazil			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. academic institution)			
NAME: JOY, R. J. T. COL				NAME ¹² Radke, M.G., LTC			
TELEPHONE: 202-576-3551				TELEPHONE:			
21. GENERAL USE				22. ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Prata, Aluizio, MD			
23. KEYWORDS (Precede each with Security Classification Code)				NAME:			
(U) Brazil; (U) Drug Development; (U) Schistosomiasis; (U) Chemotherapy							
23. TECHNICAL OBJECTIVE ¹³ 24. APPROACH, 25. PROGRAM (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Find new prophylactic and therapeutic drugs that can be used to minimize the health risk of acquiring schistosomiasis in the event U.S. military and DOD civilians are deployed in endemic areas such as South America, Caribbean, Africa, Middle East, and Far East.</p> <p>24. (U) The WRAIR US Army's Anti-Schistosomal Drug Development Program submits selected compounds for prophylactic and therapeutic testing against schistosomiasis mansoni in mice. The prophylactic mortality test uses mice exposed to a lethal dose of 3,000 or more S. mansoni cercariae, and drugs are given subcutaneously at 1280 mgs/kg. The curative test uses mice exposed to 200 cercariae, and 30 to 35 days later drugs are administered orally in 5 equal daily dosages. Prophylactic drug activity is measured by mouse survival and curative drug activity is measured by increased numbers of live and dead worms in the liver.</p> <p>25. (U) 75 07 - 76 06 This research is complementary to studies being conducted under DAOB 7294, Work Unit 086, entitled Chemotherapeutic Studies on Schistosomiasis. The laboratory Biomphalaria glabrata snail colony (Paulista Strain) maintains a daily average number of 1,000 S. mansoni cercarial shedding snails. The positive snails yield weekly collections of two million cercariae which are used to infect animals for each test group of 50 compounds. During FY 76, 1,478 selected WRAIR bottle number drugs were tested for prophylactic activity. The results were as follows -- 1) 1091 drugs were negative, 2) 377 drugs were toxic, and 3) 10 drugs were active. The 10 active drugs are commercially discreet. The 16 drugs tested in the curative test system were all negative. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 75-30 Jun 76. Support in the amount of \$6,000 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 68 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A 762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 070 Anti-schistosomal Drug Development

Investigators.

Principal: Aluizio Prata, M.D. (University of Brasilia)

Associate: LTC Myron G. Radke, MSC

Description.

Drug testing for the schistosomiasis drug development program is performed at the U. S. Army Medical Research Unit/Brasilia. Selected compounds are submitted by the Division of Medicinal Chemistry, WRAIR to our drug testing facility. Fifty drugs are tested weekly for prophylactic activity against schistosomiasis mansoni by the mouse mortality test system (Radke, et. al., 1971). In May 1976, the routine prophylactic drug testing was augmented with a curative test. Presently, there are no known drugs to prevent schistosomiasis and the few therapeutic drugs being used are expensive and toxic. Thus, any new prophylactic and therapeutic drugs found by the U. S. Army's anti-schistosomal drug development program would minimize the risk of disease in the event that it would be necessary to deploy U. S. Military and DOD Civilians in endemic areas. Drug advances in the treatment of schistosomiasis would result in reducing government expenditures of men and monies for schistosomiasis control which is endemic to the following geographic areas: South America, Caribbean, Africa, Middle East and Far East. The expertise of the U. S. Army Research schistosomiasis drug development program is directed towards finding drugs which will prevent and cure the disease. The USARMU/Brasilia test facility (having animals, snail and disease life cycle, and test system capabilities) in support of the WRAIR, Medicinal Chemistry (source of drugs, synthesized drugs, and automatic data processing) affords unique opportunities for developing and/or finding new drugs to control schistosomiasis.

Progress.

a. Laboratory Facility. The final equipment installation was completed with the 80 gallon air compressor which delivers oil/water free air to the Biomphalaria glabrata snail colony. The delay in installing the special air compressor was a result of the 220 volt 3 phase electric requirement. A special transformer was purchased to adapt the normal line voltage of 390 volts 3 phase to 220 volts 3 phase.

b. Animal Facility. Four air conditioners were installed in the Bioterio's mouse breeding facility to provide a uniform day/night temperature. Establishing a uniform ambient temperature, the mouse colony production should be stabilized. Our drug testing unit receives from the University of Brasilia's Bioterio 400, 18-23 gram mice weekly. Beginning FY 77, the mouse deliveries requested are 500/600 weekly.

c. Snail Colony. The weekly laboratory Biomphalaria glabrata snail colony production is 500, 5 - 8 mm snails of which 350/400 are individually exposed to 8 - 12 S. mansoni miracidia. Eighty-two percent of the miracidia exposed snails are surviving at 42 days (monthly snail survival was 67 to 88 percent) with a snail infection rate of 56 percent (monthly snail infection rate was 40 to 77 percent). The daily number of S. mansoni cercariae shed-
ding snails maintained was 1,239 (the daily average number of infected snails maintained per 30 days was 992 to 1,499). These infected snails provided weekly cercarial collections of 3 to 5 million. Our weekly cercarial usage is two (2) million.

d. Drug Testing. The drug testing rate was 30 drugs weekly at two dosages, 640 and 1920 mgs/kg. However, many compounds were found to be toxic when drugs were administered subcutaneously at 1920 mgs/kg and the limited 500 milligram samples forced us to lower the test dosage. In December 1975, the drug test facility began testing fifty compounds weekly at 1280 mgs/kg.

Test Procedure.

All experimental drugs are screened for prophylactic activity by the mouse mortality test system. Mice weighing 18 - 23 grams (39 - 43 days old) are exposed 45 minutes by tail immersion to 3,000 or more S. mansoni cercariae. Drugs are formulated in peanut oil at either 640 and 1920 mgs/kg or 1280 mgs/kg. Five mice are used per test dosage and drugs are administered subcutaneously two days after cercarial exposure. Any toxic drugs, that is infected treated mice dying within the first 10 days of infection, are retested at 40 and 160 mgs/kg. The number of compounds tested weekly was increased from 30 to 50. A routine test group for 50 selected compounds consisted of 325 mice; ten mice are normal, and 315 mice are exposed to 3,000 or more cercariae. The two hundred fifty (250) infected mice are used to screen 50 candidate drugs with five mice per drug for anti-schistosomal activity, five mice are given Niridazole (reference drug), and fifty mice are untreated infected controls. An active drug is one in which treated mice survive 49 days after cercarial exposure. The infected control mice start dying on the 20th day and by the 30th day all are dead. Drug activity is based upon the number of surviving mice and worm burden data as obtained by perfusing all living 49 day treated mice. In May 1976, a curative test system was added to the anti-schistosome

drug testing program. Some selected drugs and all prophylactic active drugs are tested for therapeutic activity against mice exposed to 200 *S. mansoni* cercariae. Treatment of the infected mice begins, when adult worms are present, 30 to 35 days after cercarial exposure by administering the drug orally in five equal daily dosages. Drugs are prepared in peanut oil for oral administration at either 50, 100, 160, or 320 mgs/kg. Five mice are used for each drug level. Mice are sacrificed 10 days after completing drug treatment. The liver is removed, squashed between two glass plates, and the number of live and dead worms are counted. Worms are considered dead when the addition of Serotonin fails to induce motility. A Curative test group consists of several test drugs (five mice per drug), 15 infected non-treated control mice, and 10 reference drug (Niridazole) treated mice. Drug activity is demonstrated by an increase in the number of live worms in the liver ("liver shift") and the presences of dead worms in the liver.

Results.

During FY 1976, a total of 1,290 selected WRAIR bottle numbered drugs were tested at dosages 1) 640 and 1920 mgs/kg or 2) 1280 mgs/kg against schistosomiasis mansoni by the mouse mortality test. An additional 188 drugs were retested at other dosages because of either drug toxicity or drug activity. The test results were: 1) 1091 negative drugs, 2) 377 toxic drugs, and 3) 10 active drugs. A total of 16 drugs were evaluated in the curative test. The liver squashes from four drugs had an increase in the number of live worm per mouse was greater than 10 (control animals have around five worms). However, dead worms found in the liver squash preparation from drug treated mice indicates therapeutic activity.

Project 3A 762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 070 Anti-schistosomal Drug Development

Literature Cited.

References:

1. Radke, M. G., Broome, P. B., and Belanger, G. S. : Schistosoma mansoni: Mouse Mortality Test System for Mass Screening for Prophylactic Drugs. Exp. Parasit. 30: 1-10, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OB 6526	76 07 01	DD-DR&E(AR)616	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ³	6. WORK SECURITY ⁴	7. REGRADING ⁵	8. ORG'S INSTR ⁶	9. SPECIFIC DATA - CONTRACTOR ACCESS	
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO / CODES ⁷	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62759A	3A762759A831	00	071			
b. CONTRIBUTING							
c. XXXXXXXX	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ⁸							
(U) Field Studies of Rickettsioses and Other Tropical Diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ⁹							
010100 Microbiology 002600 Biology							
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. FUNDS (in thousands)	
a. DATES/EFFECTIVE: NA				PREVIOUS			
b. NUMBER ¹⁰				FISCAL YEAR		PROFESSIONAL MAN YRS	
c. TYPE				86		5	
d. KIND OF AWARD:				77		100	
e. AMOUNT				CURRENCY			
f. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: US Army Medical Research Unit Malaysia			
ADDRESS: Washington, DC 20012				ADDRESS: Kijala Lumpur, Malaysia			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME, GRADE, and/or Institution)			
NAME: JOY, R. J. T., COL, MC				NAME: HUXSOLL, D. L., LTC, VC			
TELEPHONE: 202-576-3551				TELEPHONE			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
23. REVISIONS (Precede EACH with Security Classification Code) ¹¹ (U) R. tsutsugamushi; (U) Human infections; (U) Laboratory diagnosis; (U) Epidemiology; (U) Ecology; (U) Immunity; (U) Leptotrombidium spp							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code) ¹²							
<p>23. (U) To investigate the incidence of scrub typhus infection in selected local and foreign populations, to relate this to the prevalence of vector chiggers and their common rodent hosts, to determine the strains of R. tsutsugamushi causing human infections and relate to clinical disease, to develop improved serological methods, to study the basis for immunity, to define vector-rickettsia relationships, to develop a laboratory animal model for the disease, and to produce laboratory animals in support of the mission. Scrub typhus is an incapacitating disease of military importance.</p> <p>24. (U) Provides scientific reagents and equipment which can be best procured in the US for shipment to and use by WRAIR personnel in Malaysia functioning collaboratively with Malaysian scientists supported under USAMRDC grant reported on DA OA 7413.</p> <p>25. (U) 75 07 - 76 06 Studies of febrile patients in indigenous populations showed that scrub typhus is a common, but frequently undiagnosed, cause of febrile illness. The clinical syndrome was difficult to distinguish clinically from that due to other infections. The antigenic properties of human isolates and the antibody response to infection have shown that the Karp strain group produces the preponderance of symptomatic infections in the study area. The strain activity in humans, rodents, and chiggers in selected study sites was compared. In a study of mammals and chiggers from a primary forest only a single specimen of a known vector of R. tsutsugamushi was collected. L.L. fletcheri and L.L. arenicola colonies infected with R. tsutsugamushi were employed to study the effects of temperature on chigger development and transmission of the infection. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$21,000 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 66 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 071 Field Studies of Rickettsioses and Other Tropical Diseases

Investigators:

Principal: LTC David L. Huxsoll, VC

Associate: LTC David M. Robinson, VC; MAJ Grahaem W. Brown, RAMC; MAJ Richard D. Montrey, VC; MAJ Alexander L. Dohany, MSC; Miss Elsie Gan, B.S.

CLINICAL AND EPIDEMIOLOGICAL STUDIES OF SCRUB TYPHUS

The studies on 'normal' populations, described in the Progress Report for 1975, yielded good evidence for a high incidence of scrub typhus transmission in the areas studied. Due to the success of other studies, the surveys at Pos Iskandar, Bukit Lanjan and Elmina Estate have been discontinued for the present. Likewise, the study of indigenous soldiers has been suspended, useful data having been obtained. The investigation of febrile patients in rural Malaysia, described in the Progress Report for 1975, has been continued this year. Over 120 human isolates of *R. tsutsugamushi* have been made, and a wealth of clinical and serological data collected.

Normal Populations

Orang Asli (Aborigines): Pos Iskandar is situated in an area of secondary jungle, 35 miles N.E. of Kuala Pilah and 67 miles East of Kuala Lumpur. It is the focal point for several Orang Asli kampongs scattered around a marshy lake, Tasek Bera, and has a school, and a clinic staffed by a medical orderly. About 1000 people live in the area, subsisting largely on shifting cultivation of hill rice, tapioca, etc., in the surrounding jungle. Rubber has recently been planted in a few areas close to the villages.

Sera were collected from 208 volunteers of all ages during the first visit in January 1975. The system of agriculture necessitated families moving out of their village houses to guard the crops in the jungle ladangs for periods of several weeks at a time. Thus it was difficult to find all of the same individuals regularly. Sequential specimens of serum were collected from 127 individuals (71 twice, 44 three times, 11 four times and 1 five times), over a period of 8 months (January-August 1975). A total of 462 man-months of exposure was studied.

The age specific prevalence rates of scrub typhus antibody at Pos Iskandar are shown in Table 1, using data from the largest group bled simultaneously, in January 1975.

During the following 8 months of observation, 46 individuals showed a rise in titer (see Table 2). In 18 individuals, there was a 4-fold or greater rise in antibody to a titer of at least 1/200, and in none of these were malaria parasites or leptospiral antibody found. In 8 of the 18, the maximum titer was 1/200, including one person who showed a rise in OXK titer from 1/40 to 1/320. In 5 the titer rose to 1/400, and in a further 5, to 1/800 or more. Only 5 (28%) of these 18 people admitted having had a febrile illness during the intervals between collection of the sera. Of the remaining 28 whose antibody titer rose from <1/25 to 1/50, or from \leq 1/25 to 1/100, 6 (21%) had malaria parasites. None showed a rise in leptospiral antibody. Two (7%) showed a rise in OXK titer from 1/40 to 1/160. Only 3 (11%) admitted having had a relevant illness.

Only the 18 subjects showing a 4-fold rise in FA titer to at least 1/200 were regarded as confirmed infections. This is equivalent to an incidence of 468/thousand/annum. However, the study was carried out during only part of a year, and most of the sera were collected from January to April.

Bukit Lanjan: Bukit Lanjan is a small forested hill 5 miles West of Kuala Lumpur. Approximately 250 Orang Asli live in a kampong on the jungle fringe, and pursue a variety of urban and rural occupations, including much travel through the jungle. The preliminary survey of the population, using filter paper blood spots, was carried out in 1974, and reported in last year's Progress Report. Sequential sera and clinical data were collected from October 1974 to May 1975, by which time the numbers of volunteers had fallen to an unproductive level.

Of 61 individuals from whom sequential sera were obtained over a total of 234 man months, 8 showed a rise in FA titer (see Table 3) and 2 a concurrent rise in OXK. Of the 8, 4 recalled an illness during the relevant period, and one (H48) had been admitted to Gombak hospital for fever and chills; an isolate of R. tsutsugamushi was obtained from her by mouse inoculation. Five of the 8 had significant antibody rises, an incidence of 5/234 man months, or 256/thousand/annum.

Elmina Estate: A pilot survey using filter paper blood spots showed that 54/135 (40%) of oil-palm workers and 14/93 (15%) of rubber workers had demonstrable antibody to R. tsutsugamushi. In a longitudinal study of oil-palm laborers, sera were obtained on one occasion only from 73 workers, on 2 occasions from 26, 3 times from 12, 4 times from 11 and 5 times from 2. Thus multiple observations were made on 51 individuals over varying periods from 1 to 10 months, and a total of 273 man months of exposure were studied.

Table 1

Prevalence of Scrub Typhus Antibody at Pos Iskandar

<u>Age Group</u>	<u>Prevalence of Antibody</u> ¹
<5	1/3 (33) ²
5-9	2/28 (7)
10-14	11/44 (25)
15-24	16/37 (43)
25-34	20/34 (59)
35-44	29/38 (76)
>44	20/24 (83)

1. A titer of 1/50 or greater.
2. Positives/total (percent)

Table 2

Seroconversions to Scrub Typhus at Pos Iskandar

<u>Age Group</u>	<u>Numbers Showing Antibody Rise</u>	
	Possible ¹ Significance	Definite ² Significance
<5	1	0
5-9	9	0
10-14	7	4
15-24	6	1
25-34	1	5
35-44	3	3
<44	1	5

1. Includes rises in titer from $<1/25$ to $1/50$ and from $\leq 1/25$ to $1/100$.
2. Includes 4-fold or greater rises in titer to $1/200$ or more.

Table 3

Seroconversions to Scrub Typhus at Bukit Lanjan

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>FA</u>	<u>OxK</u>	<u>Illness</u>	<u>M.P.'s</u>
H 48	15	F	1/25 1/800	1/80 1/5120	+	-
H 130	8	F	1/50 1/800	1/40 1/40	-	<u>P. vivax</u>
H 1014	14	F	<1/25 1/50	1/40 1/40	+	-
H 1003	6	M	1/50 1/200	1/80 1/80	-	-
H 146	6	F	<1/25 1/50	1/80 1/320	+	<u>P. falciparum</u>
H 144	14	F	<1/25 1/400	1/80 1/40	+	-
H 21	8	F	<1/25 1/50	1/80 1/40	-	-
H 154	12	M	<1/25 1/100	1/80 1/80	-	-

Two workers showed a significant rise in titer of antibody to *R. tsutsugamushi* during this time (see Table 4), and two more lesser rises, one from 1/25 to 1/100, the other from <1/25 to 1/50. Three of the 4, including the 2 showing definite rises (1254 and 1225), admitted having had a febrile illness between the dates of bleeding. None of the illnesses had been prolonged or serious and none, as far as is known, required antibiotic therapy. The subject showing a rise to 1/50 did not recall any illness during the relevant period. From this data, the incidence of definite infections was calculated as 2 per 273 man months, or 88/thousand/annum.

Rodent trapping from a grid system consisting of 200 traps was accomplished at Elmina throughout the entire year. The trapping was conducted for 1 week per month with marking and releasing of the trapped rodents. A total of 696 rats were trapped from which 623 blood samples were taken for isolation studies. *R. tsutsugamushi* was isolated from only 2 (0.32%) of the specimens. Chiggers were collected from all trapped rodents. In addition to collection of chiggers from rodents, a black plate grid of 22 sites within the trapping grid was established, with 10 plates per site for a total of 220 black plates.

The primary vector found within this mature oil-palm estate was *Leptotrombidium* (L.) *deliense* (61.1%). Other *Leptotrombidium* collected were *L.* (L.) *fletcheri* (0.1%), *L.* (L.) *bodense* (0.2%) and *L.* (L.) *near-arenicola* (0.03%). Other than *L.* (L.) *deliense*, *Ascchoengastia* (L.) *indica* was the only other chigger collected in large numbers (30.0%). The remaining species were *Gahrlipeia* (W.) *lewthwaiti* (3.7%), *G.* (W.) *enode* (0.02%), *G.* (G.) *fletcheri* (0.6%), *Walchiella impar* (0.06%), and *A.* (L.) *lorius* (0.4%).

In comparing the chiggers collected from rodents with a black plate chigger index (number of chiggers collected each month divided by the total number of plates) the data tends to follow a parallel line and may prove to be a valuable tool for comparing chigger populations in the field (Fig. 1).

Military Populations (Malaysian Soldiers): Two hundred and seventy five recruits were bled before and after 6 months basic training at Port Dickson. Forty seven (17%) of the patients had FA titers of 1/25 or more. Of these, 6 (2.2%) showed a significant rise in titer between the two specimens, indicating infection in the interval. Three of the 6 admitted having had a febrile illness during training, and one man had been ill for 2 weeks. The remaining 3 men denied any illness during the study period. Four hundred and seventy six soldiers from two infantry battalions (2 Ranger and 11 MTA) were bled before and after a period of operational duty near the Thai border, in June and October 1975. On the second occasion information was obtained, by means of a questionnaire, on any illnesses experienced. Both specimens were

Table 4

Seroconversions at Elmina Estate

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Date</u>	<u>FA Titer</u>	<u>OxK Titer</u>
1254	22	F	20 January	1/50	1/40
			20 March	1/50	1/40
			19 June	1/400	1/320
			20 August	1/100	1/320
1225	23	M	20 January	<1/25	1/40
			20 March	1/200	1/40
			19 November	1/200	1/80

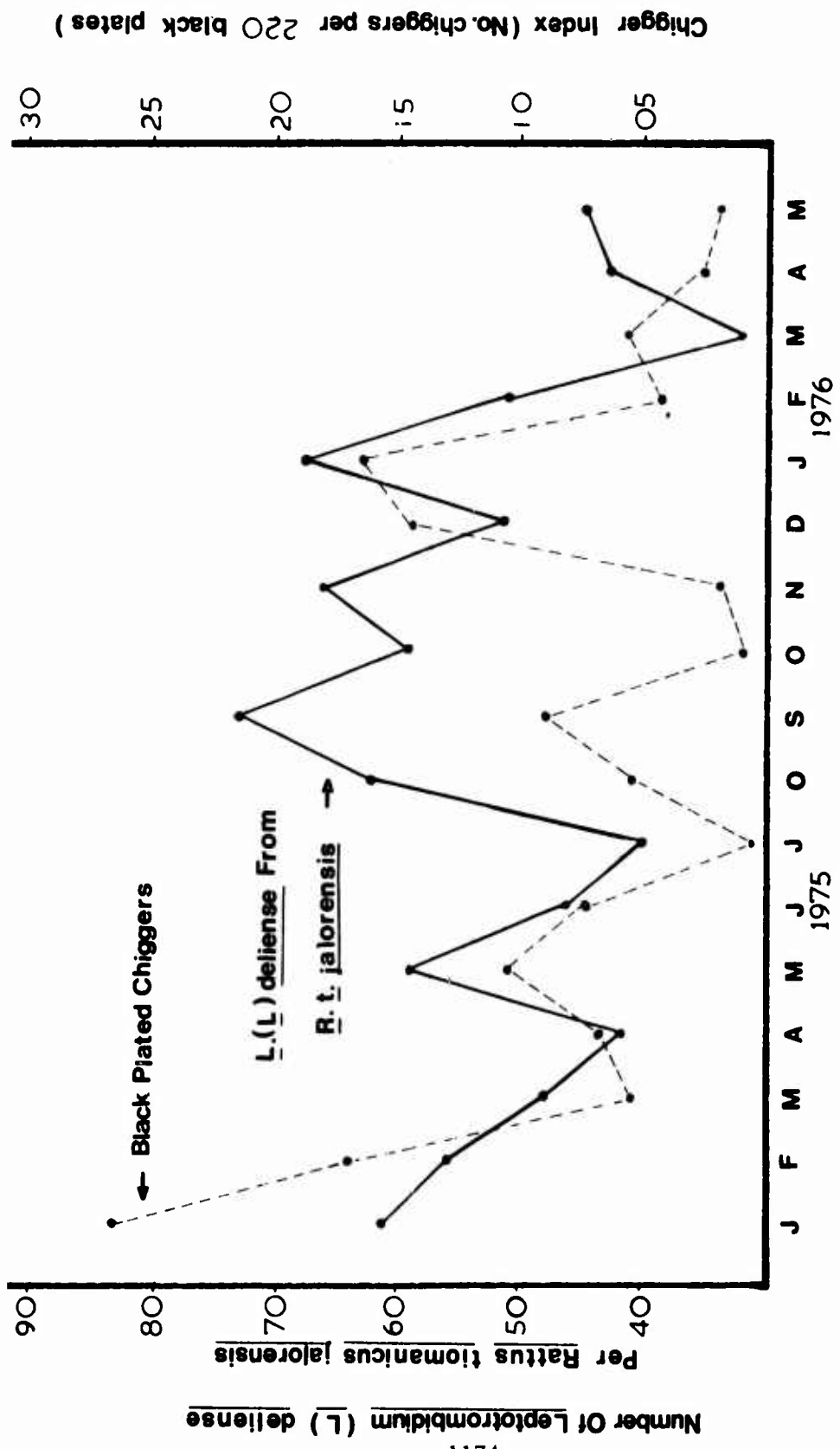


Figure 1. Comparison of black plate collections of chiggers with the number of *Leptotrombidium* (L.) *deliense* collected from trapped *Rattus tiomanicus jalorensis* within an oil-palm estate, Elmina Estate, Selangor.

negative in 235 (49%). In 210 (44%) one or both sera were positive at low levels, though no rise was detected. Eight of 262 (3.1%) of the 11 MTA group had a titer of $\geq 1/400$ in the first serum, indicating infection during the few months prior to our study. This evidence was re-inforced by OXK titers of $\geq 1/160$ (1/2560 in one) in 6 of the 8. Small rises in FA, from $< 1/25$ to $1/50$ or $\leq 1/25$ to $1/100$ occurred in 11 (2.3%) of the whole group, but in only one was this accompanied by a rise in OXK titer ($1/40$ to $1/160$). Of the 11, 5 (45%) admitted a febrile illness, though no details were available. Significant rises in FA were seen in 2 (0.4%) of the group: $1/100$ to $1/400$ in one case (no rise in OXK) and $1/50$ to $1/400$ in the other (OXK $1/80$ to $1/160$). Neither subject admitted any illness during the period.

Military Populations (British Soldiers): In last year's progress report, the occurrence of low level seroconversions amongst visiting soldiers, often without illness, was reported. This was examined further in 98 additional men, of whom 13 (13%) developed antibody to a titer of $1/50$, after a 6-8 week period of jungle training. Sera collected prior to the training exercise had no demonstrable antibody ($< 1/25$). None had clinical malaria nor serological evidence of leptospirosis. The majority denied any illness whatsoever. Blood was collected from each of the 98 men and inoculated into mice. No isolates were made from the mice and the significance of these small rises remains uncertain, as in the indigenous soldiers.

Febrile Patients

Studies were carried out at the district hospitals of Mentakab and Kuala Pilah, the Orang Asli hospital at Gombak, the General Hospital and Kinrara Military Hospital in Kuala Lumpur, and the health sub-center at Bukit Mendi.

Bukit Mendi health center is located on a Federal Land Development Authority (FELDA) oil-palm plantation in southern Pahang. An area of disturbed primary jungle, approximately eight miles by ten has been partially cleared, burned and planted over the past nine years. Most of the plantation is now in production, and approximately 10,000 people have settled, in four villages, on the scheme. Adults of both sexes work in the fields, and children sometimes accompany them. A health sub-center, visited weekly by a doctor, provides medical services for the inhabitants, and was the location for the study.

Mentakab District Hospital serves an area of central Pahang containing many similar FELDA schemes, and also a large population of rural villagers. Kuala Pilah Hospital, in Negri Sembilan, serves a rather different, semi-urban population, and rubber estate and rice field workers predominate over relatively few oil-palm laborers. Gombak Hospital admits Orang Asli patients from all over

peninsular Malaysia though many patients, including those with fever, find the long journey too difficult to make. At the General Hospital, Kuala Lumpur, the study was attempted on one of the 3rd class male medical wards and at Kinrara on febrile soldiers. However, sera were obtained from only a small number of febrile patients, and these two studies had to be abandoned after several months.

At each location, clinical and epidemiological data, and paired sera were collected from unselected febrile patients. In many instances, blood was also inoculated into mice for isolation of *R. tsutsugamushi*. Patients complaining of headaches, cough or general malaise were also included, even if not febrile at presentation. Young children, from whom venous blood could not easily be obtained, were largely excluded from the study. Convalescent sera were collected two weeks after the acute specimens wherever possible, though early discharge from hospital necessitated a shorter interval in many instances. All clinical data was gathered by the clinic and hospital staffs. With the exception of Kuala Pilah, most of the specimens were collected by our technicians.

The paired sera from all study sites were examined for *R. tsutsugamushi* antibody by indirect immunofluorescence, and for *Proteus* OXK agglutinins by the Weil-Felix test. OX2 and OX19 agglutinins were not sought. All sera were stored at -20°C from the time of separation. Results were notified to the responsible doctor as soon as possible after collection of specimens.

Isolation and identification of the organism was performed using the standard technique, in which fresh whole blood is inoculated intraperitoneally into mice. Isolates were confirmed by direct immunofluorescent examination of peritoneal mononuclear cells from the infected mice.

A compatible clinical history, plus any of the following criteria was regarded as confirming the diagnosis:

1. isolation of the organism
2. a four-fold rise in FA titer to 1/200 or more
3. a four-fold rise in OXK titer to 1/160 or more

Either of the following findings was regarded as indicating concurrent or recent infection:

1. an FA titer of at least 1/400, with no demonstrable rise
2. an OXK titer of at least 1/160, with no demonstrable rise

A summary of the results from the six studies is given in Table 5.

Table 5
Incidence of Scrub Typhus in Febrile Patients

	<u>Definite</u>	<u>Probable</u>	<u>Total</u>
Mentakab	47 (19.2) ¹	11 (4.5)	245
Kuala Pilah	33 (5.3)	20 (3.2)	621
Bt. Mendi	49 (13.1)	17 (4.5)	374
Gombak	8 (7.4)	12 (11.1)	108
G.H.K.L.	0	0	15
Kinrara	0	0	15

1. Number of patients as a percentage of the total studied at each location.

The low number of cases documented at Gombak was probably the result of self-selection by febrile Orang Asli, who appeared to stay at home rather than go to hospital. The large number of Orang Asli with high titers, but no rise, may well represent recent but not current infections. Some cases may have been treated with tetracycline at the jungle medical posts. The much higher incidence at Mentakab hospital as compared with Kuala Pilah probably reflects the difference in habitats - a lot of the area surrounding Mentakab is now planted with oil-palm, or is 'scrub' vegetation, whereas the padi fields and rubber estates so common around Kuala Pilah offer less favorable ecological conditions for the vectors.

From the results obtained, it is evident that scrub typhus is a much more common cause of illness than was previously suspected, and that the clinical syndrome, whether mild or severe, is difficult to distinguish from that due to other infections. Eschars, rashes and adenopathy were not usually observed. The infection was particularly prevalent in oil-palm workers, causing an estimated 400 cases annually in the population of 10,000 people living on the Bukit Mendi scheme alone.

Rodent and chigger collections were begun in Bukit Mendi in July 1975, with initial collections being made as a general survey of the various habitats of the entire scheme. When it was determined that a specific area (Phase III) was producing a high incidence of scrub typhus cases, study sites within this Phase were established. Grid trapping systems were established allowing for trapping, marking and releasing, and retrapping of the rodents, with collection of chiggers and blood specimens from each rodent trapped. Two such grids consisting of 100 traps each were set up in Phase III oil-palm. More recently, similar grids have been established in a Phase I oil-palm site which has yielded only a few positive cases of scrub typhus.

From the general survey prior to the establishment of the grids, a total of 10 species of small mammals were trapped, with R. tiomanicus being the dominant species within the entire area, and R. argentiventer and R. exulans being sub-predominant. The rest of the trapped animals included: Tupaia glis, R. muelleri, R. cremoriventer, Callosciurus nigrovittatus, C. notatus, C. caniceps, and Sundasciurus tenuis.

During the general survey, R. tiomanicus had an infection rate of 41.6% (32/77) from Phase I oil-palm, 37.5% (3/8) from Phase III, 37.5% (20/56) from lalang and 27.0% (10/37) from fringe habitat. In R. argentiventer only 16.7% (1/6) from lalang was positive, and none from Phase I oil palm (2) and fringe (4) was positive, thus giving an overall rate of 8.3% (1/12). R. exulans from lalang had 23.8% (5/21) positive. Two R. exulans from Phase I oil-palm and 1 from the fringe were negative. Tupaia glis 22.2% (4/18) from Phase I oil-palm and fringe habitat were found to be infected with R. tsutsugamushi. T. glis within lalang (1) and Phase III (10) were

all negative. None of the other species was found to be positive for R. tsutsugamushi.

L. (L.) deliense was the predominant vector chigger species collected from the four types of habitat during the general survey of Bukit Mendi, with 76.8% from lalang, 49.8% from forest fringe, 70.5% from Phase I and 72.6% from Phase III. L. (L.) fletcheri was also collected from the lalang habitat (14.0%). The collection of large numbers of L. (L.) deliense from the lalang demonstrates the active movement of the chigger hosts between the lalang and adjacent scrub, as L. (L.) fletcheri is the predominant species collected from black plates within West Malaysia.¹

Within the grid systems of Phase III the first area (FF) is a mixed habitat of oil-palm and adjacent swamp forest. Results of the prevalence rate of R. tsutsugamushi isolation from small mammals trapped in this area are shown in Table 6. The trapping and recapture rates were so low that it is not possible at this stage to assess any population fluctuation of any species of the small mammals obtained. Altogether eight species of small mammals were trapped, of which only two species, R. tiomanicus and R. argentiventer were found in the oil-palm habitat. The remaining six species were all from the swamp forest. R. tsutsugamushi was isolated from R. tiomanicus and R. argentiventer. The rest of the species from the swamp forests were negative. Of 60 blood samples from R. tiomanicus 8 (13.3%) were found infected and from R. argentiventer 33.3% (3/9) were infected.

The second area (YY) is exclusively planted with 3-4 year old oil-palm trees. Only two species of rats, R. tiomanicus and R. argentiventer were trapped in this area. (Table 6) Like the first area the trapping and recapture rates were very low. However, 26.3% (10/38) blood specimens collected from R. tiomanicus were positive for R. tsutsugamushi as compared to 43.48% (10/23) from R. argentiventer.

In both these study areas, R. argentiventer was shown to have a higher rate of isolation than that of R. tiomanicus, although the numbers caught were lower than R. tiomanicus.

The trees of the FELDA scheme are planted in rows approximately 6 meters apart. The litter from pruning the trees is placed in a straight line between every other row of trees. The alternate rows are left clear. This is the case in both Phase I and Phase III of Bukit Mendi. These piles of litter provide good harborage for the rodent hosts of the vectors.

The most noticeable difference between the study areas of Phase I and those of Phase III, is the lack of grass covering within Phase I sites. The covering within Phase I is primarily ferns, while that of Phase III is grass and vines.

Table 6

Prevalence of Rickettsia tsutsugamushi Isolation (Blood) from Small Animals in Phase III, Bukit Mendi.

Site	Species of Mammals	Habitat	Total Mammals Tested	Number Positive	Percent Positive
FF	<u>R. tiomanicus</u>	Oil-palm	60	8	13.3
	<u>R. argentiventer</u>	Oil-palm	9	3	33.3
	<u>R. whiteheadi</u>	Forest	4	0	0
	<u>Tupaia glis</u>	Forest	5	0	0
	<u>R. muelleri</u>	Forest	1	0	0
	<u>S. tenuis</u>	Forest	7	0	0
	<u>C. notatus</u>	Forest	2	0	0
	<u>E. gymnurus</u>	Forest	1	0	0
YY	<u>R. tiomanicus</u>	Oil-palm	38	10	26.3
	<u>R. argentiventer</u>	Oil-palm	23	10	43.5

Table 7 presents a comparison of the vector L. (L.) deliense collected from R. jalorensis from both the Phase I and Phase III study sites. During the three months from which data is available, the number of L. (L.) deliense per rodent collected from Phase I has remained fairly constant, ranging from 7.4 to 10.7, whereas the L. (L.) deliense per rodent from Phase III has fluctuated considerably, ranging from 3.6 to 27.7. During much of this time the area was experiencing extreme drought. However, just prior to the high number (27.7), showers were occurring daily for approximately 10 days. As the rodents within Phase I can only acquire the chiggers from the litter piles, and as these would probably retain a more suitable environment for the chiggers even during a dry period, one might expect the number of chiggers per rodent to remain more constant. Whereas within the Phase III area, where the rodents would not only acquire the chiggers from the litter but also from the open grassy areas, which would be highly affected by changes in moisture, a greater fluctuation of numbers of chiggers per rodent might be expected.

Weil-Felix Test

A comparison was made of three methods for the Weil-Felix test, using paired sera from 50 scrub typhus patients. The methods were:

1. the tube agglutination technique, with a 4 hour incubation period, recommended by the manufacturer of the antigen (Wellcome Reagents, Beckenham, England).
2. the standard tube agglutination technique of Shaffer and Goldin² with overnight incubation.
3. the microtiter adaptation of (1), using v-type microtiter plates (Cooke Engineering Co., Alexandria, Va) as described by Gaultney, Wende and Williams.³

The OXK results obtained by each method were essentially the same, but the microtiter method was easier to read, and quicker to perform, and much more economical of antigen and serum.

Using the microtiter technique, OXK agglutination titers were measured in 225 control subjects (see Table 8). There was confirmation of earlier observations, that rises in titer occur also in leptospirosis, and malaria. OXK titers measured in 209 sera obtained from 112 isolate positive scrub typhus patients are shown in Table 9.

LEPTOSPIROSIS

The sera collected in the Mentakab, Kuala Pilah and Bukit Mendi studies have also been examined by the HL technique for leptospiral antibody. The results are shown in Table 10. These

Table 7

Comparison of Total Chiggers and Vector Chiggers L. (L.) deliense
 Collected from Rattus tiomanicus jalorensis from Phase I and
 Phase III Oil-Palm, Bukit Mendi, Pahang.

Month	Number of Chiggers per <u>R. t. jalorensis</u>		Number of <u>L. (L.) deliense</u> per <u>R. t. jalorensis</u>	
	Phase I	Phase III	Phase I	Phase III
February	26.5	36.5	10.7	13.8
March	33.7	64.3	7.8	3.6
April	28.5	52.5	7.4	27.7

Table 8

OXK Titers in 225 Control Subjects

Source	Reciprocal Titers						Total Sera
	≤40	80	160	320	640	1280	
50 British soldiers	39	11	-	-	-	-	50
50 Malaysian soldiers	41	9	-	-	-	-	50
25 Malaria ^{1,2} patients	34	12	3	-	1	-	50
100 Leptospirosis ^{1,3} patients	66	68	51	8	6	1	200

1. Acute and convalescent sera from each patient.
2. A 4-fold rise in titer occurred in 3 patients.
3. A 4-fold rise in titer occurred in 12 patients.

Table 9
 OXK Titers in 112 Scrub Typhus Patients¹

Day of Illness ²	Reciprocal Titers						Positive ³ /Total (Percentage)
	≤40	80	160	320	640	≥1280	
1-7	42	17	16	9	6	3	34/93 (37)
8-14	4	11	13	9	10	18	50/65 (77)
15-30	3	4	6	8	8	22	44/51 (86)

1. 209 sera from 112 isolate positive patients.
2. As reported by the patients.
3. Titer $\geq 1/160$.

Table 10
Leptospirosis¹ in Unselected Febrile Patients

	Mentakab Hospital	Kuala Pilah Hospital	Bukit Mendi clinic
Leptospirosis	26 (8) ²	41 (6)	19 (4)
Total Patients ³	318	688	431

1. Criterion for diagnosis: a 4-fold or greater rise in HL titer.
2. Number of patients as a percentage of the total at each location.
3. Includes all patients with paired sera collected 3 or more days apart.

results differ from those in previously reported series from Malaysia in that these patients were not selected for symptoms, signs, age or sex. Thus a balanced indication of the overall importance of the infection as a cause of fever is obtained. Noteworthy features in this series are (1) the number of cases in the Bukit Mendi group who were treated as outpatients, often with no diagnosis or specific treatment, (2) the fact that jaundice was present in only 2 (2%) of the 86 cases, (3) no mortality was documented and (4) the majority of the cases were not suspected clinically.

IDENTIFICATION OF STRAIN ACTIVITY IN NATURALLY OCCURRING SCRUB TYPHUS INFECTIONS

Isolates recovered from human scrub typhus infections have been classified on the basis of antigenic composition and antibody response to infection with the isolates. Although the studies are still in progress, findings to date indicate that the Karp strain group produces the preponderance of symptomatic infections in the study area.

NEAR-L. (L.) arenicola

A species of chigger taxonomically intermediate to L. (L.) deliense and L. (L.) arenicola has been found to occur in several locations in the central part of West Malaysia. This species is currently being referred to as near-L. (L.) arenicola. The scutal measurements matches that of L. (L.) arenicola but the scutal setae measurements are closer to L. (L.) deliense. Traub⁴ in his original description of L. (L.) arenicola described it as having a palpal formula of NNNNN but indicated that "on an occasional specimen, the dorsal seta of the palpal tibia is frayed or, rarely, even slightly branched", thus giving a palpal formula of NNbNN. Subsequent collections of L. (L.) arenicola have shown that the palpal formula is more often NNbNN. The palpal setae of this intermediate species is also NNbNN.

The name arenicola (or "sand loving") was originally coined because the chigger was only collected from sandy beaches, not from rocky beaches in which the scrub or forest came down to the water's edge. L. (L.) arenicola has been collected throughout West Malaysia from only sandy beaches.

The chiggers which are currently being referred to as near-arenicola have not been collected from a pure sandy habitat. In fact, some of the black plate collections have been from a highly organic soil adjacent to a stream bed. This species, collected from rodents at Bukit Mendi, tends to be somewhat host specific. Of 64 collections, 58 have been from Tupaia glis, with the remaining being collected from R. tiomanicus jalorensis (4) and R. argentiventer (2). If this species is determined to be a true L. (L.) arenicola,

the significance of the finding in regards to the distribution of scrub typhus vectors is apparent.

We are currently attempting to establish a colony of this species of chigger from the central part of West Malaysia. If a colony can be established, then cross-mating of this species with L. (L.) arenicola and other taxonomically similar species can be attempted. In addition taxonomic studies of post-larval stages (nymphs and adults) can be undertaken.

AN ECOLOGICAL STUDY OF RICKETTSIA TSUTSUGAMUSHI IN THE PRIMARY FOREST OF TAMAN NEGARA, WEST MALAYSIA

There has been considerable reference to the possible existence of a jungle cycle of scrub typhus within the literature, but little actual evidence of such occurrence has been presented. To answer this question, rodents and their ectoparasites were collected from the Taman Negara, West Malaysia. Much of the area is primary forest and reachable only by foot or boat. A site at Lata Berhad located approximately 6½ miles from the park headquarters at Kuala Tahan was selected. This area was fairly accessible by boat and ranged from approximately 250 feet to 2000 feet in altitude.

The forest canopy was contiguous and the streams were mostly dry, having water in them only during the rainy season. A similar area across the Sungai Tahan was described by Soepadmo⁵ as being undulating hill or ridge dipterocarp forest.

Mammals were trapped in small animal basket traps (Harrison)⁶. The chiggers were removed and returned to the laboratory for identification. All the mammals were held in traps and returned to the laboratory for identification and serological examination. The presence of rickettsial antibody was determined by the microfluorescent antibody test and R. tsutsugamushi isolation attempts were made using the mouse passage technique (Jackson et al.)⁷.

Altogether 35 animals comprising 10 species of rats, squirrels, and tree shrews were collected (Table 11). These included: Rattus whiteheadi (8), R. surifer (6), R. rajah (4), R. cremoriventer (1), R. sabanus (10), R. muelleri (1), Tupaia glis (2), Callosciurus nigrovittatus (1), Iomys horsfieldi (1) and Lariscus insignis (1). The trapping success rate was 0.6 percent (35/5479 trapping nights) which is low compared to success rates in secondary forests in Malaysia (Lim & Heyneman)⁸, but comparable to other primary forest studies (Lim)⁹.

Two individual rodents, a R. surifer and a R. sabanus were trapped at 250 feet elevation in the fringe habitat by the side of the Kuala Tahan river. The rest were caught in primary forest. All the animals caught were lowland forms (Medway)¹⁰. R. surifer

Table 11

Mammals Collected at Varying Altitudes from Taman Negara, West Malaysia

	Altitude (ft.)						Total
	250	500-600	700-800	1000-1100	1500-1600	>1800	
<i>Rattus whiteheadi</i>	-	3	3	2	-	-	8
<i>R. surifer</i>	1	-	-	2	-	3	6
<i>R. rajah</i>	-	-	-	4	-	-	4
<i>R. cremoriventer</i>	-	-	-	1	-	-	1
<i>R. sabanus</i>	1	2	3	4	-	-	10
<i>R. muelleri</i>	-	-	1	-	-	-	1
<i>Tupaia glis</i>	-	-	1	-	1	-	2
<i>Callosciurus nigrovittatus</i>	-	-	1	-	-	-	1
<i>Iomys horsfieldi</i>	1	-	-	-	-	-	1
<i>Lariscus insignis</i>	-	1	-	-	-	-	1
	3	6	9	13	1	3	35

and Tupaia glis are known to occur from sea-level up to 6000 feet (Harrison)¹¹. R. sabanus and R. whiteheadi have been found to live as high as 2000 feet in forested areas around Kuala Lumpur (Lim)¹² but none of these two species was trapped above 1100 feet.

Of the 2,107 chiggers collected during this study (Table 12), only a single specimen of a vector, L. (L.) deliense, was taken from a R. rajah. This one chigger was the only chigger present on the animal. This animal was trapped at approximately 1000 feet in elevation. Additionally, the only other species of Leptotrombidium collected were L. (L.) bodense (243: 11.5% of the total chiggers collected) and 2 specimens of a Leptotrombidium closely related taxonomically to L. (L.) arenicola.

By far the largest number of species and specimens collected were of the genus Gahrliopia: 8 species in two different subgenera (Walchia and Gahrliopia) totalling 1205 specimens (57.2%). G. (W.) cuspidata had the largest number of specimens collected; 587 or 27.8%. Walchiella oudemansi (419: 20.0%) and Ascoschoengastia (L.) audyi (214: 10.2%) were the only other species in which large numbers of chiggers were collected.

Sera collected from the rodents were screened at 1/25 dilution by FA methods for antibody to the Karp, TA 678 and Gilliam strains of R. tsutsugamushi, epidemic typhus, R. canada, siberian tick typhus, and Q fever. Of this spectrum of antigens only two R. surifer rats were positive for Q fever antibody. No antibody was detected to any of the other strains or species of rickettsia.

Both whole blood and spleen/kidney pools were examined for R. tsutsugamushi by the standard technique. The tissue pool from a R. sabanus and both the blood and tissue pool from a Tupaia glis yielded isolates. All three isolates were antigenically similar and related to the Karp and TA 763 strains. These antigens have been the most common detected in surveys conducted in inhabited areas of the east and west coast of Peninsular Malaysia.

EFFECT OF CYCLOPHOSPHAMIDE, AZATHIOPRINE, AND 5' FLUOROURACIL ON RICKETTSIA TSUTSUGAMUSHI INFECTIONS IN MICE

The class of compounds most widely used to block the immune response to infectious and non-infectious antigens are alkylating agents. Of these the most widely studied is cyclophosphamide (CY). Kazar et al.¹³ studied the effects of CY on Rickettsia akari, R. prowazeki and Coxiella burneti infections in mice. Their studies showed that CY increased the virulence and the number of rickettsia present in tissues and was effective in suppressing antibody formation when given at the specified intervals. Tachibana and Kobayashi¹⁴ found that CY increased the virulence and enhanced the growth of R. sennetsu in mice. Maximum titers in spleen tissue were approximately two log₁₀ higher in chemoimmunosuppressed mice than in control animals.

Table 12

Species of Chiggers Collected from Mammals Trapped at Taman Negara, West Malaysia

MAMMAL SPECIES	CHIGGER SPECIES																	
	<i>L. (L.) dolosus</i>	<i>L. (L.) bodmani</i>	<i>L. (L.) near-orientalis</i>	<i>Gobletia (G.) ocapitata</i>	<i>G. (G.) laubmoisi</i>	<i>G. (G.) masoni</i>	<i>G. (G.) sumatra</i>	<i>G. (G.) fletcheri</i>	<i>G. (G.) mesolepis</i>	<i>G. (G.) patella</i>	<i>G. (G.) marginata</i>	<i>Macropus (M.) (L.) andy</i>	<i>M. (M.) ornatus</i>	<i>M. (M.) malayanus</i>	<i>M. (M.) malayanus</i>	<i>M. (M.) malayanus</i>	<i>M. (M.) malayanus</i>	Total
<i>Buteo buteo</i>	1																	19
<i>B. buteo</i>																		5
<i>B. buteo</i>		26	3		9		21	67										356
<i>B. buteo</i>			579		14	5	97	296	1									1020
<i>B. buteo</i>																		71
<i>Tapia glis</i>		216	2		14		1	6										418
<i>Callomys nigricaudatus</i>		1																5
TOTAL	1	243	2	587	66	37	5	119	369	1	21	214	2	16	419	5	5	2107

Antimetabolites such as purine and pyrimidine analogs have also been shown to have significant effects on immune response. For this study the pyrimidine analog 5 fluorouracil (5-FU) and the purine analog azathioprine were selected as representative of this group of compounds.

Many strains of *R. tsutsugamushi* are not lethal for mice, and death occurs in mice infected with virulent strains coincident with the appearance of antibody. The response of chemoimmunosuppressed mice to inoculation with a mouse virulent and a mouse avirulent strain was studied in anticipation that such studies would further elucidate the mechanisms responsible for pathogenicity.

The *Rickettsia tsutsugamushi* strains used in the study were propagated in specific pathogen free hens' eggs purchased from SPAFAS, Inc., Norwich, Connecticut. The Karp strain was uniformly lethal following inoculation, but the TA 686 strain was not lethal to our particular strain of mice. A 20% yolk sac suspension of the Karp strain contained $10^{8.5}$ LD₅₀ and a 20% suspension of the TA 686 contained $10^{7.6}$ median immunizing doses for mice (ID₅₀).

Cyclophosphamide was purchased as Endoxan-Asta^R 200 mg vials for injection from Asta-Werke Ag, Chemische Fabrik, Brackwede, Federal Republic of Germany. Azathioprine B.P. was purchased as Imuran, 50 mg tablets from Burroughs-Wellcome and Co., London. 5-fluorouracil was purchased in the injectable form from F. Hoffman-La Roche and Co. Ltd., Basle, Switzerland.

Azathioprine was employed at a dosage of 3 mg/kg/day; and 5-FU at 10 mg/kg/day. CY was given either at a continuous dosage of 45 mg/kg/day, at a single dose of 400 mg/kg, or at a dose of 400 mg/kg followed by a second dose of 200 mg/kg 3 days later depending on the experimental design.

Initial experiments were conducted to determine the effect of chemoimmunosuppression on survival following challenge with the mouse lethal Karp strain of *R. tsutsugamushi*. Administration of CY was either initiated one day post challenge and given until 7 days post challenge, or given as a single dose concurrent with challenge.

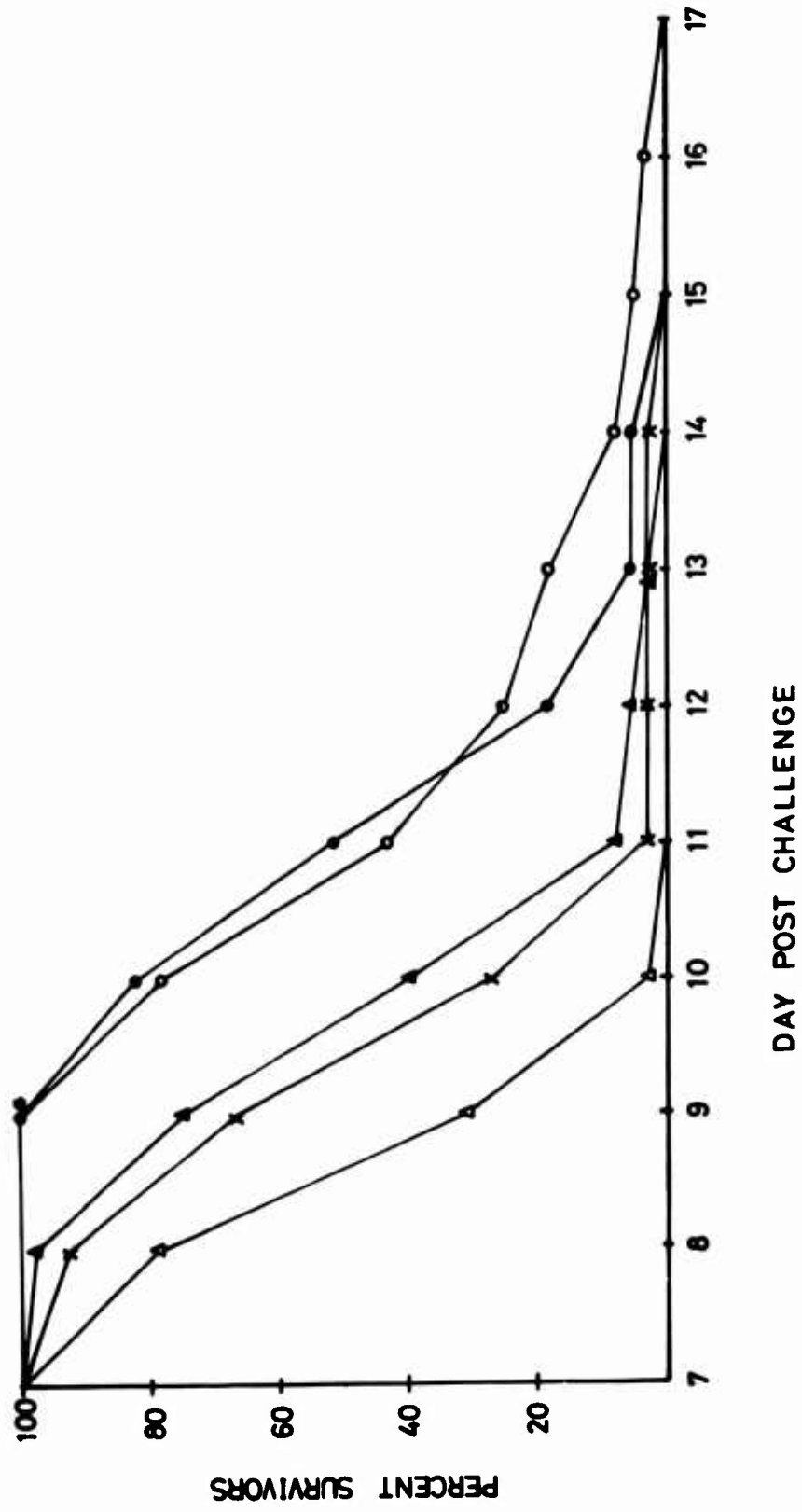
The results are shown in Figure 2. All the experimental mice died following inoculation of the rickettsia and administration of the drugs. The survival time appeared to be prolonged with azathioprine and 5-FU and shortened with daily doses of CY. These changes were consistent when the experiment was repeated. No deaths occurred in control groups administered the drugs alone.

The same parameter was studied under the same conditions following an equivalent infectious dose of the TA 686 strain. This strain is not lethal for mice, but previously exposed mice are immune to challenge with virulent strains. Since no mice died when

Caption

Figure 2. Effect of chemoimmunosuppression on infection with the mouse virulent Karp strain of R. tsutsugamushi.

△ CY daily, days 1-8 post inoculation; ▲ CY single dose on day 1 post inoculation; O azathioprine on day 1; ● FU DR on day 1; X control - Karp strain only.



inoculated with TA 686 alone or with TA 686 and azathioprine or 5-FU, only the response of mice to CY inoculation is shown in Figure 3. The mice given a single 8 mg dose of CY first evidenced signs of illness on day 10 post challenge which was approximately 2 days longer than the incubation period of the virulent Karp strain. A few mice died each day through day 15 post inoculation when 17/39 (44%) were alive. The daily administration of a small dose of CY with TA 686 produced signs of infection 1 day later, but the death patterns more nearly approximated the Karp strain in that the majority of the mice died over a three day period. At 28 days, the termination of the experiment, 10% (4/40) of the mice were alive.

The effect of varying the day of administration of the drugs on survival and antibody titers is shown in Table 13. No deaths occurred in the experimental groups inoculated with TA 686 strain and given azathioprine or 5-FU. However, CY given by either dosage schedule increased the sensitivity of the mice to this normally avirulent strain. When the single dose was given near to the time of the rickettsial challenge some mice survived, but when the CY was given between days 4 and 7 post challenge there were no survivors.

The titers of rickettsia in liver/spleen pools and in peritoneal exudate were determined when all of the CY mice were showing signs of the disease and approximately 20% had died (Table 14). At 8 days post inoculation little difference could be detected between the titers in peritoneal fluid between mice inoculated with CY and those inoculated with PBS. Also, little difference could be detected between the titers in tissues from mice treated with a single 8 mg dose on day 3 and control titers. When the 8 mg dose was followed on day 5 with a 4 mg dose the maximum mean difference was 1.0 log₁₀.

When peritoneal exudate containing cells was stained by the direct fluorescent antibody method a distinct difference was noted between CY treated mice and the other experimental groups as well as controls. The individual organisms stained much more distinctly and larger numbers were visible. We reasoned that significant levels of humoral antibody would coat the organisms and interfere with the direct staining technique. A series of exudates were stained with the direct fluorescent antibody method using conjugated rabbit origin antimouse globulin, and the rickettsia from the azathioprine, 5-FU, and control animals fluoresced indicating that they were coated with mouse globulin. However, the organisms from CY treated mice could not be observed by the use of antimouse globulin alone.

Smears were prepared from the peritoneal fluid of drug treated, infected mice and stained with monospecific conjugate. No antigens were detected in the isolates that had not been detected in the original seed material.

Caption

Figure 3. Effect of chemoimmunosuppression on infection with the mouse avirulent TA 686 strain of R. tsutsugamushi.

△ CY daily, days 1-8 post inoculation; ▲ CY single dose on day 1;
X control - TA 686 strain only.

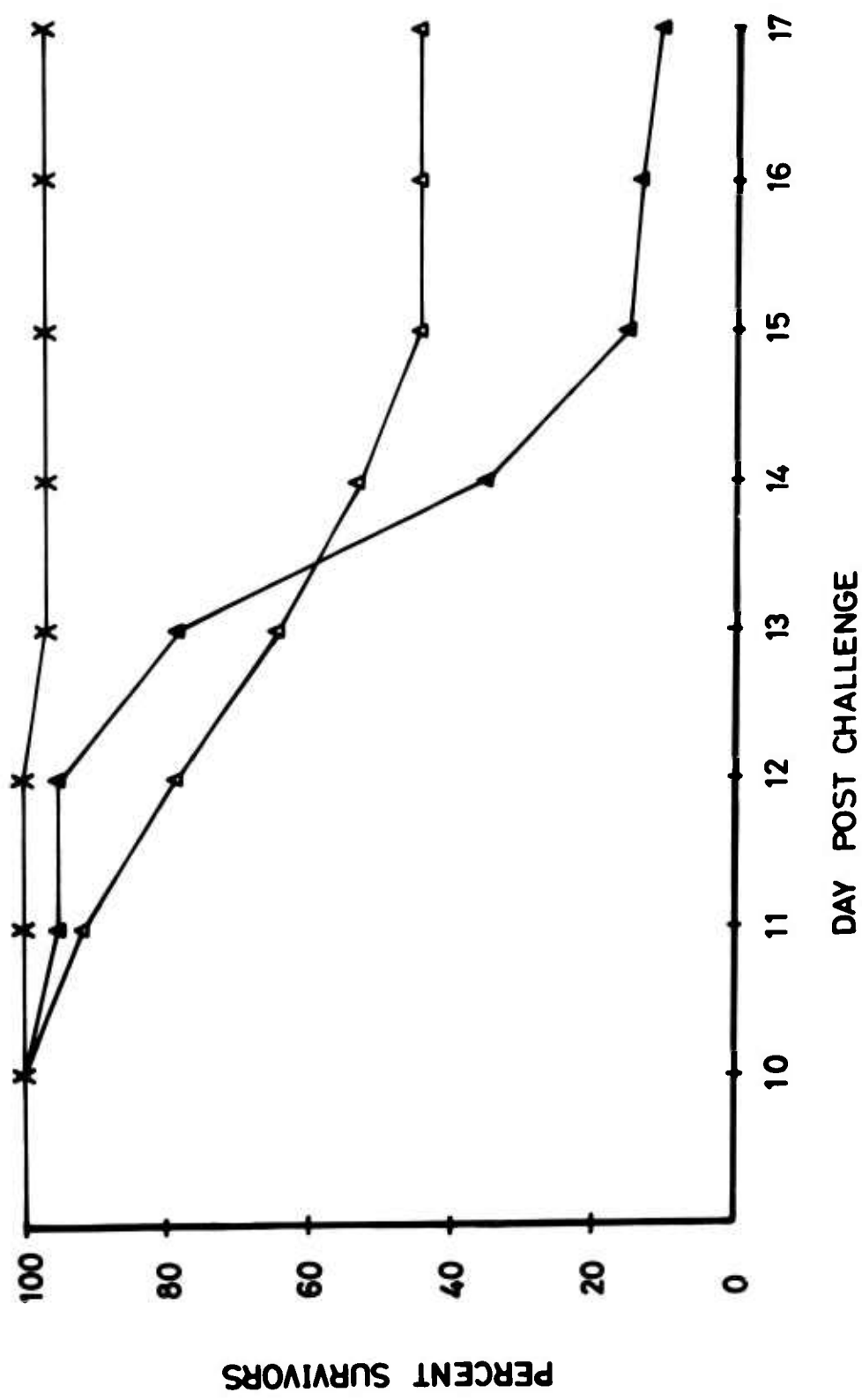


Table 13

The Effect of Varying the Day of Administration of Cyclophosphamide on Survival and IFA Titer in Mice Experimentally Infected with TA 686 Strain of *R. tsutsugamushi*

Day of Administration of drug ^a	CY				Aza	FUDR
	Single dose		Continuous			
	Survivors ^b	Titer ^c	Survivors	Titer	Titer	Titer
-1	26	20	0	-	320	80
0	33	80	15	40	640	160
1	44	80	10	40	640	160
2	36	80	20	160	640	320
3	5	80	28	160	320	160
4	0	-	33	80	320	160
5	0	-	31	80	640	160
6	0	-	5	160	640	160
7	0	-	80	80	320	160
Control	100	80	100	80	80	160

a. 0 was day of inoculation of TA 686 strain.

b. percent.

c. reciprocal homologous IFA titer of sera pool from survivors at 28 days.

Table 14

R. tsutsugamushi Titers in Normal and CY Treated Mice

Strain	PBS	CY	
		8 mg	12 mg
TA 686 (avirulent)			
Liver/spleen pools	5.6 ^a , 4.7	5.8, 5.5	6.6, 5.8
Peritoneal fluid	4.6, 4.2	5.2, 4.5	5.3, 4.8
Exp (virulent)			
Liver/spleen pools	6.3, 6.5	ND ^b	7.3, 6.0
Blood	4.5, 3.3	ND	5.4, 3.8

a. Log₁₀ values of separate experiments.

b. Not done.

The antibody titers presented in Table 13 are from sera collected at 28 days post inoculation. The titers of the 4 groups of control mice varied from 1/80 to 1/160 which was not significantly different from the titers of the groups given CY subsequent to the inoculation of organisms. Groups given CY prior to or concurrent with challenge usually had lower titers, but the small numbers of survivors in these groups may have distorted the results. Mice given azathioprine had significantly higher titers than controls at 28 days, but no difference was detected between control and experimental values following the administration of 5-FU.

The antibody response of mice following inoculation of the TA 686 strain with drug treatment was studied to determine the production of antibody to each of the 9 putative prototype strains. The results are presented in Table 15. The titers were closely associated in every case with the same 5 strains. This indicated that immunosuppression early in the course of the infection did not allow additional antigens to be expressed in comparison to control mice.

The inoculation of homologous convalescent sera had no effect on the survival of mice inoculated with CY. When CY was given 90% of the mice died following inoculation with the TA 686 regardless of the administration of 0.5 ml of homologous antibody which titered 1/320 in IFA.

The administration of CY to mice infected with an avirulent strain of *R. tsutsugamushi* increased the virulence of the strain in the treated mice. These results are similar to those found with other rickettsia (Kazar et al)¹³. The cause of the increased virulence in *R. tsutsugamushi* is not as clear as it is in the other rickettsial diseases. Increased virulence in immunosuppressed mice has been correlated with increased growth of the organism in *R. sennetsu*, *R. prowazeki*, *R. akari* and *C. burneti*; but we did not find such a correlation in *R. tsutsugamushi*. Titers in spleen cells, blood, and peritoneal fluids of immunosuppressed mice were $\leq 1 \log_{10}$ different than in control animals.

In this study CY treated mice died following inoculation of a normally non-lethal strain. Deaths were not mitigated by passive immunity, and mice which had recovered from the infection several months before became rickettsemic and a small percentage died following CY treatment. These facts indicate that the increased virulence can not be solely attributed to the inhibition of antibody production. Other investigators have postulated a nonspecific toxic effect of CY which may be important in decreasing the resistance of animals following inoculation of normally avirulent strains or species.

There is general agreement that CY is effective in aborting antibody responses when given concurrently with or soon after

Table 15

Homologous and Heterologous Antibody Responses Following Infection
with the TA 686 Strain and Drug Treatment

	Karp	Kato	Gill	TA 686	TA 716	TC 586	TA 678	TA 763	TH 1817
CY	(8) ^a	<10	<10	24	87	<10	<10	61	56
Azathioprine	(8)	<10	<10	470	79	<10	<10	14	54
5-FU	(8)	<10	<10	159	100	<10	<10	20	79
Control	(3)	<10	<10	32	50	<10	<10	10	32

a. Number of pools of mouse sera tested.

b. Reciprocal geometric mean titer.

antigen (Aisenberg)¹⁵. In our system, the titers of the survivors did not appear to be reduced when compared to controls which had not received CY. This was a result of the unavoidable bias of necessarily studying the titers in survivors and our choice of 28 days as a time to measure antibody production. Stockman et al.¹⁶ showed that mice given an equivalent dose of CY regained B cell responsiveness at 10-14 days. The organism persists for long periods of time in mice; and therefore, was able to act as an immunogen once the mouse had recovered B cell reactivity.

The increase in antibody titer following treatment with azathioprine was not expected since the drug is widely used to reduce the immune response to homografts. However, this property has been used to advantage in the laboratory to produce hyperimmune sera to several strains which are poorly antigenic.

A NEW CAPSULE FOR FEEDING CHIGGERS (ACARINA: TROMBICULIDAE)

In the life cycle of trombiculid mites, the larval stage or chigger must feed on a vertebrate host. Thus, to maintain colonies for *Rickettsia tsutsugamushi* transmission and general bionomic studies, chiggers are usually fed on laboratory white mice. Two basic feeding methods have been employed at the Institute for Medical Research in Kuala Lumpur. The ear feeding technique as described by Nadchatram¹⁷ has been used for both mass feeding and single rearing, but intensive observation of the feeding of the chiggers is difficult as the mouse must often be anesthetized. The use of a capsule (Baker et al.)¹⁸, glued to the back of a white mouse, has been useful for feeding and provides a means of close observation.

The first capsule for the feeding of chiggers was constructed from the screw top end of a toothpaste tube and was followed by the use of a small piece of laboratory glass tubing that had been flanged to provide a greater surface area for application of glue (Baker et al.)¹⁸. This type of capsule has proved effective for several years, however, a major disadvantage to this type of capsule is the small area in which the chiggers are applied (5 mm), making observation of the feeding chigger difficult.

The development of a new capsule was initiated in conjunction with systemic acaricidal studies (Dohany et al.)¹⁹. Several types of capsules were tried, including flanged tubing, the neck portion of screw cap glass shell vials and the upper portion of disposable hypodermic syringes with the rubber from the plunger acting as a cap. None of these proved satisfactory for the intended purpose of these studies. The most effective capsule proved to be a portion of a container for a disposable hypodermic syringe (Figure 4).

To prepare the capsule, the expanded portion of a 2.5 cc hypodermic syringe container was cut off and the resulting hole was smoothed of its rough edges. A nontoxic glue (Bostick Universal Cement) was applied to the turned-under edge of the

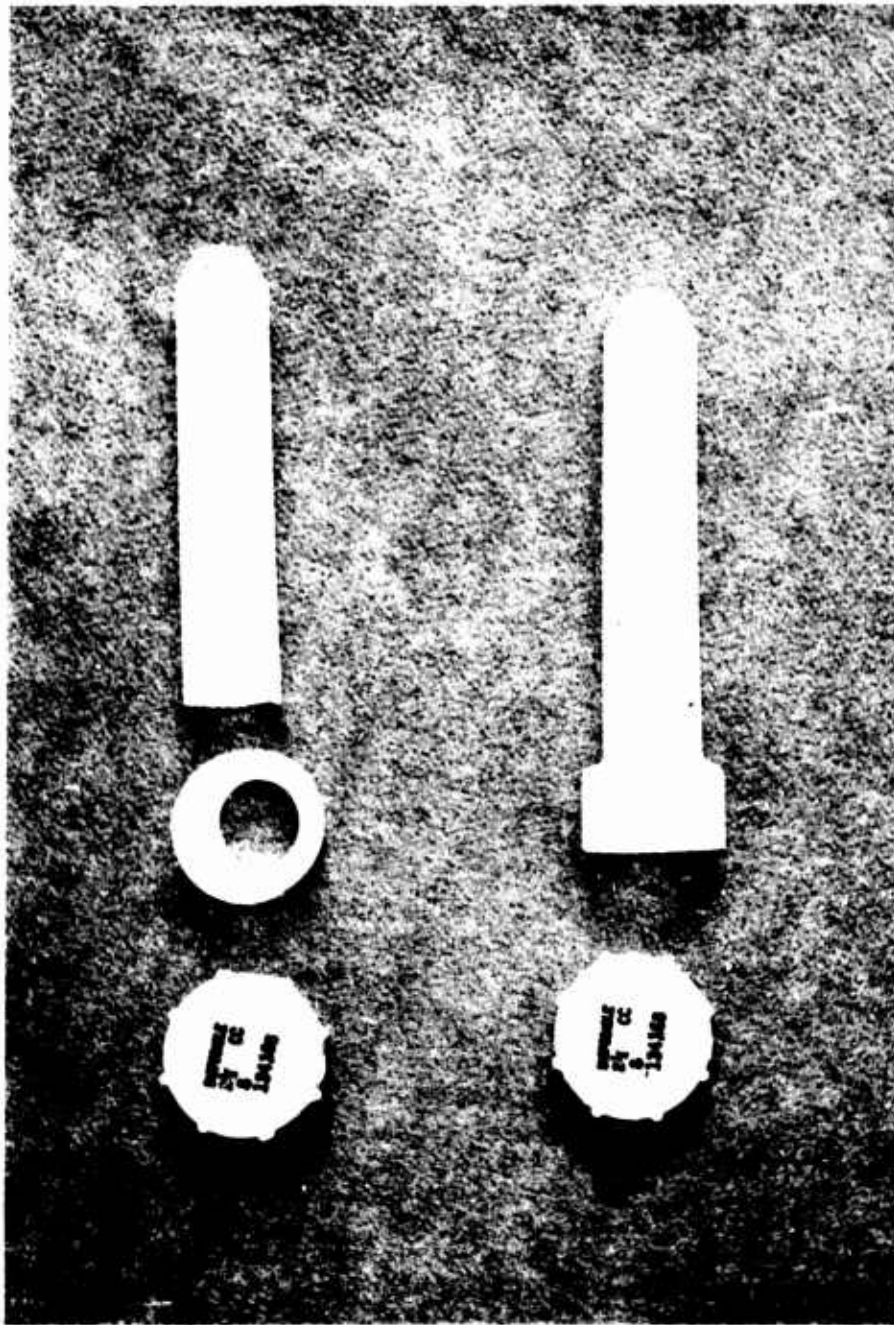


Figure 4. Construction of chigger holding capsule made from the container of a disposable hypodermic syringe.

capsule. The capsule was then applied to the previously shaved back of a guinea pig, laboratory mouse (Figure 5), or silvered leaf monkey (Figure 6). Masking tape was wrapped around the capsule and animal to hold the capsule in place until the glue had set, usually for one to two hours.

The plastic top of the container was used as the cover for the capsule. Filter paper was cut to fit into the top and was kept moist throughout its use. This moisture prevented desiccation of the chigger upon detaching.

While applying the chiggers, a piece of lens paper is used as a gasket between the cap and the capsule proper, thus preventing escape of the unengorged chiggers. For laboratory mice the cap is removed after all the chiggers attach to the host, and the engorged chiggers are collected from a pan of water as described by Baker et al.¹⁸. This method has been used for approximately one year with good success for the rearing of colonies of scrub typhus vector chiggers (*Leptotrombidium* spp.). Alternatively, the cap can be left on and the chiggers can be collected at specific intervals. Usually the chiggers move to the top of the capsule upon disengaging and become trapped in the moisture droplets of the cap, allowing for easy collection.

Advantages of this capsule over the glass tubing capsule include: (1) ease of production (used hypodermic syringe containers can be obtained from most hospitals), (2) an increased feeding area allows for an increased number of chiggers to feed and easier observation of feeding chiggers, and (3) a tight fitting cap is included.

DEVELOPMENT OF AN ANIMAL MODEL FOR R. TSUTSUGAMUSHI

The laboratory mouse has been used for many years for the isolation of R. tsutsugamushi and as an animal model for the study of R. tsutsugamushi infections. However, the requirement for a larger, intermediate animal model is well recognized. Work in this laboratory has shown that silvered leaf-monkeys, Presbytis cristatus, are susceptible to infection with R. tsutsugamushi. Following intradermal inoculation with virulent prototype strains the monkeys developed fever, eschars, lymphadenopathy, rickettsemia, and specific antibodies to the infecting strain. However, there were several drawbacks to the use of silvered leaf-monkeys: (1) the supply is not unlimited; (2) shipping of the animals would be impossible, difficult or impractical, thus geographically limiting their use; (3) some captured animals have shown evidence of prior natural infections; (4) many animals are lost (up to 40-50%) during the conditioning period; and (5) the apparent inability to restrain leaf monkeys for a sufficient period of time to allow chiggers in a capsule to feed to repletion seriously jeopardized the potential use of this animal as a satisfactory model for scrub typhus.

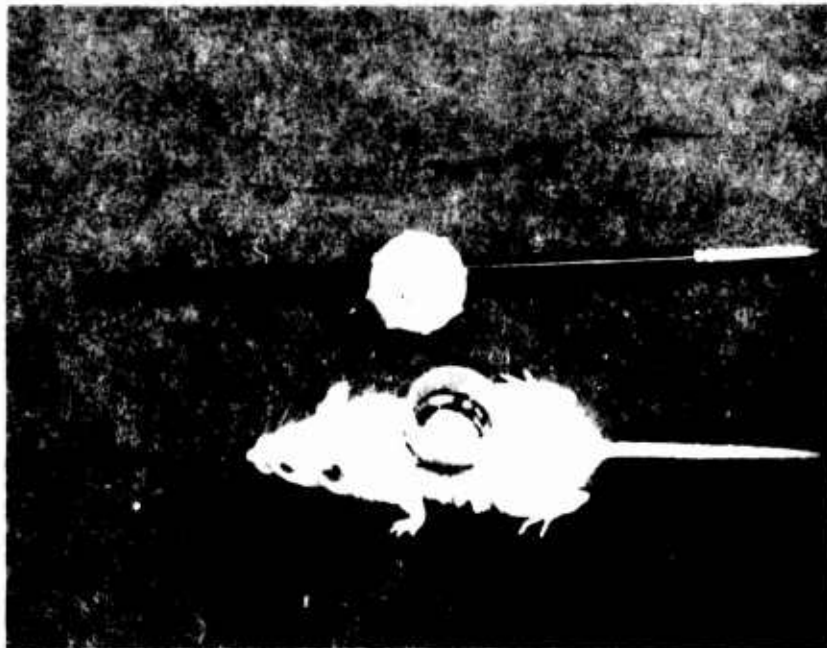


Figure 5. Chigger holding capsule attached to back of laboratory mouse.

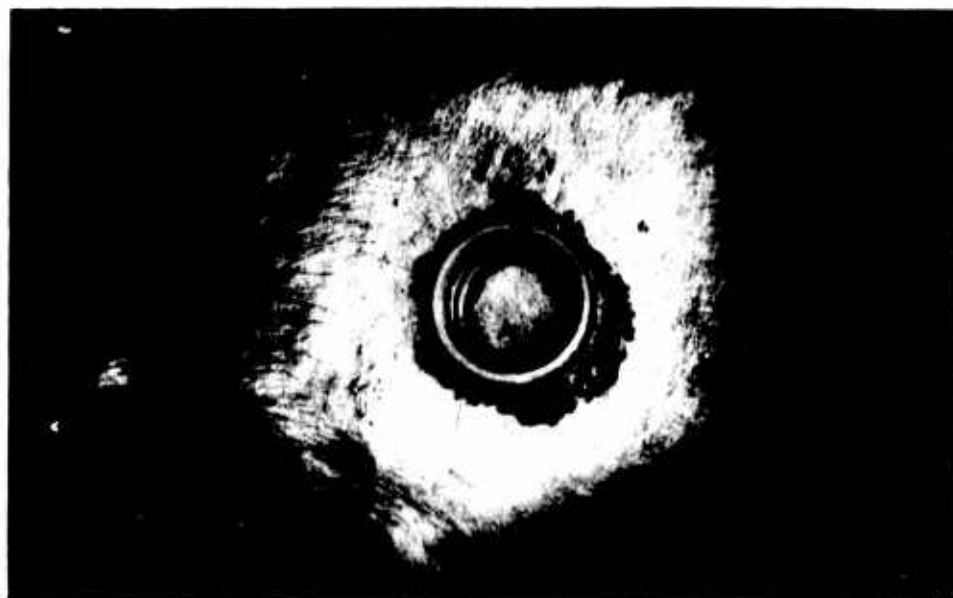


Figure 6. Chigger holding capsule attached to back of silvered leaf-monkey.

These findings prompted us to pursue the development of an intermediate animal model on a broad front. During the past year other animals have been considered and studied, and the above mentioned problems associated with the use of silvered leaf-monkeys have been reexamined with the objective of overcoming or reducing the limitations.

Serological studies²⁰ have indicated that the dog is often naturally infected in endemic areas. This observation plus the results of recent studies with *R. rickettsii* which clearly demonstrated that the dog is a good model for the human disease prompted investigation of the dog as a possible animal model for *R. tsutsugamushi* infection. Additionally, dogs are easily acquired, can be handled readily, have been previously used in well documented laboratory studies in which ectoparasites in capsules were allowed to successfully feed, and are one of the animals on which an enormous amount of basic biological data has accumulated.

In addition to the dog we have recently studied experimental infections in born- and raised-in-captivity cynomolgus monkeys. Early studies with this species indicated that it was relatively resistant to disease following experimental inoculation with *R. tsutsugamushi*, and previous reports from this laboratory indicated that the resistance to disease in wild caught cynomolgus monkeys may be due to immunity resulting from prior infection, since antibody was detectable in a large number of sera collected from wild caught animals.²¹

Studies on dogs and cynomolgus monkeys are still in progress and final conclusions on their potential as a model for *R. tsutsugamushi* must await completion of these studies.

The other approach in the development of an intermediate animal model consisted of further examination of the silvered leaf-monkey with the objective of overcoming factors which appeared to limit its use.

It is highly desirable in some studies to infect animals by the natural route (i.e. feeding of infected chiggers). Thus, a means of producing chigger-transmitted disease in controlled studies is essential to the development of a reliable intermediate animal model. As was mentioned above early attempts in this unit to develop a reliable and predictable means of producing chigger-transmitted infections in silvered leaf monkeys failed due to the inability to restrain the animals sufficiently long to allow chiggers to feed to repletion.

Studies have been initiated to develop a more suitable means of restraining silvered leaf-monkeys. In preliminary trials, using a newly designed restraint chair, silvered leaf-monkeys have been restrained for a period of 5 days, sufficient to allow chiggers to feed to repletion.

SYSTEMIC ACARICIDE TESTS USING DIMETHOATE FOR THE CONTROL OF CHIGGERS

Acaricidal spraying for the control of chiggers frequently is not feasible. The habitat for the vectors, particularly scrub habitat of *L. (L.) deliense*, is often too dense to permit ease of spraying or effective coverage. Use of a systemic acaricide, particularly in conjunction with other control measures, could prove valuable in certain situations.

Systemic insecticides are effective in the control of numerous insects affecting animals, particularly cattle grubs, fleas, lice and ticks. Dimethoate used systemically has proven successful in the control of chiggers in laboratory tests in the United States (Dohany et al, to be published) and in Malaysia (Dohany, unpublished data). Dimethoate is a general usage organophosphate insecticide which is rapidly eliminated from the animal.

An area located on the Kuala Selangor-Kuala Lumpur road was selected for a preliminary study. The site consisted of a small island of forest surrounded by lalang. A grid system of 120 traps and 50 dimethoate bait stations was established. Bait, consisting of 0.01 per cent dimethoate mixed with a 1:1 ratio of ground corn and milo, is maintained in the bait stations. The bait is removed only during trapping periods. Rodents are trapped for 4 nights of each month. The chiggers are removed from the rodents and the rodents are marked and released for retrapping. A control site separated from the study site, was established in the same general area with 70 trapping locations. Acceptance of the bait has proved to be good and initial results indicated that there is considerable reduction in the chigger population within the dimethoate area (Figure 7). Although reduction has occurred, complete control has not been obtained due to immigration of rodents from adjacent lalang and nearby forests. Future studies will be concentrated in a location in which such immigration of nontreated rodents does not occur.

DISEASES OF MILITARY WORKING DOGS

Tropical Canine Pancytopenia

Since the early 1970's USAMRU has provided minimal technical support to the Canine Unit of the Malaysian Armed Forces. The unit is located at the Jungle Warfare School, Pulada, Johor Bahru. Pulada is utilized as a home base for dogs used in operations as well as training and breeding. The initial support consisted primarily of surveillance for tropical canine pancytopenia (TCP), a disease which was responsible for the death of approximately 300 U.S. military dogs in Vietnam. In the 1960's numerous military, police, and privately owned dogs died of the disease in Singapore and Malaysia. This included a large number of dogs at the Jungle Warfare School at Pulada.

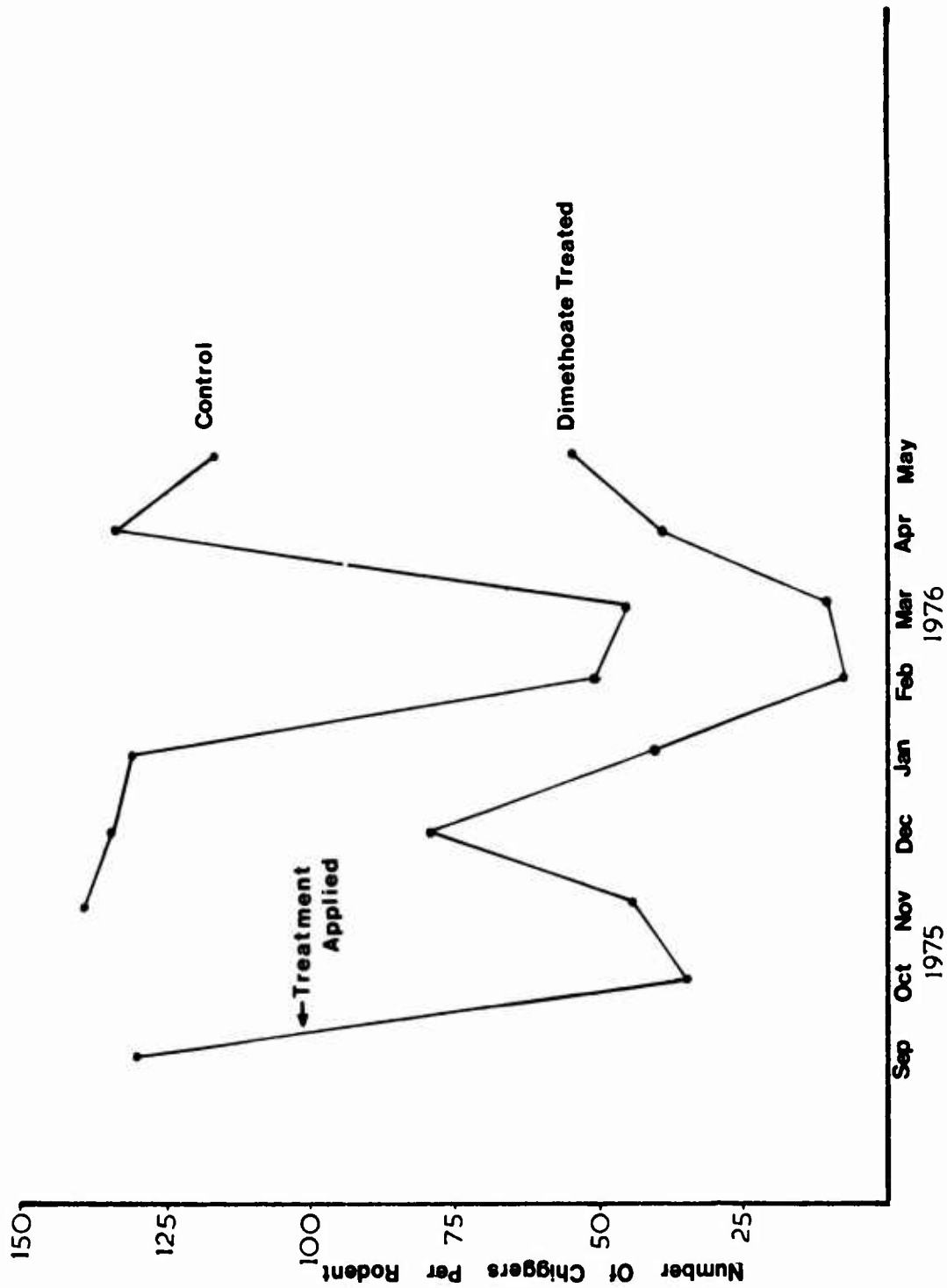


Figure 7. Control of trombiculid mites with the use of a systemic acaricide-dimethoate.

Before a serological test became available periodic hematological examinations provided the only practical means of surveillance. With the development of a serological test for Ehrlichia canis, the causative agent of TCP, a more definitive means of identifying infected animals became available. With the collaboration of investigators at WRAIR and the University of Illinois, periodic serological examinations have been made on the dogs at Pulada.

The Canine Unit at Pulada has afforded an excellent opportunity to observe and study selected clinical and epidemiological aspects of diseases of military working dogs and the efficacy of various therapeutic and prophylactic measures in treating and controlling these diseases.

Following the report of Amyx et al.²², which showed that dogs given tetracycline hydrochloride orally at a daily rate of 3 mg per pound body weight were refractory to infection with E. canis, a decision was made in 1972 to give each dog in the unit one 250 mg. capsule of tetracycline hydrochloride in its daily feed. The policy has continued for four years.

Sera collected in 1972 from 22 dogs were tested for antibodies to E. canis in the immunofluorescence test. Seven of these sera contained demonstrable antibody. Since E. canis has been shown to produce infections which persist in untreated dogs for many years, one can assume that many, if not all, of the dogs serologically positive in 1972 were indeed infected despite a lack of overt clinical signs. These findings also provide evidence that the organism is endemic in the areas in which the dogs are trained and utilized.

Since 1972 there has been no clinical evidence of infection in the unit. Recent serological studies on 50 dogs showed that only 2 dogs had titers to E. canis, and in both instances the titers were low (1:20). In addition no untoward side effects from continuous administration of tetracycline have been observed, and no impairment of training or working capabilities of the dogs has occurred.

These studies plus those recently reported from Thailand²³ have demonstrated that TCP can be controlled in military dogs by daily administration of tetracycline without untoward effects.

Chronic Anemia in Military Dogs at Pulada

During the past two years a large number of the 60 military dogs of the Malaysian Army at Pulada in Johor Bahru have developed an anemia which rendered them incapable of duty. Some dogs have had repeated episodes. Serological evidence indicated that the cause was babesiosis. Blood inoculation studies confirmed this diagnosis. Five normal dogs were inoculated with the blood of five military dogs with low packed cell volume. Of the five dogs inoculated two

developed clinical signs typical of babesiosis with Babesia gibsoni organisms visible in blood smears. Four of the five inoculated dogs converted from negative to positive for babesia on serological examination.

Babesiosis probably goes unrecognized in many pet dogs which are seldom required to do vigorous work or exercise. On the other hand, the military dog is exercised over a prescribed course on a regular basis and any loss of stamina becomes immediately evident to the dog's handler. The anemia, which is often severe renders the dog incapable of completing a course, or worse, incapable of properly performing field duty. These dogs are being utilized as tracker dogs in jungle terrain and are sent out routinely for duty. The dogs then return to Pulada to wait for reassignment. In order to combat the problem of babesiosis in this group of military dogs more information is required concerning the clinical disease, the epidemiology, and effective means of treatment or control.

MANAGEMENT OF TROPICAL LABORATORY ANIMAL RESOURCES

Responsibilities are shared with IMR for production of research animals. The major effort is related to mouse production although rabbits, guinea pigs, hamsters, rats and gerbils are also bred. Approximately 100,000 mice are produced each year and USAMRU uses about 60% of that number.

A group of silvered leaf-monkeys were acquired and conditioned for use in scrub typhus work. This species is extremely fragile, and of 51 animals acquired only 26 survived the conditioning period. Necropsies were performed on all dead animals, however, few lesions were seen. Tissues were collected from some animals for histological examination, and the cause of death was generally felt to be of enteric origin.

Cynomolgus Monkey Breeding Colony

Reports in the literature based on serological studies indicate that a large number of wild caught cynomolgus monkeys have been naturally exposed to scrub typhus. In order to supply monkeys which had not previously been exposed to scrub typhus a breeding colony was initiated. The facility used to house the colony has a concrete floor and consists of a wooden supporting frame with 2" x 2" wire mesh walls and a zinc sheet roof. Piped water is available for cleaning and general sanitation purposes. The colony consists of two groups comprised of a male and ten females. Each group is kept in an area approximately 8 feet wide, 12 feet long and 7 feet high. All breeders were tuberculin tested and found to be negative by intradermal test. In addition, each animal was cultured and found to be free of enteric bacterial pathogens and underwent a minimum of two treatments with levamisole* until no intestinal parasite ova

*Nilverm - Imperial Chemical Industries Ltd., Cheshire, England.

were present in stool samples examined by flotation.

The first group was placed together in September 1975 and the second group was placed together between December 1975 and March 1976. Some difficulty was encountered with the second group and several animals were mauled and/or killed before a compatible grouping could be arranged. Thus, in the second group there was an adjustment period over several months with culling and replacement of incompatible animals.

There have been eight births in the first group although only five are surviving. One infant was stillborn and two were killed shortly after birth. Since the last death occurred a new procedure has been instituted whereby pregnant females are placed into smaller cages within the gang cage room. They are left in the gang cage room so their association with other members of their group is not severed completely. It was felt that complete severance of this association might cause fighting when the females were reintroduced after weaning of their infants at 6 months of age.

At present there is a female in the advanced stages of pregnancy in the first group and three pregnant females in the second group.

Infants are weighed once each month and recordings are made of tooth eruptions and weights.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 071 Field Studies of Rickettsioses and Other Tropical Diseases

Literature Cited.

References:

1. Hubert, A.A., and Baker, H.J.: Studies on the habitats and population of Leptotrombidium (Leptotrombidium) akamushi and L. (L.) deliense in Malaya (Acarina: Trombiculidae). Am. J. Hyg. 78: 131-142, 1963.
2. Shaffer, J.G., and Goldin, M.: In Clinical Diagnosis by Laboratory Methods. Ed. Davidsohn and Wells, W.B. Saunders Co., Philadelphia, Ch.24, p.885, 1962.
3. Gaultney, J.B., Wende, R.D., and Williams, R.P.: Microagglutination procedures for febrile agglutination tests. Appl. Microbiol., 22: 635, 1971.
4. Traub, R.: Two new species of chiggers of the genus Leptotrombidium (Acarina, Trombiculidae). Malaysian Parasites XLV. Studies from the Institute for Medical Research, No.29: 198-204, 1960.
5. Soepadmo, E.: Plants and vegetation along the paths from Kuala Tahan to Gunung Tahan. Malay Nat. J. 24: 118-124, 1971.
6. Harrison, J.L.: A simple trap for squirrels. J. Mammal. 41: 142-143, 1960.
7. Jackson, E.B., Danauskas, J.X., Smadel, J.E., Fuller, H.S., Coale, C. and Bozeman, F.M.: Occurrence of Rickettsia tsutsugamushi in Korean rodents and chiggers. Am. J. Hyg. 66: 309-320, 1957.
8. Lim, B.L. and Heyneman, D.: A collection of small mammals from Tauran and southwest face of Mt. Kinabalu, Sabah. Sarawak Mus. J. 16: 257-276, 1968.
9. Lim, B.L.: Host relationships, and seasonal abundance of immature tick (Haemaphysalis spp. and Dermacentor spp.) in primary and mixed secondary rainforest of West Malaysia. SE Asian J. Trop. Med. Pub. Hlth. 3: 605-612, 1972.
10. Medway, L.: The wild mammals of Malaya. Oxford Univ. Press, Kuala Lumpur, 127 pp., 1966.

11. Harrison, J.L.: Introduction to the mammals of Singapore and Malaya. Singapore Branch Malayan Nat. Soc., 340 pp., 1966.
12. Lim, B.L.: Distribution, relative abundance, food habits and parasite patterns of giant rats (Rattus) in West Malaysia. *J. Mammal.* 51: 730-740, 1970.
13. Kazar, J., Brezina, R.B., and Mayer, V.: Study on the effect of cyclophosphamide on experimental rickettsial infection. *Act. Virol.* 15: 499, 1971.
14. Tachibana, N. and Kobayashi, Y.: Effect of cyclophosphamide on the growth of Rickettsia serratensis in experimentally infected mice. *Infect. Immun.*, 12: 625, 1975.
15. Aisenberg, A.C.: Studies on cyclophosphamide induced tolerance to sheep erythrocytes. *J. Exp. Med.* 125: 833, 1967.
16. Stockman, G.D., Heim, L.R., South, M.A. and Trentin, J.J.: Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. *J. Immunol.* 110: 277, 1973.
17. Nadchatram, M.: A technique for rearing trombiculid mites (Acarina) developed in a tropical laboratory. *J. Med. Ent.* 5: 465-469, 1968.
18. Baker, H.J., Hubert, A.A. and Manikumar, C.: A technique for feeding larval trombiculid mites on laboratory mice. *J. Med. Ent.* 5: 511-513, 1968.
19. Dohany, A.L., Cromroy, H.L. and Cole, M.M.: Studies of systemic acaricides in the control of chigger (Acarina: Trombiculidae) populations on rodents. *J. Med. Ent.* (in press)
20. Alexander, A.D., Binn, L.N., Elisberg, B., Husted, P., Huxsoll, D.L., Marshall, J.D., Jr., Needy, C.F. and White, A.D.: Zoonotic infections in military scout and tracker dogs in Vietnam. *Inf. Immun.* 5: 745-749, 1972.
21. Walker, J.S., Cadigan, F.C., Vosdingh, R.A., and Chan, T.C.: The silvered leaf-monkey of Malaysia, Presbytis cristatus: Disease model for human scrub typhus. *J. Inf. Dis.* 128: 223-226, 1973.
22. Amyx, H.L., Huxsoll, D.L., Zeiler, D.C. and Hildebrandt, P.K.: Therapeutic and prophylactic value of tetracycline in dogs infected with the agent of Tropical Canine Pancytopenia. *J. Am. Vet. Med. Assn.* 159: 1428-1432, 1971.
23. Davidson, D.E., Jr., Dill, G.S., Jr., Tingpalapong, M., Premabutra, S., Nguen, P.L., Stephenson, F.H., and Ristic, M.: Canine ehrlichiosis (Tropical Canine Pancytopenia) in Thailand. *SE Asian J. Trop. Med. Pub. Hlth.* 6: 540-543, 1975.

Publications:

1. Walker, J.S., Chan, C.T., Manikumar, C., and Elisberg, B.L.: Attempts to infect and demonstrate transovarial transmission of R. tsutsugamushi in three species of Leptotrombidium mites. Ann. N.Y. Acad. Sci. 266: 80-90, 1975.
2. Roberts, L.W., Gan, E., Rapmund, G., Chan, T.C., Ramasamy, S.M. and Walker, J.S.: Identification of Rickettsia tsutsugamushi in the life stages of Leptotrombidium fletcheri with isolation and immunofluorescence techniques. Ann. N.Y. Acad. Sci. 266: 73-79, 1975.
3. Dondero, T.J., Jr., Parsons, R.E. and O'Holohan, D.R.: Increased frequency of chloroquine resistant P. falciparum on a rubber estate in Peninsular Malaysia during two years of systematic chloroquine treatment. SE Asian J. Trop. Med. Pub. Hlth. 6: 488-494, 1975.
4. Robinson, D.M. and Huxsoll, D.L.: Protection against scrub typhus infection engendered by the passive transfer of immune sera. SE Asian J. Trop. Med. Pub. Hlth., 6: 477-482, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OB 6528	76 07 01	DD-DR&E(AR)836	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^b	8A. DWS ^b INSTR ^b	8B. SPECIFIC DATA CONTRACTOR ACCESS	8. 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 ^c		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY		62759A	3A762759A831	00	073		
b. CONTRIBUTING							
c. XXXXXXXX		CARDS 114F					
11. TITLE (Precede with Security Classification Code) ^d							
(U) Disease Transmission in Tropical Populations							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^e							
010100 Microbiology 002600 Biology 005900 Environmental Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
73 11		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECESSION		b. FUNDS (In thousands)	
b. NUMBER ^g				FISCAL YEAR		76	
c. TYPE				CONVERT		6	
d. KIND OF AWARD				77		5	
f. CUM. AMT.						147	
18. RESPONSIBLE DOD ORGANIZATION				19. PERFORMING ORGANIZATION			
NAME ^h : Walter Reed Army Institute of Research				NAME ^h : US Army Medical Research Unit			
ADDRESS ^h : Washington, DC 20012				ADDRESS ^h : APO New York 09675			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede SSAN if U.S. Army or Air Force Institute Code)			
NAME: JOY, R. J. T. COL				NAME ⁱ : DIXON, K. E., MAJ, MC			
TELEPHONE: 202-576-3551				TELEPHONE:			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: ROBERTS, D. R., MAJ, MSC			
				NAME: LOVELACE, J. K., CPT, MSC			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Brazil; (U) Infectious Disease; (U) Epidemiology; (U) Entomology; (U) Parasitology; (U) Virology; (U) Serology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Conduct epidemiologic studies of infectious diseases transmitted among populations along the Transamazon Highway in collaboration with the Evandro Chagas Institute. Information regarding health hazards are of importance to military personnel transiting or stationed in this geographic area.							
24. (U) Routine diagnostical, epidemiological, entomological, microbiological, serological and virological procedures are employed. Emphasis is on field studies along 800 Km of the Transamazon Highway and epidemic investigations with laboratory support.							
25. (U) 75 07 - 76 06 The surveillance program including biweekly visits to a stratified random sample of 2300 colonists along the Transamazon Highway continued. The occurrence of Toxoplasma gondii transmission and acute leptospirosis in the colonists was demonstrated. Approximately 20% of the population had antibodies to Trypanosoma cruzi but local transmission was not documented. Results from epidemiological and entomological studies during 3 epidemics of Oropouche virus incriminated Culicoides parensis and Culex pipiens quinquefasciatus as vectors. Laboratory transmission studies were begun with these 2 species. Surveillance data indicated exophilic transmission of malaria along the highway. Rearing anophelines for taxonomic studies was begun. The first case of chloroquine resistant falciparum malaria was detected in the State of Para. A program of surveillance and treatment was initiated to control malaria in a migrant work force. The program will be evaluated for its impact on malaria transmission and potential for local use in combating malaria in migrant populations. A collaborative study on the effects of simuliidae bites on newly arrived Brazilian Army personnel in the Altamira areas was begun. For technical report see Walter Reed Army Institute of Research Annual Progress Report, July 76 - 30 June 76. Support in the amount of \$34,000 from FY 77 funds is programmed for the period 1 Jul - 30 Sep 76.							

PII Redacted

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

1214

PROJECT 3A762759A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 073 Disease transmission in tropical populations

Investigators

Principal: MAJ Kenneth E. Dixon, MC; CAPT A. Lynn Hoch, MSC;
CAPT James K. Lovelace, MSC; Mr. Norman Peterson, M.S.;
MAJ Donald R. Roberts, MSC.

Associate: Amelia Andrade, B.S.; Gilberta Bensabath, M.D.; Elizabeth
Hagerby, B.S.; Mario Moraes, M.D.; SFC Peter J. Ozypowski;
Francisco Pinheiro, M.D.; SP5 Liborio C. Truncali, SP5 Jimmie
R. Tullis.

The following acronyms and Portuguese terms are used standardly in this report.

1. Bairro - a district of a city.
2. Fazenda - a ranch or a large farm.
3. FSESP - Fundação Serviço Especial de Saúde Pública; a national public health service responsible for the administration of special hospitals and research centers.
4. FUNAI - Fundação Nacional de Assistência aos Índios; national Indian agency.
5. Gleba - a standard division of land along the Transamazon highway; 5Km in width and varies in depth; divided into 10 roadfront lots and varying numbers of interior lots.
6. IEC - Instituto Evandro Chagas; FSESP research center in Belém.
7. Lote - a lot; a subdivision of a gleba, normally 1000m x 500m.
8. Município - county.
9. SUCAM -national malaria control agency.

/zcm.

INTRODUCTION

In 1970 the Brazilian government began construction of the Transamazon Highway on an east-west axis between the Belem-Brasilia Highway and the Peruvian border (see map). A government sponsored colonization program began in 1971. Colonists participating in the program originate from every state of Brazil and will undoubtedly be encountering some disease agents for the first time as well as bringing with them organisms not endemic to the Amazon region. In recognition of the potential for the study of disease transmission within a large diverse population that is in the process of settling a heretofore uninhabited area, and the similarity between the ingress of a large number of colonists and the deployment of troops into a medically unstudied region, USAMRU-Belem was established in 1973 and headquartered in the city of Belem in the state of Para. Subsequently two permanent field bases were established along the highway, one in the town of Maraba, the other in the town of Altamira (see map).

USAMRU-Belem presently consists of four sections: Epidemiology, Entomology, Laboratory, and Mammal Ecology. These sections are mutually supporting and share in the mission of:

1. Gathering baseline epidemiologic, entomologic and ecologic data from multiple sites in previously unstudied areas.
2. Rapid investigation of disease outbreaks.
3. Identification of specific research targets with potential importance for military medicine.

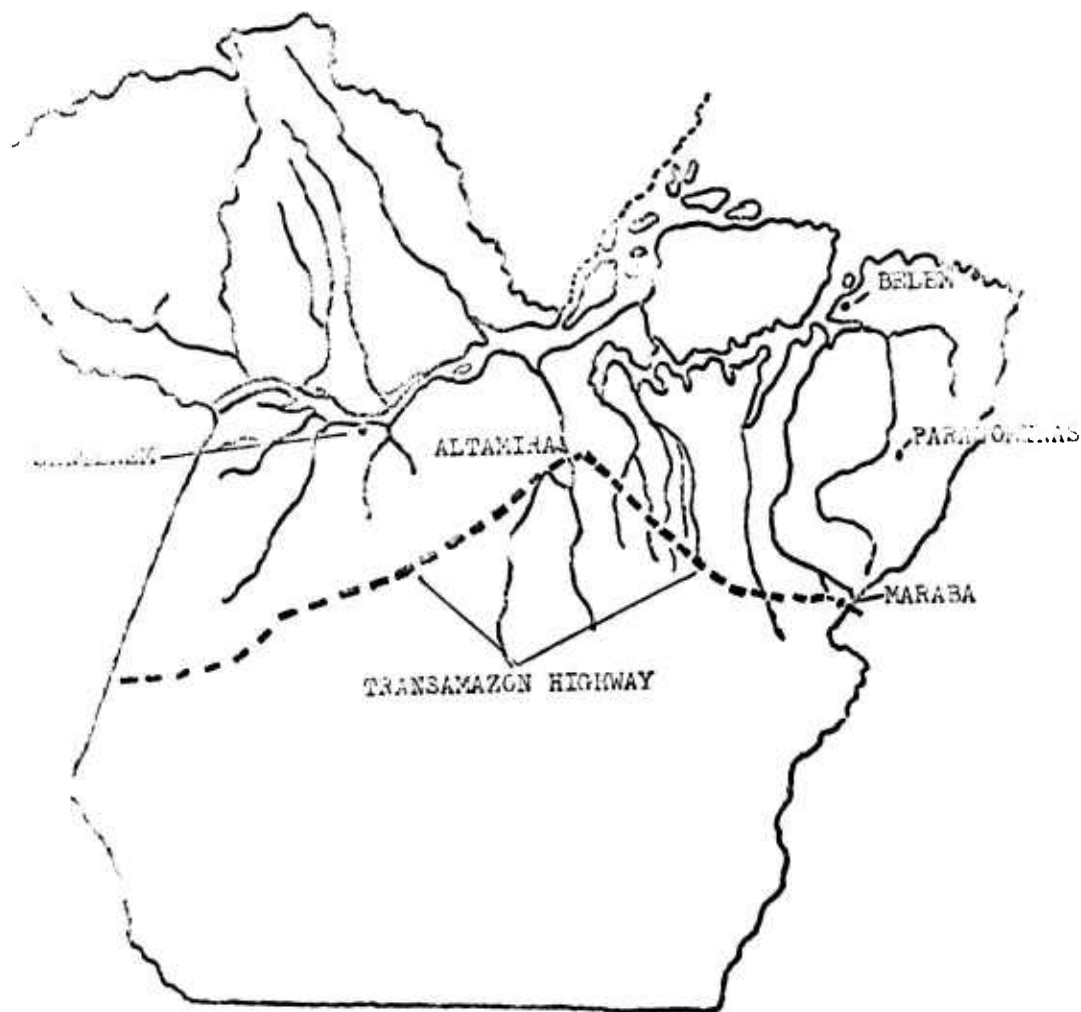
Personnel from USAMRU-Belem also participate in cooperation with researchers from the Instituto Evandro Chagas in the investigation of medical problems in areas of the Brazilian Amazon other than the Transamazon Highway.

Significant progress to date includes:

1. Establishment of a large scale malaria study project on a cattle ranch near the town of Paragominas in southern Para (see map.). The ranch is being cleared of forests by a largely itinerant work force and provides an opportunity for in-depth study of the dynamics of malaria transmission among migrant populations. Initial evidence suggests that this project will afford ample opportunity for both in vivo and in vitro drug resistance testing.
2. Field studies demonstrating the efficacy of repellent impregnated mesh jackets in reducing the number of Simuliidae bites in areas of high black-fly population density.

3. The detection of significant levels of transmission of Toxoplasma gondii and Leptospira spp. among Transamazon colonists. Further serologic testing and comparison of laboratory results with personal history data collected from colonists at regular intervals during the past 18-24 months will clarify the roles of these organisms in colonists' morbidity.
4. Culicoides paraensis Root was incriminated epidemiologically as a vector of Oropouche virus in 1975 epidemics in Para.
5. Seropositivity for plague, schistosomiasis, tularemia, and toxoplasmosis is uncommon in the mammalian specimens tested to date. Forty-five percent of the animals captured at one trapping site were seropositive for trypanosomiasis.

1219 COPY



MAP OF THE STATE OF PARA, BRAZIL, SHOWING THE MAJOR STUDY AREAS OF USAIRU-BELEN

I. EPIDEMIOLOGY

A. Passive Surveillance Program

DESCRIPTION OF PROGRAM AND OBJECTIVES: The passive surveillance program is designed to collect disease information from Brazilian organizations active in the study areas. These organizations include SUCAM, the FSESP hospitals and the town death registries. In addition blood is collected and a history taken from selected patients in the FSESP hospitals.

ANALYSIS OF DATA: Maraba: The data from the death registry in Maraba shows a decrease in deaths from malaria, hepatitis, diseases of the GI tract and diseases of the respiratory tract (TABLE 1) since 1974. The largest category of deaths (64%) was recorded as "no medical assistance" indicating that a physician was not present and no official cause for death could be given.

There were 3553 admissions to the FSESP hospital in the period covered. Aside from OB-GYN problems, malaria was the largest single reason for admission (TABLE 2). Malaria also accounted for the largest number of notifiable diseases reported by the hospital, although there was a marked decrease in malaria cases since 1974 (TABLE 3). In contrast, there were more cases of syphilis and leprosy than 1974.

A review of the SUCAM records showed 3949 positive malaria slides in the current reporting period, a slight decrease from the 4089 positive slides reported in 1974. There was much regional variation, however. The municipios of Itupiranga, Jacunda and Maraba, all of which are within the study area of the USAMRU epidemiology program, showed a marked decrease in malaria cases. Tukurui, also within the study area, showed little change. Several municipios south of Maraba, and outside the study area, showed a large increase in malaria since 1974 (TABLE 4). The data from the highway itself shows a much higher number of cases from Maraba south to Paranorte than in the stretch between Maraba and Altamira which includes the USAMRU study area (TABLE 5).

Between 1 July 1975 and 20 March 1976, history forms were filled out on 944 patients in the FSESP hospital, 70 of whom were febrile at the time of admission. Blood for serology and virus isolation was drawn from 585 patients, including 42 of the 70 febrile patients.

Altamira: The numbers of deaths recorded in the death registry is shown in TABLE 6. As was found in Maraba, the number of deaths due to malaria in Altamira was lower than in 1974. Deaths from diseases of the respiratory tract were also lower, but there was an increase in deaths due to gastrointestinal diseases.

The admissions to the FSESP hospital are shown in TABLE 7. Little data is available from 1974, but the decrease in admissions for malaria and the increase in gastrointestinal disease is supporting evidence for the trends noted above in death rates from these diseases. The decrease in malaria is even more evident in the records of notifiable diseases (TABLE 8). The number of malaria cases dropped from 928 in 1974 to 85 in the 11 month period covered in the table. There was a decrease in hepatitis and, as was found in Maraba, an increase in syphilis and leprosy.

A total of 386 history forms were filled out on patients admitted to the FSESP hospital in Altamira, 114 of whom were febrile. A blood sample for virus isolation, serology and a malaria slide was obtained from 370 of the 386 patients.

TABLE 1. Number of Deaths in the Maraba Jurisdiction July 1975-June 1976 by Diagnosis and Month.

MONTH	MALARIA	HEPATITIS	DISEASES OF THE GI TRACT	DISEASES OF THE RESPIRATORY TRACT	No MEDICAL ASSISTANCE	OTHER
July	3	0	1	6	8	16
August	1	2	2	2	5	12
September	1	0	3	3	6	9
October	0	0	1	7	17	11
November	0	4	2	4	16	13
December	0	1	3	2	5	20
January	1	1	1	3	8	8
February	2	1	0	3	3	13
March	3	0	1	4	6	11
April	2	1	1	3	5	15
May	0	0	2	3	9	16
June	1	0	3	5	4	12
TOTAL	14	9	17	43	87	136
1974 TOTAL	28	12	42	61	61	102

/zcm.

TABLE 2. Admissions to the FSESP Hospital in Maraba, July 1975-June 1976 by Diagnosis and Month

MONTH	MALARIA	TRAUMA	GASTROENTERITIS	RESPIRATORY DISEASE	OB-GYN	OTHER	TOTAL
July	45	17	30	24	121	78	315
August	52	20	28	22	124	60	306
September	19	16	29	28	136	87	315
October	47	19	30	31	102	77	306
November	43	14	33	19	122	71	302
December	41	20	36	15	112	72	296
January	42	26	8	18	106	77	277
February	52	8	4	7	84	97	252
March	35	4	7	15	118	119	298
April	32	17	8	14	108	78	257
May	32	3	1	16	127	149	328
June	37	4	1	8	122	129	301
TOTAL	477	168	215	217	1382	1094	3553

TABLE 3. Reportable Diseases Seen at the FSESP Hospital in Maraba, July 1975-June 1976 by Diagnosis and Month

MONTH	MALARIA	HEPATITIS	SYPHILIS	TETANUS	TB	MEASLES	POLIO	LEPROSY	LEISHMANIASIS	YELLOW FEVER
July	45	0	7	0	5	0	0	0	1	0
August	52	0	5	0	7	0	0	0	0	0
September	16	6	11	1	6	0	0	0	0	0
October	45	8	32	2	4	0	0	0	0	0
November	35	9	11	1	6	2	0	6	0	0
December	42	7	4	0	6	1	0	5	0	0
January	34	4	8	2	5	0	0	2	0	0
February	52	2	0	0	3	0	0	4	0	0
March	36	3	2	0	6	0	0	5	0	0
April	28	6	3	1	3	0	0	0	0	0
May	39	8	5	3	1	1	0	11	0	0
June	36	8	10	1	5	0	0	9	0	0
TOTAL	460	61	98	11	57	4	0	36	1	0
1974 TOTAL	928	80	34	8	41	12	2	3	5	0

TABLE 4. Positive Malaria Slides Reported by SUCAM in the Maraba Jurisdiction, July 1975-June 1976 by Municipio (Country).

MONTH	CONCEIÇÃO	ITUPIRANGA	JACUNDA	MARABA	S. JOAO	SANTANA	TUCURUI
July	50	7	2	170	65	48	11
August	65	--	14	--	84	--	9
September	21	--	7	161	85	13	15
October	16	9	2	178	52	17	13
November	54	3	13	181	75	46	15
December	85	3	--	117	65	213	15
January	186	--	8	178	29	198	1
February	7	4	7	109	53	34	44
March	21	4	7	146	50	30	75
April	91	2	4	77	57	90	11
May	21	1	0	0	1	119	5
June	179	0	--	3	3	86	9
TOTAL	796	33	64	1320	619	894	223
1974 TOTAL	119	156	169	2374	795	269	199

/zcm.

TABLE 5. Positive Malaria Slides Reported by SUCAM along the Transamazon Highway July 1975-June 1976.

MONTH	PARANORTE-MARABA	MARABA-JATOBAL	JATOBAL-ALTAMIRA
July	87	10	11
August	116	2	9
September	116	1	15
October	83	12	13
November	120	1	24
December	103	2	16
January	83	1	1
February	88	3	44
March	66	4	75
April	61	2	44
May	31	1	5
June	66	0	9

/zcm.

TABLE 6. Number of Deaths in the Altamira Jurisdiction July 1975-June 1976 by Diagnosis and Month.

MONTH	MALARIA	HEPATITIS	DISEASES OF THE GI TRACT	DISEASES OF THE RESPIRATORY TRACT	No MEDICAL ASSISTANCE	OTHER
July	0	1	2	4	18	5
August	0	4	3	3	18	11
September	0	3	4	5	13	6
October	1	7	3	1	14	3
November	0	0	0	2	12	8
December	0	6	7	0	4	5
January	0	0	5	0	16	9
February	0	1	6	3	19	4
March	0	3	2	1	13	6
April	0	0	2	1	9	8
May	1	0	1	2	12	7
June	1	2	3	0	14	8
TOTAL	3	27	38	22	162	80
1974 TOTAL	17	29	14	44	186	--

/zcm.

TABLE 7. Admission to the FSESP Hospital in Altamira, July 1975-June 1976, by Diagnosis and Month

MONTH	MALARIA	TRAUMA	GASTROENTERITIS	RESPIRATORY DISEASE	OB-GYN	OTHER	TOTAL
July	7	8	5	6	81	67	174
August	11	3	2	9	90	60	175
September	5	16	4	11	96	66	198
October	10	23	3	8	82	72	198
November	4	11	4	9	86	62	172
December	7	21	3	0	31	53	115
January	6	8	14	21	66	22	137
February	15	7	14	18	70	58	182
March	14	9	11	14	86	48	168
April	5	7	13	8	88	53	174
May	8	9	12	16	95	68	208
June	6	11	16	13	64	186	290
TOTAL	98	133	101	133	935	815	--
1974 TOTAL	250	--	55	--	--	--	--

/zcm.

TABLE 8. Reportable Diseases Seen at the FSESP Hospital in Altamira, July 1975-June 1976 by Diagnosis and Month.

MONTH	MALARIA	HEPATITIS	SYPHILIS	TETANUS	TB	MEASLES	POLIO	LEPROSY	LEISHMANIASIS	YELLOW FEVER
July	7	0	3	0	3	0	0	0	0	0
August	2	0	14	0	4	1	1	0	0	0
September	5	0	16	0	6	0	1	0	0	0
October	14	0	4	0	2	1	0	0	0	0
November	5	0	10	0	2	0	1	0	1	0
December	10	0	6	0	2	0	0	0	1	0
January	2	4	11	1	4	1	0	2	0	0
February	15	6	9	1	2	1	1	5	2	0
March	14	0	11	6	4	0	0	4	0	0
April	6	1	6	0	3	0	0	2	0	0
May	5	2	9	0	3	1	0	7	1	0
June	--	--	--	--	--	--	--	--	--	--
TOTAL	85	13	99	8	35	5	4	20	5	0
1974 TOTAL	928	80	34	8	41	12	2	3	5	0

/zcm.

B. Active Surveillance Program

DESCRIPTION OF PROGRAM AND OBJECTIVES: The surveillance program along the Transamazon Highway is designed to provide:

1. Prevalence data on the sample population every six months.
2. Incidence data on the same population.
3. Rapid identification of disease outbreaks so that intensive investigations may be started at the earliest possible moment.

The study population is composed of persons living along the road and persons living away from the road. The former were chosen by randomly selecting a 20% sample of roadfront lots (2 in each gleba) along one side of the highway and then including an equal number of lots directly across the road. This was done from Maraba west to Aratau (approximately 270 Km.) and from the city of Altamira west to the end of the Altamira jurisdiction (about 250 Km.). In the Altamira area, many people live off roadfront, in agrovilas (small villages of approximately 60 houses). Six of these agrovilas were chosen for study, and all families living in them were included in the sample.

On the first visit to a family, blood is drawn from each family member for serological studies, a malaria smear is made and questionnaires are filled out. Every six months thereafter, blood is drawn from each person and the data on the questionnaires is updated. Every two weeks after the original survey, each family is visited by the field teams. If any member of a family has had an illness episode during the previous two weeks, the details are noted on a questionnaire. At that time, blood is drawn for virus isolation, serology and a malaria slide. Liquid nitrogen is used for holding and transport of specimens.

Information on the number of illnesses encountered, types of illness and results of malaria slides is summarized on a spot map each week by the field supervisor and transmitted to Belem. If it appears that a disease outbreak is in progress, supplementary data from the records of FSESP, SUCAM and the death registry, together with more detailed information from the colonists, may be requested. If these data indicate that a disease outbreak is in progress, an investigating team is sent from Belem.

Each of the two field sites has a field supervisor and a simple laboratory equipped with laboratory benches, stools, a desk, a file cabinet, equipment for separating serum and staining slides and enough supplies to last three months. In Maraba there are three field workers and one vehicle; in Altamira there are four field workers and two vehicles.

The initial results of the active surveillance program were presented in the annual report for July 1974 to June 1975. These data included the age-sex distribution of the colonists and of our sample; the state of origin; number of illnesses encountered on each visit; numbers of colonists in each lot and agrovila; and examples of each of the forms used in the field work.

WORK ACCOMPLISHED: In July 1975, the field workers completed the 12 month visit in Maraba and the six month visit in Altamira (TABLE 1). In February 1976, they completed the 18 month collection in Maraba and the 12 month collection in Altamira. The situation in the agrovilas was complicated by the fact that many of the families originally enrolled moved from their houses in the agrovilas to their lots (TABLE 2). The number of illnesses reported in each of the bi-weekly visits is shown in TABLE 3 and 4.

DATA OBTAINED: Data on the symptomatology of each disease episode was collected and is being coded for computer analysis. This data will be correlated with the results of serological tests for parasitic, bacterial and viral pathogens.

Arboviral serology has been done on the sera from the original visits in Maraba and Altamira and the sera from the first six month visit in Maraba. In Maraba, 13% of the sera were positive for group A arbovirus and 38% were positive for group B. Many of the later group are reactions to the 17-D vaccine for yellow fever. Nine percent of the sera were positive for miscellaneous arboviruses (see TABLE 5), principally Guaroa and Itupiranga. About 1% of the sera reacted with Oropouche antigen. There were 11 sera that converted from negative to a positive titer for Group A, 30 that converted to a positive titer for group B and 17 that converted to a positive titer for miscellaneous arboviruses.

Of the 451 sera tested from the original visit in Altamira, 34 (7.5%) were positive for a group A virus, 80 (17.7%) were positive for a group B virus and 18 (4.0%) were positive for other arboviruses.

Mayaro was the Group A virus most commonly encountered, with 43 (59%) of the 73 positive sera from the original sample in Maraba reacting only to Mayaro. The affected colonists came from various areas of Brazil and are spread out along the entire study area. Twenty-seven (63%) were male and 36 (84%) were greater than 15 years of age. Six (1.2%) of the 485 sera collected during the six month visit represented seroconversions to Mayaro. A similar situation was found in Altamira where 29 (85%) of the sera positive for Group A virus reacted only to Mayaro. The affected colonists came from various areas of Brazil and are spread out along the entire study area. Twenty-five (86%) were male and 24 (83%) were greater than 15 years of age. It would appear that the disease is transmitted along the Transamazon Highway and, since it primarily affects adult males, is probably transmitted in the forest.

Aside from the arboviruses in Group A and Group B, Guaroa was the virus encountered most frequently in the active surveillance program, with 19 (3.8%) of the original sample in Maraba reacting only to Guaroa. Twelve (63%) of the 19 were male and the youngest was 33 years old. Twelve (63%) moved to the Transamazon from the state of Maranhao. In comparison 33% of the sera tested at the time of the original visit came from people who originated in Maranhao. A chi-square test was performed and the difference was significant ($p < .01$). In the original visit in Altamira, 12 (2.3%) of the 449 persons whose sera were tested reacted only to Guaroa. All were males and only two were under the age of fifteen. Of the six sera drawn from people who had come from the state of Maranhao, two (33%) were positive for Guaroa. Although transmission of this arboviruses does occur

along the highway, it would appear that many of the colonists with antibody to Guaroa had contracted their infection in Maranhao.

TABLE 1. Number of sera obtained during the routine semi-annual visits in Maraba and Altamira.

12 month collection	Maraba	
Sera collected	703	(87%)
Absent	81	(10%)
Refusal	23	(3%)
TOTAL	803	
18 month collection	Maraba	
Sera collected	643	(79.5%)
Absent	120	(14.8%)
Refusal	46	(5.7%)
TOTAL	809	
6 month collection	Altamira	
Sera collected	634	(78.6%)
Absent	131	(16.2%)
Refusal	42	(5.2%)
TOTAL	807	
12 month collection	Altamira	
Sera collected	539	(66%)
Absent	141	(17.2%)
Refusal	137	(16.8%)
TOTAL	817	

/zcm.

table 2. Population and Number of Sera Collected from Agrovilas During the Routine Semi-Annual Visits.

	6 months	12 month visit
Sera collected	614 (71%)	292 (64.9%)
Absent	237 (27.4%)	119 (26.4%)
Refusal	14 (1.6%)	39 (8.7%)
TOTAL	865	450

/zcm.

TABLE 3. Number of Illnesses and Number of Febrile Illnesses Seen on Each of the Biweekly visits in Maraba, 1 July 1975-30 June 1976.

VISIT N ^o	DATE STARTED	NUMBER OF DAYS SINCE START OF PREVIOUS VISIT	TOTAL ILLNESSES	FEBRILE ILLNESSES
A - 9	29 Jun 75	20	8	7
A - 10	15 Jul 75	16	5	5
B(six month visit)	11 Aug 75	27	3	3
B - 1	24 Aug 75	13	0	0
B - 2	16 Sep 75	23	3	3
B - 3	03 Oct 75	17	1	1
B - 4	28 Oct 75	25	4	4
B - 5	15 Nov 75	18	6	6
B - 6	08 Dec 75	23	3	3
B - 7	23 Dec 75	15	6	6
B - 8	22 Jan 76	30	2	2
C(six month visit)	09 Feb 76	18	6	6
C - 1	16 Mar 76	35	8	8
C - 2	11 Apr 76	26	0	0
C - 3	25 Apr 76	14	8	7
C - 4	11 May 76	17	4	4
C - 5	26 May 76	15	0	0
C - 6	09 Jun 76	14	3	1
C - 7	28 Jun 76	19	1	1

/zcm.

TABLE 4. Number of Illnesses and Number of Febrile Illnesses Seen on Each of the Biweekly Visits in Altamira 1 July 1975-30 June 1976.

VISIT Nº	DATE STARTED	NUMBER OF DAYS SINCE START OF PREVIOUS VISIT	TOTAL ILLNESSES	FEBRILE ILLNESSES
A - 1	07 Jul 75	32	7	4
A - 2	21 Jul 75	14	4	4
A - 3	08 Aug 75	17	0	0
A - 4	27 Aug 75	19	1	1
A - 5	12 Sep 75	17	5	5
A - 6	01 Oct 75	19	7	7
A - 7	13 Oct 75	12	11	10
A - 8	21 Oct 75	8	11	10
A - 9	05 Nov 75	14	9	7
A - 10	17 Nov 75	12	24	17
A - 11	03 Dec 75	16	10	6
B	21 Jan 76	49	2	2
B - 1	20 Feb 76	29	3	2
B - 2	11 Mar 76	21	7	5
B - 3	22 Mar 76	11	7	5
B - 4	05 Apr 76	14	4	4
B - 5	27 Apr 76	22	4	4
B - 6	17 May 76	21	1	1
B - 7	02 Jun 76	15	0	0

/zcm.

TABLE 5. Results of Arbovirus Serology on Specimens Collected on the Original and Six-Month Visits in Maraba.

Group A Arbovirus

	Original Visit	6 Month Visit
Total	589	485
Positive	73	63
%	12.4%	13.0%

Group B Arbovirus

	Original Visit	6 Month Visit
Total	589	485
Positive	224	174
%	38.2%	36.0%

Other Arbovirus

	Original Visit	6 Month Visit
Total	589	485
Positive	55	44
%	9.4%	9.1%

/zcm.

II. ORPOUCHE VIRUS

BACKGROUND: Oropouche virus is the causative agent of many urban epidemics in the Amazon basin. Until recently, the vector of this virus was unknown. Joint studies by the USAMRU entomology and epidemiology sections, in collaboration with the IEC virology section, have incriminated Culicoides paraensis as the major vector in urban areas.

Preliminary data were reported on this subject in the 1975 annual report. Some of those observations will be repeated in this section with additional data to finalize the studies undertaken. These data were collected during 3 epidemic in 1975.

A. Santarem Epidemic

In the first quarter of 1975 there was an epidemic of oropouche fever in the town of Mojui dos Campos, about 40 Km south of the city of Santarem. As this time there were no cases in the city of Belterra and only scattered suspected cases in Santarem. The physicians at the FSESP Hospital in Santarem were asked to be on the alert for new outbreaks in the area, and to report any cases immediately to the Instituto Evandro Chagas.

During the last week of June, information reached the I.E.C. and USAMRU-Belem from two different sources that there was an increase of cases with the symptoms of oropouche being seen in Santarem. A team composed of MAJ. Dixon, MAJ. Roberts, CAPT. Lovelace, Dr. Francisco Pinheiro and three technicians travelled to Santarem to make initial inquiries.

The physicians in Santarem indicated that the first cases in the city had been seen over two months before, in the last week of April, and that new cases continued to appear. Most of the convalescent cases interviewed had been ill in May or June. In Belterra, the outbreak had occurred earlier and been more explosive. Most convalescent cases had first become ill in March and all the illness episodes since that time appeared to be recrudescences. Many people spoke of reoccurrence of symptoms 2-4 weeks after the first attack. In some instances, a third attack occurred.

An analysis of the SUCAM data showed an increase of non-malaria fever in April and May in most of the municipios (counties) served by the Santarem headquarters. There was no such increase reported in the city itself. Data from the FSESP Hospital showed a slightly increased number of admissions for fever of unknown origin and meningismus. No consistent pattern emerged from the outpatient data.

During 4-8 July, three technicians remained in Santarem to collect school absentee data and draw bloods from a random sample of six different areas of the city. Boundaries were drawn so that the city was divided into three riverfront areas and three inland areas. There was a gradient of relatively dry, somewhat higher land on the east to lower wetter areas in the western edge of the city. Thirteen to twenty-one families were selected from each area and blood was drawn from all family members.

During meetings between USAMRU and I.E.C. personnel in Belem, a plan for coordinated work in Santarem was devised. The I.E.C. took responsibility

for clinical studies of acute cases, entomologic captures in areas from which fresh cases were identified, and field transmission studies using freshly caught Culicoides, the suspected vector, and hamsters. USAMRU was responsible for a broad-based epidemiologic survey including collection and analysis of school absentee data and collection of demographic information, environmental data and blood specimens from a randomly selected sample of the urban population; and for a broad-based entomologic survey of the entire city.

Analysis of the school data from the beginning of school in March to the summer vacation in July showed a uniform pattern of increased absences from late April and early May to the end of classes. The only exceptions were schools from which the data was too scanty for accurate analysis and one private school attended by older and wealthier students. When absentee rates were collected separately for morning, afternoon and evening classes, it was seen that the highest rates were found in pupils attending evening classes and the lowest rates in those attending afternoon classes. This supports our previously formed hypothesis that attendance in the afternoon, during the peak of the Culicoides biting cycle, should confer some protection against a disease transmitted by Culicoides. Attendance in the morning, when the insects are less active should confer less protection, and students going to school at night, and therefore exposed during the entire biting cycle of Culicoides, would be expected to have the highest absentee rates.

Blood for serology was drawn from a total of 93 families with 430 members living in the city of Santarem. Blood was also obtained from 10 families with 56 members living in two villages, Jacumin and Maruru, along the main highway out of Santarem. In September a second blood specimen was obtained from 50% of the original sample. The following seropositivity rates for oropouche were found:

<u>AREA</u>	<u>DESCRIPTION</u>	<u>1st VISIT</u>	<u>2nd VISIT</u>
1	inland, east	1.35%	0%
2	inland, central	3.44%	3.44%
3	inland, west	40.47%	44%
4	riverfront, east	0%	0%
5	riverfront, central	22.38%	34.14%
6	riverfront, west	37.34%	38.63%
Jacumin	outside Santarem	0%	0%
Maruru	outside Santarem	0%	0%

There was a clear division of areas into a group with a very low attack rate (0-3.44%) and a group with an attack rate of 22-44%. When this latter group was analyzed in more detail, it was found that attack rates differed by sex as shown in TABLE 1 (χ^2 10.5, $p < .001$) but did not differ significantly by age.

Twenty-two of the 514 persons interviewed in the city of Santarem reported a febrile illness accompanied by headache or muscular pains during the previous two months. Eight (36%) of the persons with a history of fever were found to have antibodies against oropouche. Seventy-six (19%) of the 408 persons with no history of fever had antibodies against oropouche.

TABLE 1. Santarem, 1975 - Oropouche
Age/Sex Attack Rate in Areas 3, 5, and 6.

AGE GROUP		M	F
0 - 4#	pos	2	2
	total	11	8
	% attack rate	18,18	25
5 - 9#	pos	3	5
	total	18	17
	% attack rate	16,16	29,41
10 - 19#	pos	6	18
	total	28	38
	% attack rate	21,42	47,36
20 - 29#	pos	2	15
	total	6	35
	% attack rate	33,33	42,85
30#	pos	7	21
	total	28	45
	% attack rate	25	46,66
TOTALS #	pos	20	61
	Total	91	143

/zcm.

B. Oropouche Epidemic in Itupiranga

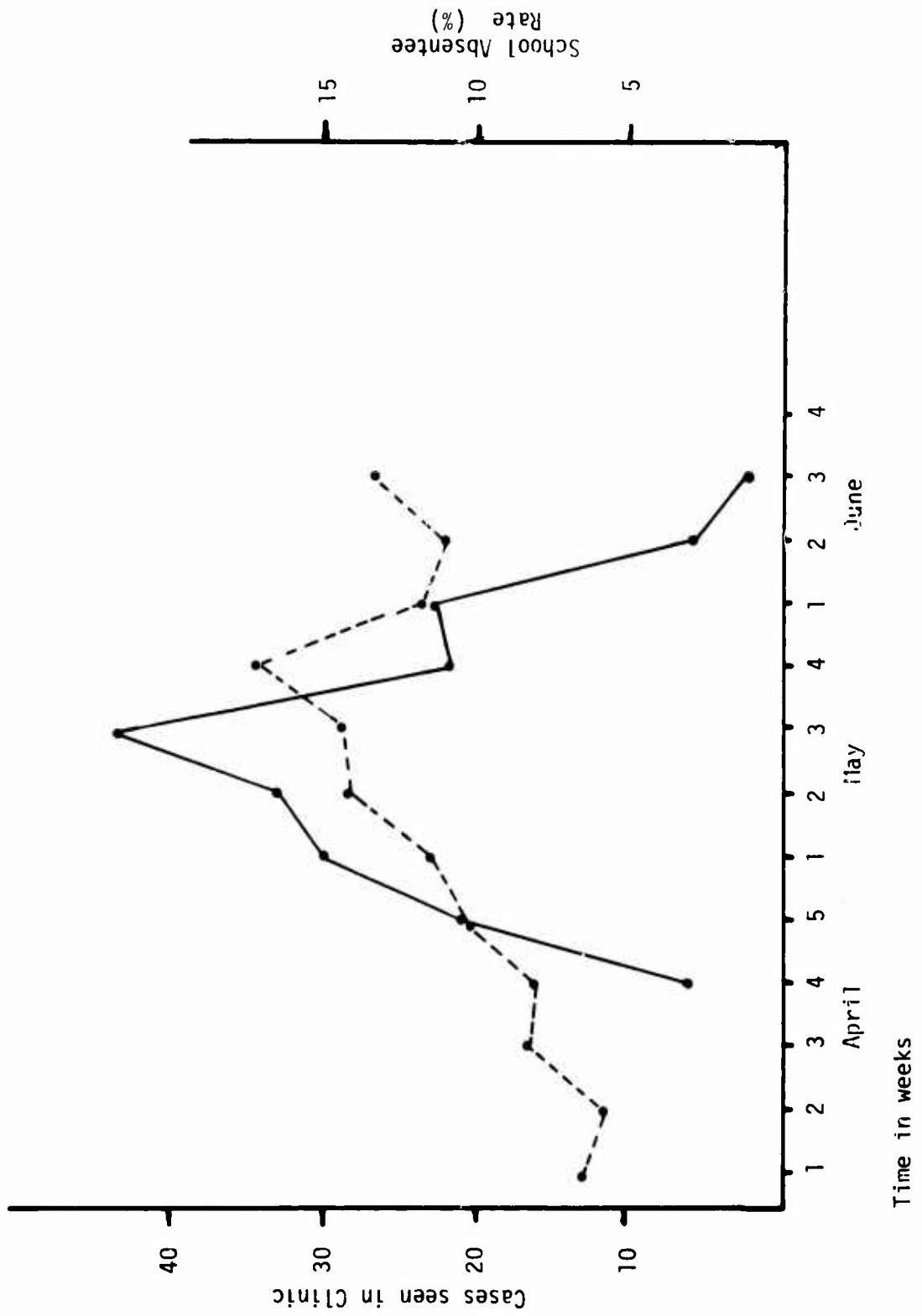
During the first week of June, 1975, the USAMRU team in Maraba received word of an epidemic in the nearby town of Itupiranga. The symptoms of the disease were fever, headache, muscle pain, pains in the eyes and a burning sensation of the skin. The earliest cases were reported to have occurred in the first week of April.

During the preliminary investigation blood for serology was drawn from 59 persons who claimed to have had a recent febrile illness. Thirty-nine of the sera had antibodies to oropouche virus, most with a titer of 1:80. A review of the cases presenting at the clinic with a history of fever, headache and muscle pains showed that the epidemic started in the latter part of April and reached a peak in the third week of May. School absentee data followed the same general curve (FIGURE 1).

A map of the city was made and 45 families were randomly selected. Environmental data, a history of recent febrile disease and 20 cc of blood was collected from each person in the sample.

Six-two (41.3%) of the 150 persons from whom blood was drawn had antibodies to oropouche. There were no significant differences in attack rates for different ages or sexes. There also appeared to be no significant differences in attack rates for different areas of the town, with the exception that those families living on the extreme edge of the town, farthest from the river, had a lower incidence (about 20%).

FIGURE 1. Number of Cases of Fever Seen in Clinic and School Absentee Rate in Itupiranga, April - June 1975.



C. Oropouche, Entomologic Studies

The first epidemic was studied at Mojui dos Campos, Para, in February, 1975. The village is 33 Km southeast of Santarem with a population of about 2000. The second epidemic was studied at Itupiranga, Para, in June, 1975, and the third was studied at Santarem, Para, in July, 1975. The collections relative to this presentation were conducted as follows:

1. Mojui dos Campos: Hourly collections were made in the early morning and late afternoon at 3 houses each day. The collections were conducted inside and outside the house simultaneously. Collections were conducted 3-14 March, 1975.
2. Itupiranga: Continuous landing captures were conducted in the back yards of selected houses within the city from 0600 to 2000 hrs. Collections were made by 2-man teams that rotated in 3 hour shifts. Landing captures were also conducted at the margin of the Tocantins River at sunset. Shannon and CDC light trap collections were made within the city.
3. Santarem: The city was divided into 6 sampling areas and collections were made at 6 randomly selected houses in each area. (FIG. 1). Collections were made in the backyards and were conducted continuously from 1400-2000 hr. for 3 days at each house. Twelve teams of collectors were employed (2 men per team) and 2 areas were covered simultaneously. Each team was visited hourly by trained entomology technicians. During the visits, the hourly collections were gathered and new collection containers were left with the teams. The collections were returned to the laboratory where the C. paraensis were killed, identified, enumerated and grouped for virus isolation. Mosquitoes were preserved in liquid N₂ for later processing. The teams were rotated between houses each day. Forms were also completed for each house on environmental variables, e.g., types of trees, domestic water source, sanitary facilities, etc., to correlate with the absence or presence of C. paraensis.
4. Belem: Belem has been the site of two epidemics of Oropouche virus since 1961. Six barrios in Belem were selected for study in the manner employed in Santarem. This study was undertaken to see if there was a correlation in occurrence of C. paraensis in a similar type of urban area as was found in Santarem and specially for future reference when Oropouche again occurs in the city

PROGRESS TO DATE: Culex pipiens quinquefasciatus and Culicoides paraensis were abundant during the epidemic at Mojui dos Campos. In 36 paired, indoor-outdoor collections of C. paraensis 29% of the total number were collected inside the houses. The documentation of this species as readily

seeking blood meals indoors was done in small domestic lots and probably reflects the proximity of breeding sites and abundant vegetation for protected resting sites. Studies at Palestina demonstrated the anthropophilic-endophilic behavior of Cx. pipiens quinquefasciatus (see Malaria).

Several hematophagous insects were abundant during the epidemic at Itupiranga. These included black flies, C. paraensis, Cx. pipiens quinquefasciatus, and Mansonia titillans (TABLE 1). Black flies and C. paraensis were day active; whereas the mosquito species were crepuscular (TABLE 2). Culicoides paraensis had a pronounced peak in biting activity between 1700 and 1800 hr (FIG. 2).

Several species were collected from Santarem; but, again, only C. paraensis and Cx. pipiens quinquefasciatus were present in dense populations (TABLE 3). The other species were more abundant in rural areas away from the city (TABLE 4).

Dense populations of C. paraensis and Cx. pipiens quinquefasciatus correlated with the prevalence of human Oropouche seropositivity (TABLE 5). The best numerical correlation was obtained with the populations of C. paraensis (FIGS. 3 and 4). Culicoides paraensis, unlike Cx. pipiens quinquefasciatus were also abundant in the rural areas along the Santarem-Curu-Una highways. The absence of human Oropouche seropositivity in the rural areas may be a product of low human population density that did not support the spread of the virus.

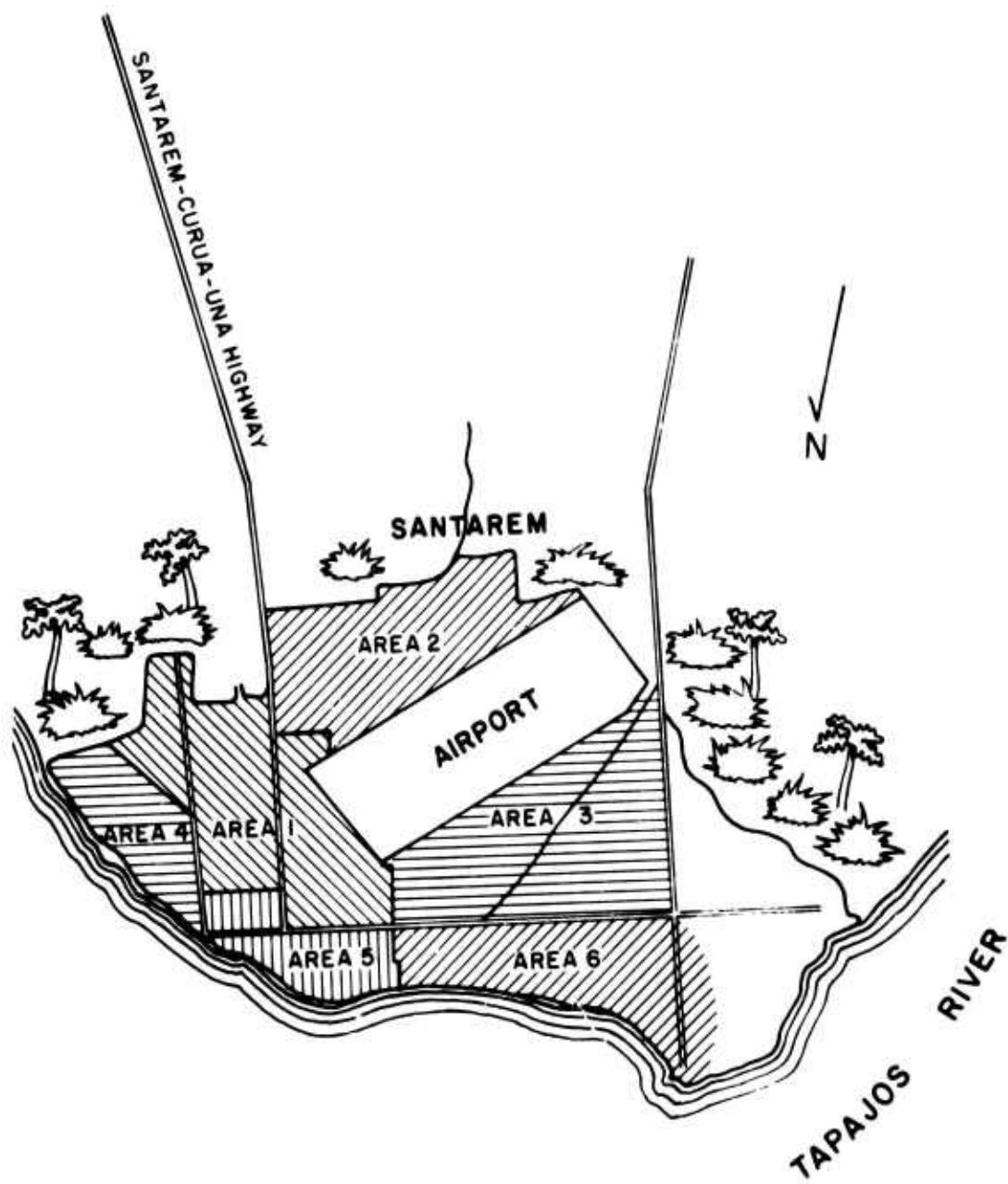
The distribution of specific populations could not be correlated with the environmental variables included on the questionnaires completed for each house in the survey. This was primarily a result of poor quantification of the variables listed. A one-way analysis of variance test did show a significantly ($P < 0.05$) greater number of C. paraensis associated with lots that were 5 or more years old. Since C. paraensis is a tree hole or container breeder this may be related to the presence of more and larger trees on older lots.

Past epidemics in Belem, Brazil have occurred in the low socio-economic suburban areas and did not occur in the residential old city ("cidade velha") nor in the commercial center. Several mosquito species were collected during the survey of 6 bairros in January (TABLE 6). The best represented species were C. paraensis and Cx. pipiens quinquefasciatus. These species were least abundant in the "cidade velha" and commercial area (TABLE 7). Maximum number of cases of Oropouche was detected in area #9 during the 1968 epidemic. Of the areas surveyed, area #9 presented the largest numbers of Cx. pipiens quinquefasciatus and C. paraensis.

The available data indicate a consistent association in within-city distribution by areas of C. paraensis and Cx. pipiens quinquefasciatus population densities. However, there is no correlation in densities by house. Their presence in the same general areas is probably a result of socio-economic factors in the human population.

FIGURE 1

Six Study Areas Within the City of Santarem, Para, Brazil.



AMAZON RIVER

FIGURE 2

Temporal Distribution of Host-seeking Culicoides paraensis
(Goeldi) in a Peridomiliary Environment, Itupiranga, Brazil.

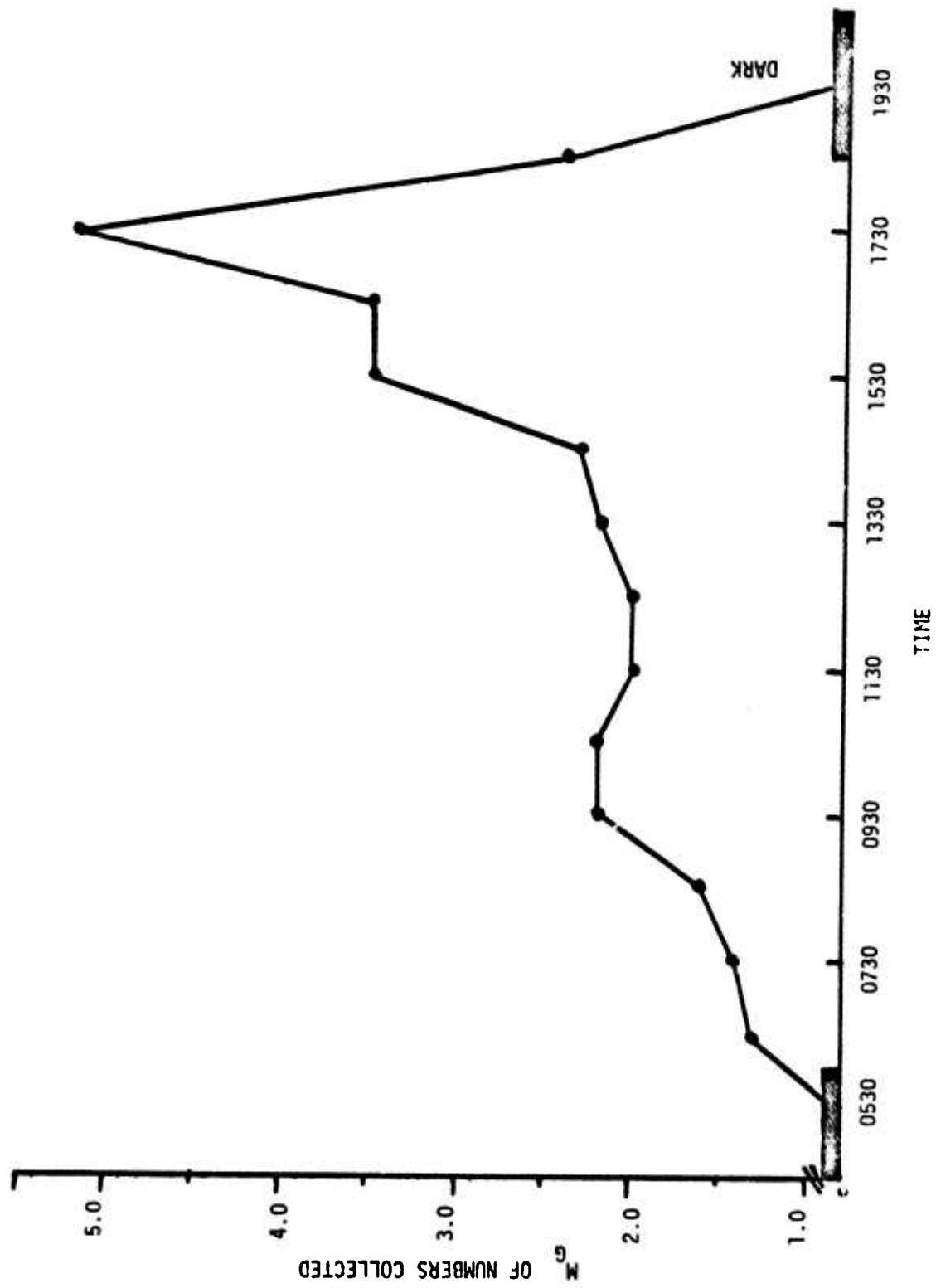
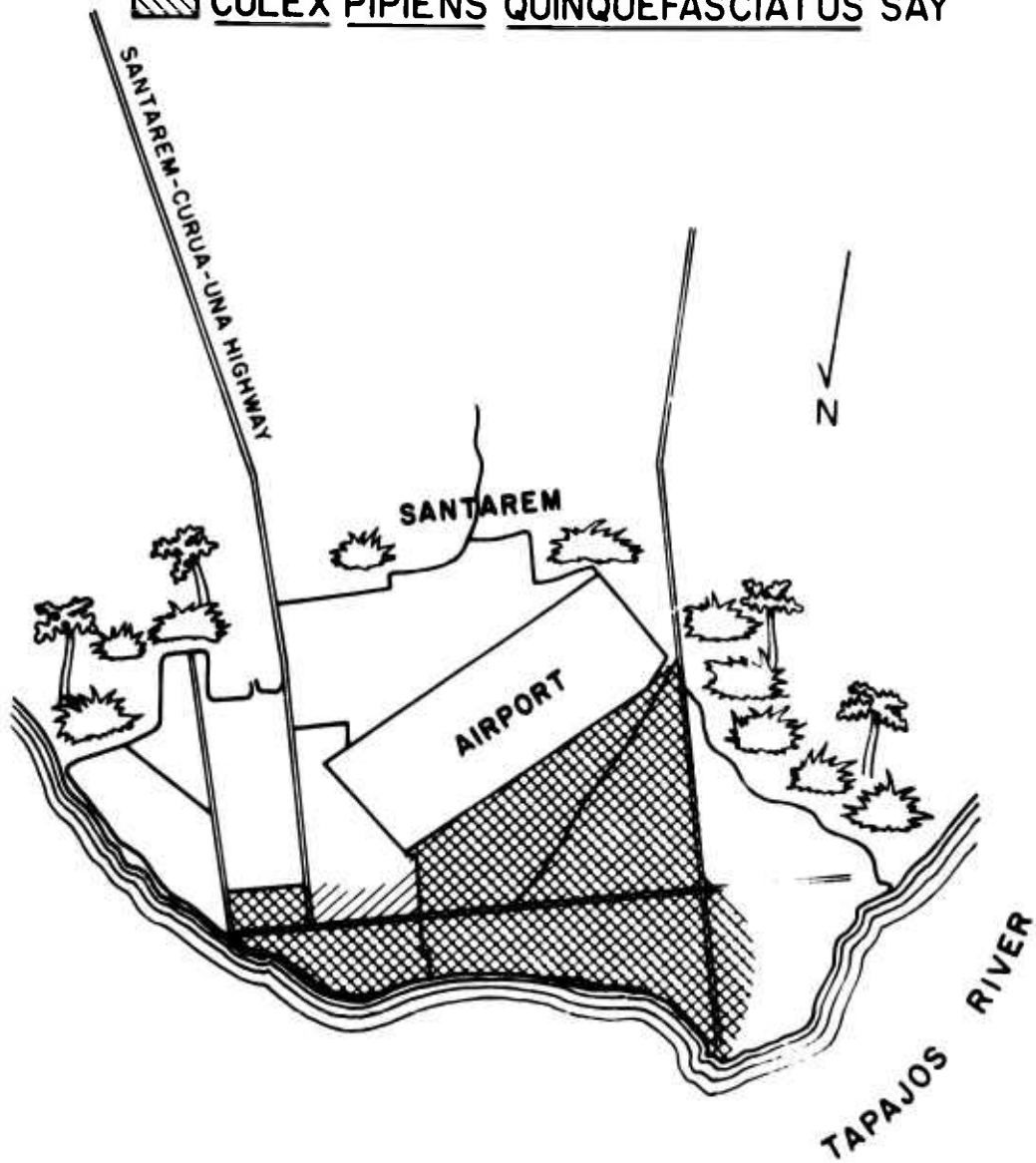


FIGURE 3

DISTRIBUTION OF DENSE POPULATIONS
OF ANTHROPOPHILIC INSECTS BY AREA

-  CULICOIDES PARAENSIS (GOELDI)
-  CULEX PIPIENS QUINQUEFASCIATUS SAY



AMAZON RIVER

FIGURE 4

PERCENTAGES OF HI POSITIVE SERA BY AREA

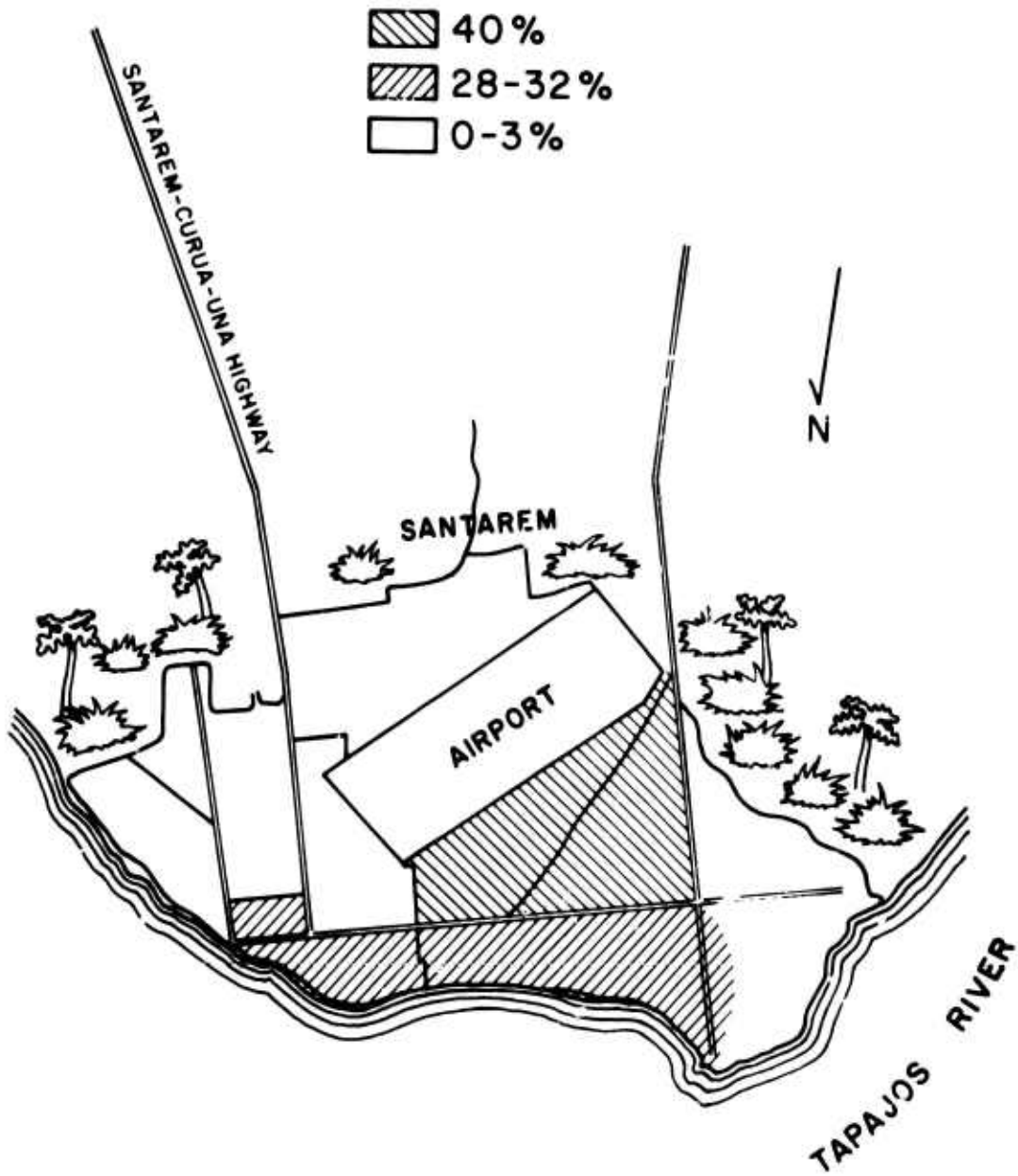


TABLE 1. Numbers of Insects Obtained by 3 Collection Methods in Itupiranga, Para, Brazil, in June 1975.

SPECIES	LANDING CAPTURE ^a AT EDGE OF RIVER	SHANNON TRAP ^b CAPTURE	CDC ^b
<u>Ad. souamipes</u>	0	0	6 (0.22)
<u>An. (Nys.) aquasalis</u>	3 (1.0) ^c	0	0
<u>An. (Nys.) nuneztovari</u>	3 (1.0)	0	1 (0.03)
<u>An. (Nys.) triannulatus</u>	0	1 (0.25)	0
<u>Cx. (Mel.) taeniopus</u>	0	0	1 (0.03)
<u>Cx. (Cux.) coronator</u>	0	11 (2.75)	5 (0.18)
<u>Cx. (Cux.) corniger</u>	0	1 (0.25)	30 (1.07)
<u>Cx. (Cux.) pipiens quinquefasciatus</u>	0	0	27 (1.0)
<u>Culex sp. B #19</u>	0	4 (1.0)	2 (0.07)
<u>Ma. humeralis</u>	150 (37.5)	9 (2.25)	1 (0.03)
<u>Ma. titillans</u>	262 (174.6)	2 (0.5)	4 (0.14)
<u>Anopheles spp.</u>	57	2	0
<u>Culex spp.</u>	2	25	37
<u>Uranotaenia spp.</u>	0	9	2
<u>Culicoides spp.</u>	0	1	0
Number of collections	3 (30 min. each)	4 (1 hr each)	27

^a Collections conducted at the edge of the Tocantins river (which is bordered by the village) from 1845-1915.

^b Collections were made for 1 hr within the village of Itupiranga.

^c Probably represents a morphologic variant of *An. nuneztovari* or is a new species.

/zcm.

TABLE 2. Numbers of Insects Collected in Hourly Landing Captures During an Epidemic of Oropouche Virus in Itupiranga, Para, Brazil of June 1975. Each value is based on 25 man-hr of Collecting Time and were Continuous from 0600 to 2050 hr.

SPECIES	NUMBERS COLLECTED/HR															
	0600-0650	0700-0750	0800-0850	0900-0950	1000-1050	1100-1150	1200-1250	1300-1350	1400-1450	1500-1550	1600-1650	1700-1750	1800-1850	1900-1950	2000-2050	
<u>An. (Nys.) nuneztovari</u>	17	0	0	0	0	0	0	0	0	0	0	0	55	4	0	
<u>An. (Nys.) triannulatus</u>	9	0	0	0	0	0	0	0	0	0	0	0	52	30	2	
<u>Cx. (Cux.) pipiens quinquefasciatus</u>	2	0	0	0	0	0	0	0	0	0	0	0	67	103	307	
<u>Ma. humeralis</u>	0	0	0	0	0	0	0	0	0	0	1	0	28	12	7	
<u>Ma. titillans</u>	29	1	2	1	1	1	0	0	1	0	2	7	382	203	89	
<u>Culicoides paraensis</u>	23	26	34	64	62	57	55	64	88	139	106	225	82	0	0	
<u>Simuliidae</u>	528	1097	2015	1106	652	367	597	1219	1194	1066	1454	1728	21	0	0	
Total number of Culicidae collected	57	27	2	1	1	1	0	0	1	0	4	7	669	399	428	

/zcm.

TABLE 3. Average Numbers of the Most Abundant Species in Landing Collections Conducted in Santarem, Para, Brazil, July 1975.

SPECIES	^a AVERAGE NUMBER COLLECTED						
	Areas	1	2	3	4	5	6
Culicidae							
<u>An. (Nys.) albitarsis</u>		0	0	1.0	0	0	0.5
<u>An. (Nys.) triannunatus</u>		2.2	6.0	0	0	0.3	0
<u>Cx. (Cux.) coronator</u>		0.5	2.5	0.7	1.0	0.3	0
<u>Cx. (Cux.) pipiens quinquefasciatus</u>		4.2	0.7	26.7	7.2	24.5	31.3
<u>Ma. amazonensis</u>		8.2	6.0	0	0	0.3	0
<u>Ma. titillans</u>		1.3	1.0	1.5	0.5	0	6.8

^a Average collected from 6 sites in each area.

/zcm.

TABLE 4. Relative Densities of 7 Species in the Rural and Urban Areas of Santarem, Para, Brazil. The Landing Collections were Made in July 1975.

SPECIES	AVERAGE COLLECTED PER HOUSE	
	a WITHIN CITY OF SANTAREM	b RURAL AREA ALONG THE SANTAREM-CURU-UNA HIGHWAY
<u>Culicidae</u>		
<u>An. (Nys.) albitarsis</u>	0.2	6.6
<u>An. (Nys.) triannulatus</u>	1.4	29.1
<u>Cx. (Cux.) coronator</u>	0.6	2.25
<u>Cx. (Cux.) pipiens quinquefasciatus</u>	15.5	1.0
<u>Ma. amazonensis</u>	1.6	0.5
<u>Ma. titillans</u>	1.8	10.5
<u>Ceratopogonidae</u>		
<u>Culicoides paraensis</u>	9.5	32.0

^a Each value is based on 36 man-hr of collections at each of 39 houses.

^b Each value is based on 36 man-hr of collections at each of 9 houses.

/zcm.

TABLE 5. Population Densities of *Culicoides paraensis* (Goeldi) within the City of Santarem, Para, Brazil, July 1975.

TOTAL COLLECTED BY AREAS						
AREAS						
	4	2	1	5	6	3
	5	5	55 ^a	1	38	42
	0	0	46 ^a	52 ^b	27	17
	0	9	4	20	37	170
	3	1	4	18	11	41
	4	6	3	17	9	36
	2	1	1	1	54	55
TOTAL COLLECTED	14	22	113	109	176	361

^a Houses in proximity to areas 5 and 6.

^b House located adjacent to area 6.

/zcm.

TABLE 6. List of Species and Numbers in Landing Collections Conducted in January and February 1976 in Belem, Para, Brazil.

Culicidae	Number Collected
<u>Ae. (Uch.) digopistus</u>	1
<u>Ae. (Och.) scapularis</u>	15
<u>Ae. (Och.) serratus</u>	1
<u>Ae. (Och.) taeniorhynchus</u>	62
<u>An. (Ano.) intermedius</u>	1
<u>An. (Nys.) aquasalis</u>	98
<u>An. (Nys.) nuneztovari</u>	1
<u>Cq. venezuelensis</u>	168
<u>Cx. (Cux.) corniger</u>	2
<u>Cx. (Cux.) coronator</u>	345
<u>Cx. (Cux.) pipiens quinquefasciatus</u>	1117
<u>Cx. (Mel.) spissipes</u>	1
<u>Cx. (Mel.) taeniopus</u>	2
<u>Ma. (Man.) amazonensis</u>	108
<u>Ps. (Gra.) cingulata</u>	27
<u>Ps. (Jan.) ferox</u>	5
<u>Li. durhami</u>	10
<u>Culex sp. B #21</u>	1
<u>Culex (Mel.) spp.</u>	1
<u>Coquillettidia spp.</u>	4
<u>Culicoides paraensis</u>	1396
TOTAL	3366

/zcm.

TABLE 7. Average Number of 6 Species Collected per Day in 6 Bairros (Areas) in Belem, Para, Brazil.

SPECIES	AVERAGE NUMBER COLLECTED PER DAY PER AREA											
	AREA #7		#8		#9		#20		#2/3		#12	
	DAY 1	2	1	2	1	2	1	2	1	2	1	2
Culicidae												
<u>An. (Nys) aquasalis</u>	0.2	0	4.5	3.3	2.7	0.8	1.3	1.2	0.7	1.5	0	0
<u>Cx. (Cux.) coronator</u>	3.8	3.2	0	16.8	33.7	0	0	0	0	0	0	0
<u>Cq. venezuelensis</u>	5.5	4.0	0.5	0.3	4.2	3.3	5.3	1.5	1.2	2.0	0	0.2
<u>Ma. amazonensis</u>	3.3	1.0	2.8	0.2	3.8	6.0	0.2	0.2	0.3	0	0	0.2
<u>Cx. (cux.) pipiens quinquefasciatus</u>	14.5	12.7	24.2	17.3	28.8	26	20.5	28	3.7	4.5	4.7	1.3
Ceratopogonidae												
<u>Culicoides paraensis</u>	15.8	6.3	2.5	0.2	83	38	23	9.5	4.5	3.2	21.8	39.8

/zcm.

III. MALARIA

BACKGROUND: Malaria continues to be a major health problem in Brazil. It is recognized as 1 of the 5 most important diseases in the country and is clearly the major problem in the Amazon basin. During the 2 years of this project there has been accumulated considerable epidemiological evidence that malaria is being transmitted in some rural areas in the absence of recognized primary or secondary vectors.

Road construction throughout the region is opening new rural areas for occupation. The rural and semi-rural populations are rapidly increasing with access to remote areas, and the mobility of migratory work forces has likewise increased. Both phenomena now present particularly acute problems to the control of malaria.

For obvious reasons, highways are generally constructed through areas away from the rivers and riverine habitats. Since the recognized vectors seem predominantly riverine in occurrence, it is reasonable that any non-riverine, secondary vectors will become increasingly important with the current shift in populations to non-riverine areas. Thus, knowledge of any potential secondary vectors is important in assessing the impact of the conventional control procedures being employed by SUCAM.

A. Entomology Program

Objectives for the entomology program relating to malaria are:

1. Document, over a large geographical area, the spatial and temporal distribution of the various anopheline species with the use of the routine surveillance program.
2. Study specific behavior, e.g. endophily, of species suspected as secondary vectors.
3. Document natural transmission by secondary vectors in areas with active malaria transmission.
4. Conduct a program of rearing taxonomic specimens to resolve the taxonomic difficulties in the Nyssorhynchus subgenus.

PROGRESS TO DATE: No recognized malaria vectors have been found at the routinely visited sites along the Transamazon Highway. In August 1975, 4 teenage members of a colonist family in Gleba 36 became ill with malaria (3 with falciparum and 1 vivax); all denied recent travel from their lote. Migratory workers had been employed on the lote during the preceding 2-3 months. Two weeks of collections at that site revealed no known vector species and dense populations of Anopheles (Nys.) nuneztovari Gabaldon and An. (Nys.) oswaldoi (Peruassu). Results from the routine surveillance program likewise revealed an absence of known vector species in the vicinity of that lote.

A site was established in Palestina (a village 100 Km southwest of Maraba) to study the behavior of the common anopheline and culicine species. The

program was initiated in March 1975 and consisted of conducting hourly landing collections throughout the night. The collections were for 30 min. each hour and were conducted simultaneously inside and outside the house. In addition, a 10 min. resting collection was conducted inside the house at the end of each hour. Other collections were made nightly with the Shannon trap, CDC light trap, and a pyrethrin spray inside one room from 0630-0635 hrs.

The dominant species collected by the landing and resting collections were Anopheles nuneztovari Gabaldon, Coquillettidia venezuelensis (Theobald), Culex pipiens quinquefasciatus Say and Mansonia titillans (Walker) (TABLE 1). The outdoor collections were considerably more productive with all species but Cx. pipiens quinquefasciatus. The "O/I ratio" for the 9 most abundant species are as follows:

<u>SPECIES</u>	<u>O/I ratio</u>
<u>Cx. pipiens quinquefasciatus</u>	0.42
<u>Ma. titillans</u>	1.96
<u>An. aquasalis</u>	3.9
<u>Cq. venezuelensis</u>	4.0
<u>Ps. cingulata</u>	5.1
<u>An. albitarsis</u>	5.3
<u>Cq. nigricans</u>	5.4
<u>An. triannulatus</u>	5.5
<u>Ae. serratus</u>	7.8

Based on these data, the 3 common anophelines in Palestina do not readily enter houses to feed or rest. The specimens identified as An. aquasalis are probably morphological variants of An. nuneztovari or a new species. This species (?) was more likely to enter houses than the other anophelines and seemed relatively more abundant in collections at 0530-0600 hrs (TABLE 2).

Specimens of Ma. titillans were frequently encountered inside the house.

The Cx. pipiens quinquefasciatus were the only populations that demonstrated a real preference for entering, feeding, and resting inside the house.

The peak period of biting activity for the 5 most abundant exophilic species occurred at sunset (TABLE 2). Coquillettidia venezuelensis and An. triannulatus had small, but pronounced, secondary peaks at sunrise.

The 3 species represented in greatest numbers in the Shannon trap collections were Cq. venezuelensis, Cx. coronator and An. triannulatus. The CDC light trap collections conducted on the same nights in the same locale produced highest numbers of Cx. corniger, Cx. coronator and An. triannulatus. Other than the quantitative differences, the traps were qualitatively about equal, i.e., most species were represented in both traps.

A program to rear taxonomic specimens was initiated in February 1976. Material has been collected from the Palestina, Maraba and Itupiranga areas. The reared material is being sent to Dr. Ronald Ward, Project Manager,

Medical Entomology Project at the Smithsonian Institution for taxonomic work-up.

COMMENTS: Plans for studying the behavior of An. darlingi at Agrovila Uniao were not realized because populations of this species disappeared after the program was initiated. A continuation of these studies is planned at a fazenda site near Paragominas. With the abundance of malaria cases, the presence of An. darlingi and other species make this site ideal for future studies. Efforts will also be made to infect potential vectors and to detect natural transmission by secondary species along the Transamazon highway.

TABLE 1. Numbers of Insects Collected in 72 all-night Collections in Palestina, Brazil, March 1975-April 1976, Hourly 30-min Collections of Insects Landing on man were Made Simultaneously Outside and Inside the House. Collections of Insects Resting on Walls were Made During the Last 10 min. of Each hr.

	<u>OUTSIDE</u>	<u>INSIDE</u>	<u>RESTING</u>	<u>TOTALS</u>
<u>Culicidae</u>				
<u>An. (Nys.) albitarsis</u>	105	20	4	129
* <u>An. (Nys.) aquasalis</u>	47	12	2	61
<u>An. (Nys.) nuneztovari</u>	6	0	1	7
<u>An. (Nys.) oswaldoi</u>	19	5	1	24
<u>An. (Nys.) triannulatus</u>	406	74	7	487
<u>Ae. (Och.) fulvus</u>	1	0	0	1
<u>Ae. (Och.) scapularis</u>	4	2	0	6
<u>Ae. (Och.) serratus</u>	55	7	4	66
<u>Cq. albicosta</u>	4	1	0	5
<u>Cq. arribalzagai</u>	1	0	0	1
<u>Cq. nigricans</u>	45	8	10	63
<u>Cq. venezuelensis</u>	501	126	39	568
<u>Cx. (Mel.) spissipes</u>	4	2	1	7
<u>Cx. (Mel.) taeniopus</u>	3	1	0	4
<u>Cx. (Mel.) vomerifer</u>	2	1	0	3
<u>Cx. (Cux.) corniger</u>	2	3	14	19
<u>Cx. (Cux.) coronator</u>	13	2	30	45
<u>Cx. (Cux.) declarator</u>	0	1	0	1
<u>Cx. (Cux.) pipiens</u>				
<u>quinquefasciatus</u>	273	616	752	1641
<u>Culex sp. B# 19</u>	9	6	10	25
<u>Ma. (Man.) humeralis</u>	8	7	1	16
<u>Ma. (Man.) pseudotitillans</u>	4	0	0	4
<u>Ma. (Man.) titillans</u>	184	94	28	306
<u>Ps. (Gra.) cingulata</u>	112	22	13	147
<u>Ps. (Jan.) ferox</u>	3	1	0	4
<u>Tr. (trc.) digitatum</u>	0	0	1	1
<u>Anopheles spp.</u>	236	10	12	258
<u>Aedes spp.</u>	0	7	0	7
<u>Coquillettia spp.</u>	2	4	1	7
<u>Culex spp.</u>	43	11	52	106
<u>Mansonia spp.</u>	8	0	3	11
<u>Psorophora spp.</u>	6	0	0	6
<u>Uranotaenia spp.</u>	4	0	0	4
<u>Wyeomyia spp.</u>	0	1	0	1
TOTALS	2110	1039	986	4037

TABLE 1. (Cont.)

	<u>OUTSIDE</u>	<u>INSIDE</u>	<u>RESTING</u>	<u>TOTALS</u>
Culicidae				
TOTALS	2110	1039	986	4037
Simuliidae	21	0	0	21
Ceratopogonidae	4	0	0	4
TOTALS	25	0	0	25
GRAND TOTALS	2135	1039	986	4062
Number of man-hours	432	432	144	
Ave. number collected/man-hr	4.94	2.4	6.85	

*Probably represents a morphological variant of An. nuneztovari or a new species.

/zcm.

TABLE 2. Numbers of 5 Mosquito Species Collected in Landing Captures at Palestina, Brazil, March 1975-April 1976. Each Value is Based on 36 Man-hr of Collecting Time Within 10 m of the House.

	<u>Cg.</u> <u>venezuelensis</u>	<u>An.</u> <u>triannulatus</u>	<u>Ma.</u> <u>titillans</u>	<u>Ps.</u> <u>cingulata</u>	<u>An.</u> <u>albipennis</u>	TOTALS
1130- 1800	2	2	2	0	2	7
1830- 1900	345	297	145	62	94	943
1930- 2000	31	28	13	7	3	82
2030- 2100	22	17	4	12	2	57
2130- 2200	15	6	4	10	0	35
2230- 2300	1	7	1	7	1	17
2330- 2400	7	1	2	2	0	12
0030- 0100	6	2	3	2	0	13
0130- 0200	10	3	0	1	0	14
0230- 0300	9	0	0	2	0	11
0330- 0400	13	4	5	1	0	23
0430- 0500	5	1	1	3	0	10
0530- 0600	35	39	4	3	4	84
TOTALS	601	406	184	112	105	1308

B. Investigation of Malaria Epidemic at Vila Nova

In August, 1975, USAMRU-Belem was asked to investigate an epidemic of febrile disease which had started approximately one month previously in Vila Nova, Para. During the intervening period there had been ten deaths. Fever and jaundice were symptoms common to the ten, and yellow fever was suspected to be the cause.

Vila Nova is a town of about 500 inhabitants, located 950 Km south of Belem and 14 Km inland from Sao Geraldo and Xambioa on the Araguaia River. The houses are all constructed of mud and the only water in the immediate area is a small stream which flows past one end of the town. The area as a whole is dry.

On July 20th, 58 persons with fever were examined and blood was drawn for virus isolation, serology and a malaria slide. Demographic information and symptomatology were recorded, temperature measured, and abdomen examined for a palpable spleen. Thirty-two persons had a temperature greater than 37°C and eight had a palpable spleen. Almost all reported an intermittent fever of long duration with epigastric pains, headache and sometimes vomiting. Many reported remissions after taking chloroquin, usually in non-curative doses.

Blood was drawn and information taken from an additional 12 families with 60 members randomly selected from the village.

About two hours were spent doing insect resting captures inside houses and 90 man-hours doing biting collections inside houses, outside houses and on the banks of the stream that flows past the village. Total captures were 5 anophelines and about 50 culicines. All the mosquitos were captured on the banks of the stream.

The two physicians in Xambioa, the nearest large town, claimed to have treated 120 cases of malaria, most of which they diagnosed as *P. vivax*. They also reported three cases of yellow fever, two of which died. These cases were diagnosed clinically. Blood was not taken for serology nor were examinations for malaria made. No liver specimens were taken from the two fatalities. There were no reports of dead monkeys in the area, and there appeared to be few live ones.

Upon return to Belem, blood slides were examined for malaria parasites. Twenty-nine of the 58 persons with a history of recent fever were positive for malaria, including seven of the eight with a palpable spleen. Three of these were identified as *vivax*; the rest were *falciparum*. Five of the 60 persons constituting the random sample had a positive malaria slide. All five were identified as *falciparum*. Since only one slide was taken from each person, and since it is a common practice to take small doses of chloroquine for any fever, the actual prevalence of malaria is probably higher than is indicated by the number of positive slides.

All five anophelines were identified as *Anopheles darlingi*.

Virus isolation and serology studies revealed no evidence for the presence of yellow fever or other arboviral disease.

C. Malaria Control in Migrant Workers

Malaria among migrant workers, especially on fazendas in the states of Para and Maranhao, is considered a major threat to Brazil's national malaria control program. These workers often are singled out as the source of reintroduction of the infection into areas where malaria control programs have greatly reduced or eradicated the disease. Large numbers of itinerant laborers are hired for forest clearing, grass planting, fencing, and construction on the many existing and developing fazendas in the Amazon region. The workers normally live in primitive, wallless shelters and sleep in hammocks. The forest clearers usually live furthest from the roads in areas that are difficult to reach by spraying, examination, and treatment teams. The national malaria control organization (SUCAM) has the task of developing proposals for combating malaria among migrant workers, primarily at the fazenda level. Malaria rate estimation in itinerant populations and evaluations of control procedures under fazenda conditions are important types of information for developing a rational protocol for malaria control. These data are not available for the Amazon region.

BACKGROUND: In mid-February, 1976, a program was initiated by Fazenda Uraim near Paragominas in the state of Para; to combat an increasingly serious malaria problem. The program was designed by the USAMRU-Belem staff and financed by Fazenda Uraim. This fazenda was opened in virgin jungle and was initially accessible only by river. During the first year of work there were no malaria problems among the workers. A road (92 Km in length) was constructed into the fazenda the following year. During the second year cases of malaria began to appear and continued to increase during the third year (1975).

A visit was made to the fazenda in July 1975 to evaluate the area for studying malaria in the migrant labor. Malaria was a major problem in the areas where the forest was being cleared. Populations of Anopheles darlingi Root were not found. A second trip in October, 1975, reaffirmed the seriousness of malaria among the forest workers and revealed the presence of An. darlingi at a riverine area where the forest was being cleared. In January, 1976, An. darlingi was discovered throughout the fazenda in both cleared and partially uncleared areas. It was on the basis of these visits that an experimental program was presented to the fazenda management for malaria control. The program design included a malaria detection - treatment team and 2 men for vector surveillance. Vector surveillance was subsequently discontinued and certain types of entomologic data are not available. All persons on the fazenda are examined biweekly (each biweekly survey is called a visit), and all malaria positives are treated. A preliminary analysis of data was made at the end of the third visit; and a ten visit analysis is being done, but the results are not presently available. Positivity rates for ten visits will, however, be presented.

PROGRESS TO DATE: The maximum number of persons examined during the first 3 visits was 437 (TABLE 1). The actual population size of the fazenda is unknown since there are insufficient controls on exiting personnel. Consequently, the efficiency of coverage can not be evaluated. Percentages derived from frequency distributions of persons seen during each of the 3 visits (TABLE 2) indicate that a mean of 30.3% of all persons were not seen during preceding or subsequent visits. This approximates a 60% turnover of the entire fazenda population per month.

A mean number of 72 cases of malaria were detected during each visit (TABLE 3). Calculations based on data in TABLE 1 would give 17% positivity for the fazenda population. The lowest number of positive cases (15% positivity) and particularly of cases of falciparum, was found during the third visit. A comparison of the percentage of persons seen only once in visits 1 and 2 with the percentage of total cases of malaria contributed by these people is presented in TABLE 4. This group represented 28% of the population surveyed and contributed an average of 44% of the malaria cases.

It appears that case treatment is relatively effective. From cases included in subsequent visits, 17% of falciparum cases (total of 64 cases) and 10% of vivax cases were positive at the next biweekly visit. The results are probably due to 1) not taking medicine as prescribed 2) some cases of falciparum that were initially under dosed.

Crude estimates of transmission rates were derived from persons examined during two or more visits to determine the percentage of persons converting from negative to positive. The transmission rate of falciparum was reduced between the first and third visits (TABLE 5). Transmission of vivax malaria remained more or less constant. The net effect was a reduction of transmission from 19% during the initial visit to 12% during the second and third visits. The bulk of these cases probably represent on-fazenda transmission. There was no reduction in total number of cases during the first three visits; however, the reduction in transmission rate seems to indicate that a true decrease in total cases will occur.

Results from the first ten visits: The impact of the active surveillance and treatment program became obvious during the fourth visit. The positivity rate decreased with each visit and was lowest at the eighth visit. An increase in the number of cases during the ninth and tenth visits corresponds with the reinitiation of forest clearing (the first since 1975). This increase is a result of the inaccessibility of forest workers and their hesitance to walk 4-6 Km for examination and treatment.

Since the program has been in effect, each person applying for work has been examined for malaria before he enters the fazenda. The overall positivity rate of applicants for February-May was 9.7% most of which (5.7%) was falciparum malaria.

COMMENTS: The Fazenda Uraim program is providing much malariogenic data that is relevant to the most pressing problem faced by the malaria control effort, viz. malaria control in the migratory labor force employed on fazendas. For a variety of social and cultural reasons, this problem may have strong influence on the development of chloroquine resistance in this region. A detailed analysis of the first 10 visits is being conducted, to include effectiveness of treatment.

Work at the fazenda affords an opportunity to conduct a variety of studies on malaria that would otherwise be difficult. Future plans include continuation of past studies on chloroquin resistente falciparum malaria, efforts at detecting natural infections in secondary vectors, testing the susceptibility of various species to infection, and study of the behavior of the principal vector - An. darlingi.

TABLE 1. Total Number of Persons Examined During Visits 1, 2, and 3 at Fazenda Uraim.

VISITS	NUMBERS OF PERSONS EXAMINED
1	1424
2	397
3	437

/zcm.

TABLE 2. Frequency Distrubution of Persons Examined by Visits 1, 2, and 3 at Fazenda Uraim.

	VISITS						
	1,2,3	1,2	1	2,3	2	3	3,2
Numbers of persons examined	121	92	136	85	99	156	75

/zcm.

TABLE 3. Numbers of Cases of Falciparum and Vivax Malaria Detected During Visits 1, 2, and 3 at Fazenda Uraim

MALARIA TYPE	VISITS		
	1	2	3
Vivax	20	19	28
Falciparum	54	57	38

/zcm.

TABLE 4. Percent of Population Seen 1 Time Only During visits 1 and 2, with Percent of Total Number of Malaria Cases Represented in These Individuals. Data Collected at Fazenda Uraim, Paragominas, Brazil

	VISITS	
	1	2
% population seen 1 time only during visits 1 and 2	32%	24%
% of total cases of malaria on the Fazenda represented in the "seen 1 time only" population	39%	49%

/zcm.

TABLE 5. Percent of Individuals that Convert (Negative to Positive) on Subsequent Visits at Fazenda Uraim.

MALARIA TYPES	VISITS		
	1-2	2-3	1-3
Falciparum	14%	7%	8%
Vivax	5%	5%	10%
Combined %	19%	12%	18%

zcm.

TABLE 6. Malaria Positivity Rates for the 1st 10 Visits at Fazenda Uraim, Paragominas, Brazil.

TYPE OF MALARIA	% POSITIVE BY VISIT									
	1	2	3	4	5	6	7	8	9	10
Vivax	5	5	6	4	4	2	1.5	1	3	4
Falciparum	13	14	9	4	4	3	2.5	2	3	3
TOTAL	18	19	15	8	8	5	4	3	6	7
TOTAL NUMBER EXAMINED	424	397	437	347	442	486	528	487	587	654

/zcm.

IV. LABORATORY PROGRAM

BACKGROUND: The primary functions of the laboratory section of USAMRU-Belem are the technical support of the epidemiology surveillance program and the provision of diagnostic capabilities in parasitology, bacteriology, and a variety of clinical lab procedures as required in epidemic investigation, special projects, and the mammalian ecology program. The laboratory section is also responsible for the receipt, recording, and labeling of sera and whole blood specimens collected by epidemiology and ecology field teams.

A. Serology

The fundamental emphasis of the laboratory program has been the serologic analysis of specimens collected in the epidemiology surveillance program. A screening procedure was designed to facilitate determination of antibody prevalences and detection of titer rises and seroconversions (TABLE 1). At present sera are being tested for antibodies to the following agents:

Toxoplasma gondii, Brucella abortus, Leptospira spp, Entamoeba histolytica, Trypanosoma cruzi, Schistosoma mansoni, Trichinella spiralis, and Echinococcus granulosus.

A screening test for antibodies, to Leishmania spp and confirmatory tests for T. cruzi and S. mansoni are being developed.

PROGRESS TO DATE: Maraba: To date, 690 Transamazon colonists in the area of Maraba have been studied. This number represents all the colonists, for whom two or more sera are available, presently enrolled in the epidemiology surveillance program in that region.

LEPTOSPIRA: Sera were tested for antibodies to six pools of Leptospira spp (TABLE 2) using the macroagglutination test (Difco). Results of these tests are presented in TABLES 3 and 4.

Original sera of colonists showing positivity to one or more pools at twelve months were tested concurrently with 12 months specimens in order to detect seroconversions.

Seventy-two colonists, 10.4% of the individuals tested, showed seroconversions to at least one Leptospira pool. Seroconversions to pools 3 and 4 were most common. Preliminary analysis indicates that the seroconversions tend to be localized within families or among neighboring lots in the same gleba. Fifty-six colonists showed reactivity of both original and 12 month sera.

BRUCELLA: Six hundred and seventy-nine (679) Maraba 12 month sera were tested for antibodies to Brucella abortus and B. melitensis by the macroagglutination test (Difco antigen). Of these, 6 sera (less than 1%) were reactive. Since original sera from these colonists were also reactive, transmission of this agent in the Maraba area study population was not demonstrable.

SCHISTOSOMIASIS: Schistosoma mansoni is not endemic in the Amazon region of Brazil; however, at least one planorbid species capable of serving as

intermediate host for this parasite has been found in this area (Lacaz et.al., 1972). Several hundred stool positive immigrants from endemic areas of the country have been identified and treated by SUCAM.

The sera of 666 colonists were tested for antibodies to *S. mansoni* using the indirect hemagglutination (IHA) test. The distribution of the IHA titers for the 12 month sera is given in TABLE 5. Only 17.2% of the sera were reactive, and there were no titers greater than 1:64. The accepted diagnostic titer for this test is 1:256, but titers greater than or equal to 1:16 are probably specific for exposure to *Schistosoma* spp. (Cuadrado and Kagan, 1967). These titers apparently represent residual antibody from exposure occurring prior to the arrival of the colonists in the Transamazon.

One four-fold titer increase (1:8 to 1:32) was recorded between the original and 12 month sera of a single colonist. Repeated IHA testing confirmed this titer increase. The colonist is an adult female who immigrated to Para in 1972 after having lived in the state of Espirito Santo, an endemic area for *S. mansoni*, her entire life. Her original serum was drawn in June, 1974, approximately 20 months after her arrival in the region. The low 12-month titer (1:32) makes it impossible to state with certainty that this titer increase represents infection with human schistosomiasis. Future sera and stool exams will be done to clarify the nature of this case.

AMOEBIASIS: It is assumed that *Entamoeba histolytica* infection occurs throughout Brazil, but data on the prevalence of the parasite in rural areas are almost nonexistent. Prevalence data acquired by stool examination, moreover, do not serve as an index for the occurrence of serious amoebic disease, the latter being dependent upon a variety of host factors as well as the pathogenicity of the amoeba strain. Serologic tests have the advantage of being an objective and rapid method for screening large populations for the presence of invasive amoebiasis. A variety of serologic techniques were tested, including a commercial latex agglutination test and the indirect hemagglutination (IHA) test using either antigen sensitized human - O or sheep erythrocytes. The latex agglutination test proved unsatisfactory because of its extremely low sensitivity. The IHA test using sheep erythrocytes yielded more consistently reproducible results than did the same test using human cells, and is therefore the test being used in this laboratory.

TABLE 6 shows the IHA titer distribution of 686 twelve month sera from Maraba area colonists. Twenty sera were reactive, and only two of these were found to have the minimum significant titer of 1:256. The reactive 12 month sera were retested concurrently with homologous original sera. There was no evidence of significant titer increases or seroconversions. In view of this low positivity rate, 200 sera were tested concurrently by this laboratory and by the Department of Immunology, WRAIR. Comparable results affirmed the validity of the test being performed at this laboratory.

The IHA titers of persons with invasive amoebiasis remain high over a relatively long time period (Healy et. al., 1970). It is therefore unlikely that a high incidence of this disease would have been missed by serologic screening. The absence of invasive amoebiasis in a population in which, judging from prevalence of the other intestinal parasites, there

exist suitable conditions for transmission of E. histolytica can be explained if a) the parasite does not occur in the population; b) the parasite does occur but is not a highly pathogenic strain. Host factors, which are highly variable and hard to identify, are not being considered. The virtual absence of antibody and the varied physical and nutritional states within this large population would apparently preclude host immunity and resistance as significant factors. Discussions with laboratory personnel at SUCAM and the FSESP Hospital in Maraba indicate that diagnosis of E. histolytica is fairly common at these facilities. Unfortunately no records of stool examinations are maintained. Both laboratories are being requested to maintain a ledger of the number of stool exams performed and the number of diagnosis of E. histolytica for a definite time period in order that an estimate of parasite prevalence may be made. It will then be possible to conclude whether low parasite prevalence or low pathogenicity of amoeba strains accounts for the absence of significant antibody titers.

CHAGAS DISEASE: Although an important disease on a national basis, infection with Trypanosoma cruzi has not been reported from Para. Six hundred and fifty-five 12 month specimens were tested for antibodies to T. cruzi using a latex agglutination test. Six hundred and three (92.1%) were non-reactive. Forty-six colonists (7.0%) had reactive sera at both 0 and 12 months. Six colonists (0.92%) showed seroconversions. These colonists were dispersed over a wide area and no two were from the same family. In only one case did another family member show seropositivity.

In order to determine the specificity of the test system, a battery of antisera to various Leishmania species (TABLE 7) was tested. The Chagas antigen cross reacted only with serum from a cutaneous case of L. brasiliensis brasiliensis. No data on false positive reactions due to exposure to animal trypanosoma is available. This test system appears to be suitable for screening procedures, however, the complement fixation test will be used for future confirmatory testing. C.F. testing of future sera from the apparently seropositive colonists will be required before the significance of the latex agglutination seroconversions can be known.

TOXOPLASMOSIS: TABLE 8 gives the IHA titer distribution of 679 Maraba area 12 month sera. FIGURE 1 is a graph of the frequency distribution of the same group of sera. Exposure to Toxoplasma gondii was demonstrated in 36.5% of the population, with past or present infection (titer greater than or equal to 256) in 21.6%. Slightly more than 7.0% of the population had experienced relatively recent infection (titer greater or equal to 2048). Forty-one seroconversions or four-fold titer increases were demonstrated between original and 12 month sera. Seroconversions were not localized in any one area of the Transamazon, but did tend to occur in groups in families. The frequency of high IHA titers indicates the high transmission efficiency of the parasite in this region. A serologic study of domestic animals will be undertaken to elucidate the epidemiology of T. gondii infection along the highway.

ECHINOCOCCOSIS: The latex agglutination test was used to screen 431 sera for antibodies to Echinococcus granulosus. Five sera (1.16%) were reactive. Data on this infection do not exist for humans in the Amazon region. Since patients with cysticercosis, collagen diseases, and hepatic cirrhosis react positively to Echinococcus antigen, further study will be required to determine the significance of positive reactions among Transamazon colonists.

TRICHINOSIS: A latex agglutination test was used to screen 194 sera for antibodies to *Trichinella spiralis*. Fifty-five sera (28.3%) reacted positively, and 15 sera (7.6%) were weakly positive. The importance of this infection in the Amazon region is unknown. Dietary habits would provide ample opportunity for transmission, but there is little information on infections of wild and domestic animals in the region. No extensive program of study will be conducted in relation to this infection. Twenty percent of the human study population will be tested routinely, and a serologic survey of a discrete number of possible wild and domestic reservoirs will be conducted.

PROGRESS TO DATE: Altamira: At present sera from the 12 months collection along the Transamazon from Altamira and the Altamira area agrovilas are being screened for reactivity to the standard battery of antigens. TABLE 9 lists the reactions of the sera already screened. All positive sera will be retested concurrently with homologous original sera, and titers will be determined.

COMMENTS: Preliminary comparisons between Altamira and Maraba show similar positivity rates to all antigens tested so far. Complete data analysis will not be possible until information on the personal history of the colonists is made accessible by computer. At present a manual data recovery system is allowing study of the complete serologic data for each individual colonist and enables the geographical pinpointing of possible areas of transmission of the organisms being studied. Study of serologic data from colonists and domestic and sylvatic animals will hopefully elucidate transmission cycles of some of the organisms in this region.

TABLE 1. Serologic Testing Scheme

Screen 12 months specimens for antibodies to:

Schistosomiasis
Toxoplasmosis
Amoebiasis
Leptospirosis
Brucellosis

Screen 20% of 12 months specimens for antibodies to:

Trichinosis
Echinococcosis

Positives at 12 months screen will be processed according to system set forth below. Negatives will serve as base for testing 24 month sera.

Testing scheme

- | | |
|---------------------------------|--|
| 1) 12 month (+) | 2) 12 months (+) |
| Original (-) | Original (+) |
| 6 mnth (+) | If 12 mnth titer is 4 x greater than original test |
| Test acutes between 0 & 6 | 6 mnth + appropriate acutes |
| 3) 12 mnth (+) | 4) 12 mnth (-) |
| Original (-) | No further testing of previous sera at present |
| Test acutes between 6 & 12 mnth | |

/zcm.

TABLE 2. Leptospirosis Macroagglutination Test.

DIFCO Leptospiral Antigen Pools Contain the Following Antigens:

Pool 1

L. ballum
L. canicola
L. icterohemorrhagiae

Pool 5

L. cynopteri
L. celledoni
L. javanica

Pool 2

L. bataviae
L. grippotyphosa
L. pyrogenes

Pool 6

L. cynopteri
L. panama
L. shermani
L. ictero - kremastos pool
L. kremastos
L. mendanensis
L. seiroe
L. biflexa
L. biflexa patoc

Pool 3

L. autumnalis
L. pomona
L. wolffii

Pool 4

L. australis
L. hyos
L. mini, georgia

TABLE 3. Leptospirosis Serology: Maraba 12 Month Specimens.

Number of sera tested:

Number of sera reactive - 1 pool	52	(40.6%)
2 pools	45	(35.1%)
3 pools	15	(11.7%)
4 pools	7	(5.4%)
5 pools	2	(1.5%)
6 pools	7	(5.4%)
Total reactive sera	<u>128</u>	(18.5%)
Non-reactive sera	562	(81.5%)

Seroconversions (0 - 12 months)

Individuals seroconverting for 1 pool	-	41
Individuals seroconverting for > 1 pool	-	31
Total number individuals seroconverting	-	72

TABLE 4. Leptospirosis Serology: Pool Distributions of Reactions
Maraba 12 Months Sera.

Single pool reactions only

<u>Pool 1</u>	<u>Pool 2</u>	<u>Pool 3</u>	<u>Pool 4</u>	<u>Pool 5</u>	<u>Pool 6</u>
1	4	34	11	3	1

Multiple pool reactions

<u>Pool 1</u>	<u>Pool 2</u>	<u>Pool 3</u>	<u>Pool 4</u>	<u>Pool 5</u>	<u>Pool 6</u>
11	20	71	66	22	11

Pool distribution of seroconversion reactions

Conversion to one pool reactive

<u>Pool 1</u>	<u>Pool 2</u>	<u>Pool 3</u>	<u>Pool 4</u>	<u>Pool 5</u>	<u>Pool 6</u>
0	1	21	12	4	1

Conversion to > 1 pool reactive

<u>Pool 1</u>	<u>Pool 2</u>	<u>Pool 3</u>	<u>Pool 4</u>	<u>Pool 5</u>	<u>Pool 6</u>
8	12	23	25	13	7

TABLE 5. Distribution of Schistosoma mansoni IHA titers, Maraba 12 Month Sera

RECIPROCAL TITER	< 8	8	16	32	64	≥ 128
Number of sera	552	95	13	3	3	0
Percent of total	82.8	14.2	1.9	0.45	0.45	-

TABLE 6. Distribution of *E. histolytica* IHA Titers Among 12 Month Sera of Maraba Area Transamazon Colonists.

Total sera tested - 686

Total sera reactive - 20 (2.9%)

RECIPROCAL TITER	< 16	16	32	64	128	≥ 256
NUMBER OF SERA	666	12	5	1	0	2
	(97.1%)	(1.7%)	(0.72%)	(0.15%)	(0)	(0.30%)

/zcm.

TABLE 7. Cross Reactivity of Leishmania Species Antisera with T. cruzi Latex Agglutination Antigen.

SPECIES	REACTION
1. <u>L. mexicani amazonensis</u> (Diffuse cutaneous case)	Negative
2. <u>L. brasiliensis brasiliensis</u> (Mucocutaneous case)	Negative
3. <u>L. brasiliensis guyanensis</u> (Cutaneous case)	Negative
4. <u>L. brasiliensis brasiliensis</u> (Cutaneous case)	Positive
5. <u>L. mexicani amazonensis</u> (Cutaneous case)	Negative

/zcm.

TABLE 8. Toxoplasma IHA Titer Distribution, 12 Month Sera, Maraba

RECIPROCAL TITER	< 64	64	128	256	512	1024	≥ 2048
Number of Sera	431	55	47	36	35	26	50
% of Total	63.5	8.1	6.9	5.3	5.2	3.8	7.2

Total no. sera tested: 679

No. titers ≥ 256: 147 (21.6%)

Titer rises (4x) or seroconversions: 41 (6.0%)

/zcm.

FIGURE 1.
Toxoplasma IHA titer frequency distribution
Maraba 12 months sera

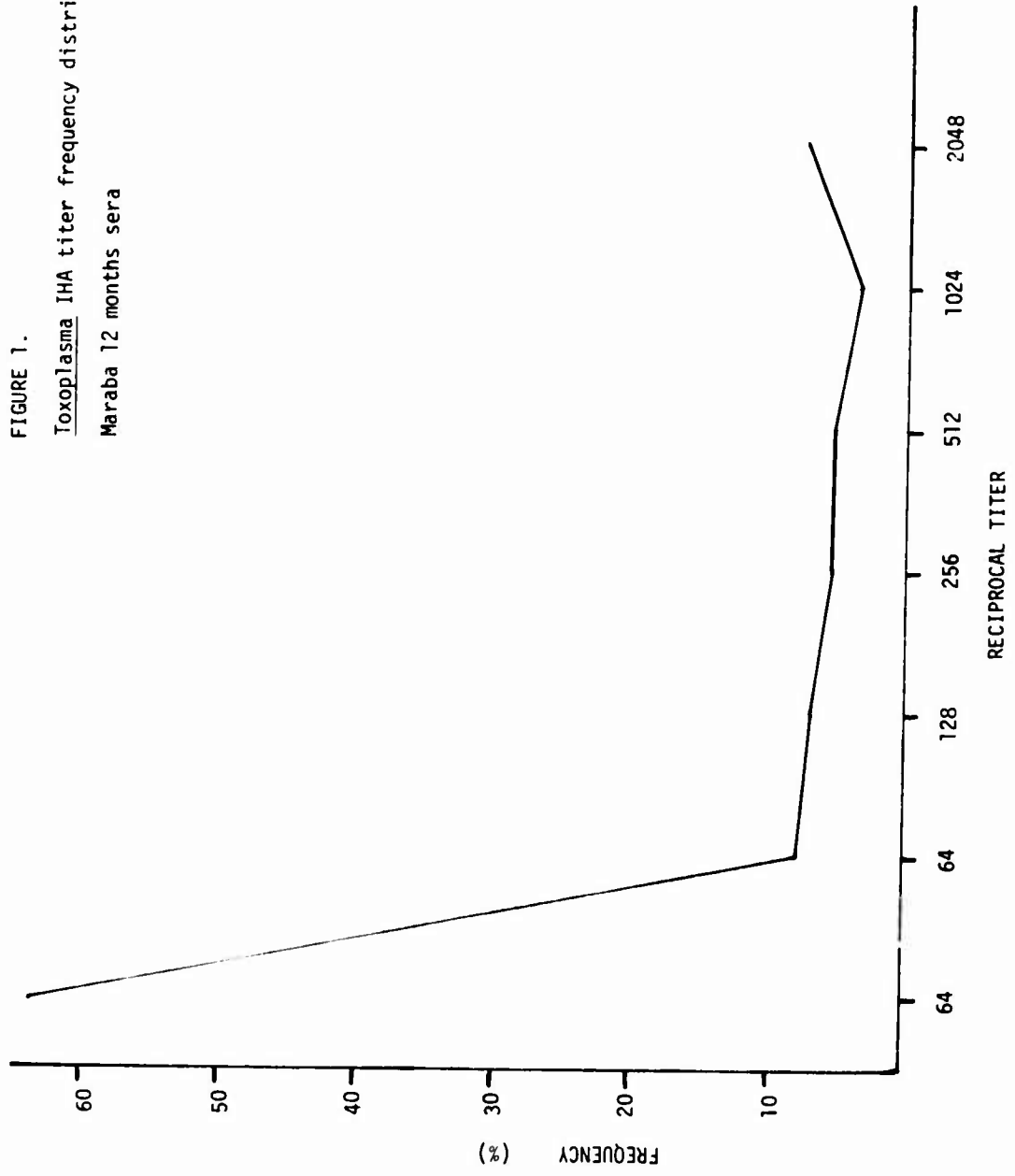


TABLE 9. Results of Serologic Screening of Altamira 12 Month Sera.

ANTIGEN	POSITIVE SERA	NEGATIVE SERA	TOTAL SERA
<u>Toxoplasma gondii</u>	210 (49.8%)	212	422
<u>Entamoeba histolytica</u>	25 (5.9%)	397	422
<u>Trypanosoma cruzi</u>	33 (7.8%)	389	422
<u>Schistosoma mansoni</u>	70 (16.6%)	352	422
<u>Leptospira spp</u>	270 (33.9%)	525	795

/zcm.

B. Toxoplasmosis among Ticuna Indians

In May 1975, one member of USAMRU-Belem (CPT Lovelace) accompanied three investigators from the Instituto Evandro Chagas to an area of the Upper Solimoes (Amazon) River near the Colombian frontier (FIGURE 1) in order to clarify reports of the occurrence of Onchocerca volvulus infection among the Ticuna Indians in that region. Onchocerciasis was not seen in this population, but a high incidence of the non-pathogenic filarid Mansonella ozzardi was found in all the villages studied (Appendix 1). In five locations, venous blood was drawn from approximately 10% of the village population and prepared for serologic testing for antibodies to other disease agents.

The Ticunas comprise a group of approximately 20,000 people, about half of whom live in Brazil. Because of their proximity to major waterways, the Ticunas are acculturated in many respects, and most have gathered into villages associated with missionary activity. Agriculture and animal husbandry are not widely practiced. The Ticuna diet consists preferentially of fish and manioc. This diet is supplemented during infrequent periods of poor fishing by hunting monkeys, small deer, and paca. With the exception of one village, distances from cattle rearing areas prohibit the consumption of beef. Dogs and cats are not kept.

TABLE 1 lists the Toxoplasma IHA titer distributions for each Ticuna village and for the entire population. Since participation in this program was voluntary and the adults were hesitant to coax their children to submit to venipuncture, data for Ticunas in the 0-9 age group is virtually non-existent. The villages of Umariacu and Nova Italia exhibit the highest geometric mean titer (GMRT) and the largest percentage of sera with titers greater than or equal to 256, the "significant" titer for this test (Walls and Kagan, 1967). The relatively more efficient transmission of T. gondii in these villages can be related to various factors. Umariacu is located within easy canoeing distance of Tabatinga and Benjamin Constant, Brazil, and Leticia, Colombia. There is frequent travel to these cities where beef and pork are readily available. Nova Italia, on the other hand, is an isolated village off the main course of the Solimoes. This village had the largest population of chickens and pigs, and it is assumed that both animal husbandry and varied diet provide exposure to T. gondii in this village.

The frequency distribution of the entire population (FIGURE 2) reflects exposure of the population to Toxoplasma, but the unimodality of the graph indicates a low prevalence of successful infection. The small peak of titers at ≥ 2048 indicates a number of relatively recent infections, the majority of which are in Umariacu and Nova Italia. It must be assumed that the restricted Ticuna diet and the paucity of animal husbandry limits their contact with T. gondii infected animals. Hunting, dressing, and eating wild game, keeping of yard animals, and supplementing their diet with beef and pork seems to provide the Ticunas with opportunities for exposure and to a lesser extent infection with this parasite.

The Ticuna sera will be further tested for evidence of other parasitic infections. Results will be compared with those of other tribal groups who live in various geographical areas of north Brazil and who have different life styles and dietary habits.

TABLE 1. Toxoplasma IHA Titer Distribution of Ticuna Indians from 5 Villages on the Upper Solimoes River

<u>VILA BETANIA</u>							
RECIPROCAL TITER	< 64	64	128	256	512	1024	≥ 2048
AGE							
1 - 9	1						
10 - 19	11		2		1		1
20 - 29	11	1			1		1
30 - 39	12	1	2	2	1		
40 - 49	12	2		2			
≥ 50	11	1		1			
TOTAL	58	5	4	5	3		2

Nº sera ≥ 256 - 10 (12.9%)

Geometric mean reciprocal titer (GMRT) - 220 (titers < 64 excluded from calculations)

<u>CAMPO ALEGRE</u>							
RECIPROCAL TITER	< 64	64	128	256	512	1024	≥ 2048
AGE							
10 - 19	5	4	1				1
20 - 29	15	3	4	2	1		
30 - 39	19	2	1	1		1	
40 - 49	15	5					2
50	15	9	3	5			3
TOTAL	69	23	9	8	1	1	6

Nº sera ≥ 256 - 16 (16.9%)

GMRT - 190

<u>BELEM DOS SOLIMOES</u>							
RECIPROCAL TITER	< 64	64	128	256	512	1024	≥ 2048
AGE							
10 - 19	10	2		1	1		1
20 - 29	11	6	3		2	2	
30 - 39	7	1	1		1		
40 - 49	4	1		2			
50	7	2	5	1	1		
TOTAL	39	12	9	4	5	2	2

Nº sera ≥ 256 - 13 (17.8%)

GMRT - 190

TABLE 1. Toxoplasma IHA titer distribution of Ticuna Indians from 5 Villages on the Upper Solimoes River - (Cont.)

<u>NOVA ITALIA</u>							
RECIPROCAL TITER	< 64	64	128	256	512	1024	≥ 2048
AGE 10 - 19	6			1	1	1	1
20 - 29	6		2	1		1	
30 - 39	3	1	2	1	1		2
40 - 49	2	2	1	1		1	3
50	3	2	1		1	1	1
TOTAL	20	5	6	4	3	4	7

Nº sera ≥ 256 - 18 (36.7%)
GMRT - 370

<u>UMARIAÇU</u>							
RECIPROCAL TITER	< 64	64	128	256	512	1024	≥ 2048
AGE 10 - 19	12					1	2
20 - 29	13	1	3	4		1	2
30 - 39	7	1		1	2	1	
40 - 49	13	1			1	1	1
50	12	1	2	2	2	1	4
TOTAL	57	4	5	7	5	5	9

Nº sera ≥ 256 - 26 (28.3%)
GMRT - 452

<u>TOTAL TICUNA POPULATION</u>							
RECIPROCAL TITER	< 64	64	128	256	512	1024	≥ 2048
AGE 1 - 9	1	--	--	--	--	--	--
10 - 19	44	6	3	2	3	2	6
20 - 29	56	11	12	7	4	4	4
30 - 39	48	6	6	5	5	2	2
40 - 49	46	11	1	5	1	2	6
50	48	14	11	9	4	2	8
TOTAL	243	48	33	28	14	12	26
PERCENT	60.1	11.9	8.2	6.9	3.5	2.9	6.4

TABLE 1. Toxoplasma IHA Titer Distribution of Ticuna Indians from 5 Villages on the Upper Solimoes River - (Cont.)

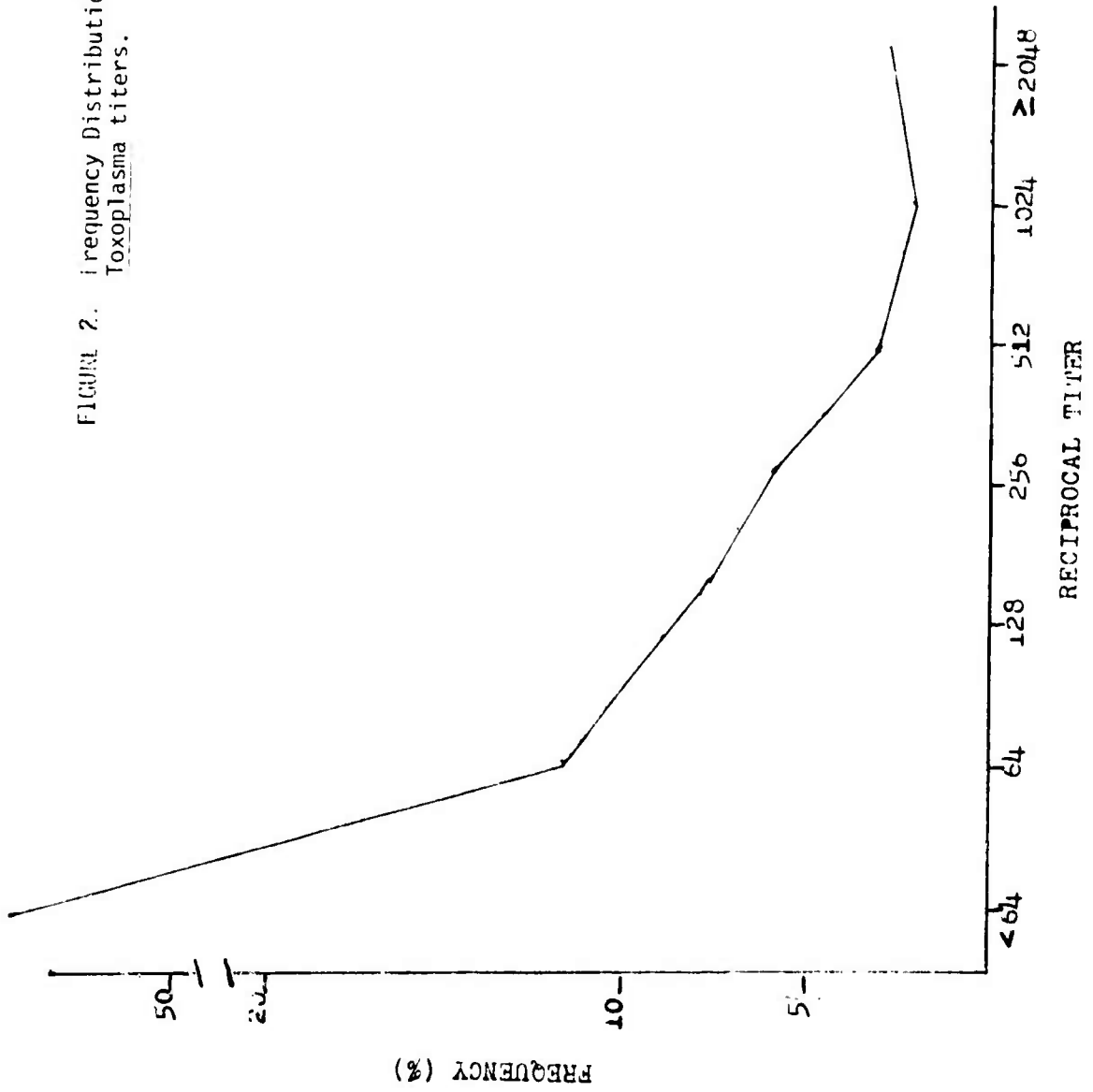
FREQUENCY (%) DISTRIBUTION OF TOTAL POPULATION BY AGE							
RECIPROCAL TITER	64	64	128	256	512	1024	2048
AGE 10 - 19	66.6	9.1	4.5	3.0	4.5	3.0	9.1
20 - 29	57.1	11.2	12.2	7.1	4.1	4.1	4.1
30 - 39	64.9	8.1	8.1	6.8	6.8	2.7	2.7
40 - 49	63.9	15.3	1.4	6.9	1.4	2.8	8.3
50	50.0	14.6	11.5	9.4	4.2	2.1	8.3

/zcm.



FIGURE 1. Map of Brazil Showing Location of Major Ticuna Villages (circle)

FIGURE 2. Frequency Distribution of *Ticuna* *Toxoplasma* titers.



BEST AVAILABLE COPY

APPENDIX 1



Boletim
Epidemiológico

VOLUNTARI VIANAS

MINISTERIO DA SAUDE
FUNDAÇÃO SESP

DIVISÃO DE EPIDEMIOLOGIA
ESTATÍSTICA E INFORMAÇÃO

Semanas Nº 506-(1976)

Este boletim contém dados recebidos do Ministério da Saúde, dos Secretários de Saúde dos Estados, Territórios e Distrito Federal, e de outros entes locais. DIVISÃO DE EPIDEMIOLOGIA, ESTATÍSTICA E INFORMAÇÃO
Av. Rio Branco, 251-12º andar, Caixa Postal, 2530, Telegrafas "FSESP", Tel. 232-8066, Rio de Janeiro, RJ - BRASIL

PORTE	PAGO
IMPRESSO	- C
CENTRAL	- RJ

HAISONELLA OZZARDI ENTRE OS ÍNDIOS TICUNAS

A existência de *H. ozzardi* no Brasil foi assinalada, pela primeira vez, em 1949, por M. P. Deane, na cidade de Manaus, Amazonas. A partir daí, vários inquéritos, feitos pela FSESP e pelo antigo Serviço Nacional de Malária, mostraram que ela ocorre principalmente a oeste daquela Capital, em localidades às margens do rio Solimões e seus tributários mais importantes. Fora do Estado do Amazonas, está presente apenas, de modo muito limitado, no norte de Mato Grosso (alto rio Xingú) e no Território de Roraima.

H. ozzardi é filária nativa do continente americano. Além do Brasil, sua área de distribuição compreende o México (península do Iucatán), Panamá, Colômbia, Venezuela, República da Guiné, Suriname, Guiné Francesa, Antilhas e Norte da Argentina. Por ser autóctona, tem prevalência mais elevada justamente entre populações indígenas ou com predominância de sangue índio.

Considerada como não patogênica, pouca importância se atribuiu a este filário. Aíros, por isso, têm sido os estudos

epidemiológicos e clínicos levados a cabo, até agora, e, em seu respeito, em nosso País, apesar da larga distribuição que ele apresenta no Estado do Amazonas. Tais estudos poderiam, entretanto, lançar muita luz sobre alguns problemas ainda não completamente resolvidos, como o do aparecimento de certas manifestações de hipersensibilidade em indivíduos parasitados, e o dos vetores responsáveis pela transmissão de *H. ozzardi* no Brasil.

No decorrer do ano de 1975, com o propósito principal de investigar a presença de oncocercose em tribos indígenas amazônicas, fora do grupo Ianomama, realizou o Instituto Evandro Chagas, com o auxílio da SUCAM, inquéritos para filaríose entre os índios Ticunas e Mura Piraná, do Estado do Amazonas, e Tiriós, do Pará.

Não se encontraram microfílarias, quer de *O. volvulus* quer de *H. ozzardi*, em 48 índios Tiriós (rio Paru do Oeste, e em 53 habitantes da localidade Maici (vila Mura Piraná, no rio Maici). Entre os Ticunas, um grupo cujas aldeias se estendem pelo alto Solimões, desde Santo Antônio do Içá até a fronteira do Brasil com a Colômbia e o Peru, a pesquisa não revelou *O. volvulus*, porém 45,7% dos indígenas examinados, pertencentes a sete aldeias, eram portadores de microfílarias de *H. ozzardi*. Os resultados desta investigação estão resumidos nos quadros seguintes:

continua na pág. 27

continuação da pág. 25

QUADRO I
PREVALÊNCIA DE *M. ozzardi* EM SETE LOCALIDADES
TICUNAS DO ALTO SOLIMÕES

LOCALIDADES	EXAMINADOS	POSITIVOS	MICROFILAREMIA
Vila Betânia	121	40	33,0
Nova Itália	68	31	57,3
Campo Alegre	145	70	48,2
Vendoval	94	37	39,3
Belém	117	54	46,1
Feijoaí	120	65	54,1
Umarinaçu	135	61	45,1
TOTAL	800	366	45,7

QUADRO II
MICROFILAREMIA POR *M. ozzardi* EM INDIOS TICUNAS
DISTRIBUIÇÃO POR SEXO E IDADE

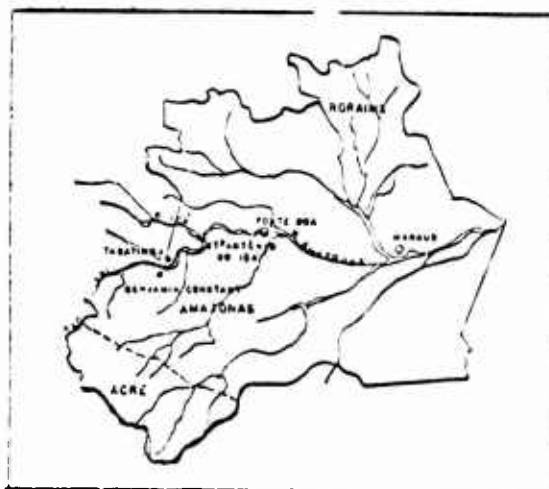
IDADE	HOMENS			MULHERES		
	Exam.	Posit.	Microf.	Exam.	Posit.	Microf.
0 - 4	38	4	10,5	25	1	04,0
5 - 9	54	9	16,5	33	6	18,1
10 - 19	67	19	28,3	67	21	31,3
20 - 29	90	44	48,8	72	42	58,3
30 - 39	69	46	66,6	60	28	46,6
40 - 49	46	32	69,5	41	23	56,0
50 - 59	27	20	74,0	32	17	53,1
60 e +	35	31	88,5	39	23	58,9
TOTAL	426	205	48,1	374	161	43,0

QUADRO III
MICROFILAREMIA POR *M. ozzardi* EM INDIOS TICUNAS
DISTRIBUIÇÃO POR IDADE

IDADE	EXAMINADOS	POSITIVOS	MICROFILAREMIA
0 - 4	63	5	7,9
5 - 9	92	15	16,3
10 - 19	134	40	29,8
20 - 29	162	66	53,0
30 - 39	129	74	57,3
40 - 49	87	55	63,2
50 - 59	59	37	62,7
60 e +	74	54	72,9
TOTAL	800	366	45,7

MAPA DO ESTADO DO AMAZONAS

AS ALDEIAS TICUNAS SE ESTENDEM DESDE SANTO ANTONIO DO IÇA
ATE A FRONTEIRA COM A COLOMBIA E O PERU



A microfilaremia variou de 33% a 57,3% nas sete localidades. Os índices mais elevados (acima de 50%) foram encontrados em Nova Itália e Feijoaí, aldeias situadas, a primeira, no rio Amaturá e, a segunda, no rio Solimões, próxima a Vendovalin Constant.

Quanto ao sexo, a prevalência global mostrou-se ligeiramente maior no sexo masculino do que no feminino. Como a diferença não é significativa, pode-se deduzir que o risco de exposição é praticamente o mesmo para ambos os sexos. Há, entretanto, uma diferença no que se refere à idade em que a microfilaria é adquirida. Enquanto no sexo masculino a microfilaria cresce de maneira uniforme até atingir cerca de 90% nos indivíduos com 60 anos ou mais, no sexo feminino, após alcançar quase 60% na terceira década da vida, mantém-se, a partir daí, no mesmo nível até os 60 ou mais anos. A diferença entre os dois sexos foi bastante significativa nos grupos etários de 30 a 39 anos e 60 e mais anos. Uma explicação para o fato de as mulheres só adquirirem a doença nas primeiras décadas da vida seria a mudança de hábitos após o casamento, fazendo diminuir a exposição ao vetor. Em outras palavras, as atividades domésticas, depois dos 20 anos, afastam as mulheres dos locais de transmissão, de modo que a prevalência entre elas, a partir daí, não se modificaria muito. Isso parece

continua na pág. 26

continuação da pág. 27

indicar que o vetor de *M. oasardi* é um inseto silvestre e a transmissão ocorre somente quando os indivíduos deixam as casas na cidade e vão à mata. H. L. Curqueira e A. D. Shelley já demonstraram, aliás, que *G. crassifemur*, espécie de simuliídeo

é muito abundante no alto Solimões, é um vetor apropriado do desenvolvimento das microfilárias de *M. oasardi*.

FONTE: Mário A. P. Moraes, Geovane N. Chaves, Margarida N. R. Alves e James Lovelace, do Instituto Evandro Chagas, Fiocruz, Belém, Pará.

NOTA INTERNACIONAL

MERCÚRIO NOS PEIXES

A poluição e a contaminação com mercúrio constituem uma história interessante e complexa, porque no sentido toxicológico há "dois mercúrios". De um lado, o clorato mercúrico e o metilmercúrio que são altamente venenosos. Os compostos de mercúrio, especialmente nos peixes e nos mariscos, têm causado ampla publicidade de incidentes de envenenamento de criaturas humanas. De outro, o clorato mercúrico foi usado como laxativo e seus compostos são empregados na quimioterapia, em diuréticos e pelos dentistas.

Todos os envenenamentos por mercúrio foram trágicos; alguns foram causados por negligência, fraude e irresponsável, pela indústria; outros, por analfabetismo ou mesmo estupidez.

Entre os minerais que os rios carregam para o mar está o mercúrio, que tem sido levado para os oceanos por milhões de séculos; ele também chega aos oceanos procedentes dos vulcões. Atualmente, cerca de 10.000 toneladas de mercúrio correm para o mar anualmente, sendo uma metade de origem natural e outra das atividades industriais modernas desenvolvidas durante os últimos 200 anos. Os efeitos foram desastrosos quando o mercúrio industrial chegou aos estuários na forma tóxica de metilmercúrio e os níveis tóxicos se acumularam no peixe e no marisco. Entretanto, de acordo com Schroeder, apenas 0,03% do mercúrio que chega ao oceano permanece em solução; o restante é isolado e afunda, lá permanecendo.

A preocupação atual com o mercúrio surgiu principalmente do envenenamento da baía de Minamata, no Japão. O metilmercúrio estava presente no resíduo de lama de uma fábrica que usava clorato mercúrico como catalizador na produção de clorato de vinil. Foi descoberto, em 1958, que o marisco do rio Minamata continha cerca de 17 ppm de mercúrio na base de peso úmido. De 1953 a 1960, foi relatado que 111 pessoas haviam sido envenenadas por haver comido peixe e marisco da área contaminada, inclusive 19 crianças nascidas de mães que tinham comido tais alimentos. Um segundo incidente semelhante ocorreu em Niigata, Japão, em 1965.

O tratamento de sementes de cereais com dicloromercúrio (Panogen) foi amplamente praticado na década de 1960, especialmente na Suécia. O tratamento evitava o mau crescimento da ferrugem e os pulgões nas mudas de trigo e de outros cereais. Os ovos das águas e dos falcoes exóticos na Suécia não chegavam a chocar, e isto foi atribuído ao consumo de pássaros comedores de sementes pelos pássaros predadores. As penas dos falcoes continham elevadas quantidades de mercúrio. Foi verificado, inesperadamente, que o peixe de água doce continha altos níveis de mercúrio, atribuídos à contaminação indireta dos abastecimentos de água. As fontes incluíam a manufatura de cloro e álcalis pela eletrólise por pilhas de mercúrio e o uso de produtos para o controle de algas e bactérias na indústria do papel. A Suécia proibiu o uso dos compostos de algum mercúrio como agentes para proteção de sementes. Não obstante, o envenenamento com sementes tratadas continuaram a ocorrer, apesar dos avisos. Alguns casos resultaram do consumo direto de tais cereais e, em um incidente em novo México, os grãos tratados foram utilizados na alimentação de porcos e a carne comida pela família.

continuação da pág. 28

C. Salmonellosis among Paracanas Indians

In November 1975, the deaths of two indian children of the tribe Paracanas were reported. Both children, as well as a third who survived, were less than 3 years old and had been admitted to the Maraba FSESP Hospital with symptoms of diarrhea, bloody vomitus, and convulsions. A diagnosis of "nonspecific encephalitis" was made by the attending physicians. The entire tribe of approximately 100 persons was evacuated from its village and temporarily relocated at a FUNAI post in the town of Tukururi near the Transamazon highway where medical care was more accessible. Subsequently, a visit to the tribe was made by CPT Lovelace and a medical student from the Instituto Evandro Chagas.

This group of Paracanas has originally occupied a small village 15 Km from the Transamazon highway at a point approximately 120 Km from Maraba. The village had consisted of 3 malocas (palm leaf huts) in which the tribe lived communally. The Indians practiced rudimentary agriculture, but their staple diet was wild game cooked hastily over an open fire with minimal or no dressing or cleaning. Questioning of the Indians and the FUNAI worker who lived with them revealed a high incidence of diarrhea in the tribe during the period in which the deaths of the children occurred (TABLE 1 and FIGURE 1). Twenty-seven random stool cultures were made. Salmonella spp. was isolated from 10 stools and Shigella spp. from three (11.1%). Four of the Salmonella isolates were from children 0-5 years old, three from children 6-10 years old and three from age groups older than 10 years. Two Shigella isolates were from 1 year old children; the other from a 32 year old man.

Diarrhea was common in all age groups, but during the period of the USAMRU visit symptoms were most pronounced in infants and young children. Most cases from which intestinal pathogens were isolated had lived in a single maloca. The two dead children had lived in this maloca also. The high prevalence of Salmonella in the population, and symptomatology compatible with bacterial food poisoning indicate that the probable cause of death in the cases of the two children was Salmonellosis. Observation of the Paracanas' dietary habits lead one to assume that the original source of infection was a forest mammal that had been killed for food.

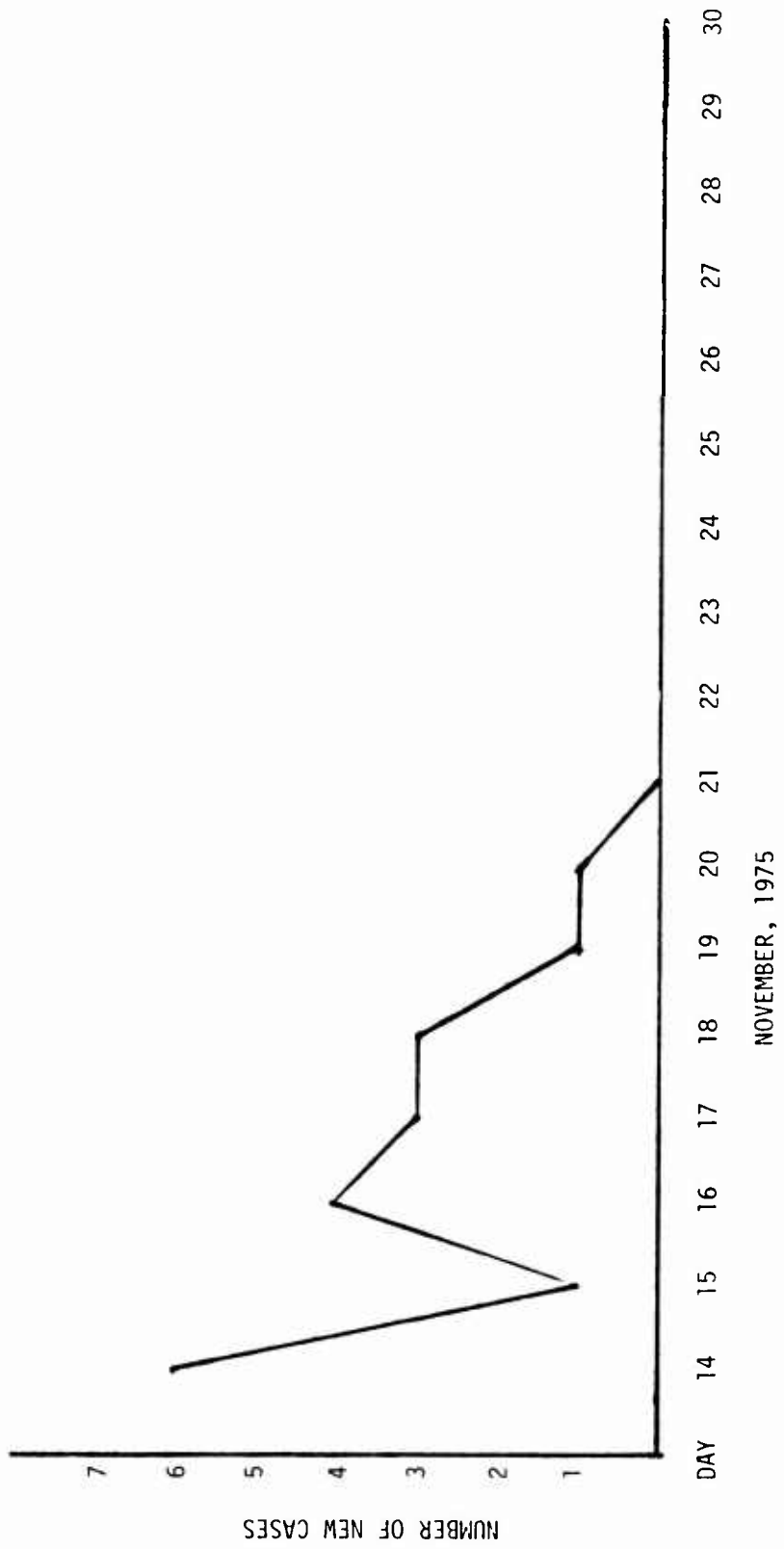
Contamination of living areas by wild mammals could also be a possible source. Human infections initially acquired from lower mammalian sources would be multiplied in the Indian population by promiscuous defecation around the maloca by persons, especially children, with diarrhea.

TABLE 1. Attack Rates of Diarrhea in Paracanas Children, November 1975.

No. of cases of diarrhea, females < 1 year	$\frac{3}{3}$	= 100%
No. of females < 1 year in tribe		
No. cases of diarrhea, males < 1 year	$\frac{4}{4}$	= 100%
No. of males < 1 year in tribe		
No. cases diarrhea, females 1-4 years	$\frac{6}{8}$	= 75%
N. females 1-4 years in tribe		
No. cases diarrhea, males 1-4 years	$\frac{5}{6}$	= 83.3%
No. males 1-4 years in tribe		

/zcm.

FIGURE 1.
DAILY NUMBER OF NEW CASES OF DIARRHEA
PARACANAS INDIANS, 14 - 30 NOVEMBER, 1975



D. Altamira Soldier Study

In January, 1976, two companies of Brazilian soldiers garrisoned in Altamira were enrolled in a study in cooperation with the Brazilian army, FSESP, and SUCAM to determine what pathogens recruits might encounter during their six months training period.

The soldiers were studied in two groups, by company, both of which underwent identical training, including jungle exercises, but on a different schedule. In January initial stool specimens were collected from 42 soldiers, examined for intestinal parasites, and cultured for bacterial pathogens. A follow-up examination was performed on the twenty-seven soldiers of this group who were still in the area in June. During the same time period a monthly platelet count was performed on 47 other soldiers. Skin scrapings from the axillary region and left plantar surface of the same group of soldiers were cultured for mycoses in January and June.

TABLE 1 lists the most common parasites and bacteria recovered from stools during the two collections. There was an obvious increase in the raw number of intestinal parasite and bacteria infections despite the smaller number of soldiers examined in June. The absence of hookworm infections in June is mysterious and can not be attributed solely to the detrition of soldiers from the study.

TABLE 2 shows the most common mycotic infections encountered in January. The follow-up cultures, initiated in June, have not been completed.

Monthly platelet counts for two companies (1 Companhia and C.C.S.) are shown in TABLE 3. The mean platelet counts for each company are compared with black fly population counts in FIGURE 1. Platelet counts remained within the normal range despite increases in black fly population densities. Previous observations on Altamira hemorrhagic syndrome would lead one to anticipate an inverse relationship between mean platelet count and black fly numbers. This relationship was not observed in the present study. It is probable that limited geographic distribution of the black flies populations accounts for the observations. Captures of black flies were made at sites in the forest at a minimum of 20 Km outside Altamira. Fly densities in the city and at the military garrison were subjectively lower than the capture sites and lower than densities of previous years according to residents. The soldiers would consequently not be subjected to constant bites while on post or in the city.

TABLE 1. Intestinal Parasites and Bacteria recovered from Altamira Soldiers.

SPECIES	NO OF INFECTIONS	
	JAN (42 soldiers)	JUNE (27 soldiers)
<u>Parasite</u>		
<u>Ascaris lumbricoides</u>	13	16
<u>Trichurus trichiurus</u>	5	11
<u>Hookworm</u>	24	0
<u>Entamoeba histolytica</u>	0	2
<u>Iodamoeba butschlii</u>	1	0
<u>Giardia lamblia</u>	0	3
<u>Strongyloides stercoralis</u>	0	2
No parasites	9	2
<u>Bacteria</u>		
<u>Protens sp</u>	6	5
<u>Salmonella sp</u>	4	11
<u>Shigella sp</u>	1	0
Normal flora	31	10

/zcm.

TABLE 2. Mycotic Organisms Isolated from the Axillary Region and Plantar Surface of Altamira Soldiers, January 1976.

Phialophora gougerotti

Candida albicans

Monosporum apiosperum

Aspergillus fumigatus

Actinomyces sp.

Cladosporum werneckii

Sporotrichum schenckii

Cephalosporum sp.

Nocardia asteroides

Torulopsis glabata

Trichosporon beigelli

Epidermophyton floccosum

Coccidioides immitis

Trichophyton schoenleinii

Geotrichum sp.

Aspergillus flavus

Nocardia brasiliensis

Rhizopus sp.

/zcm.

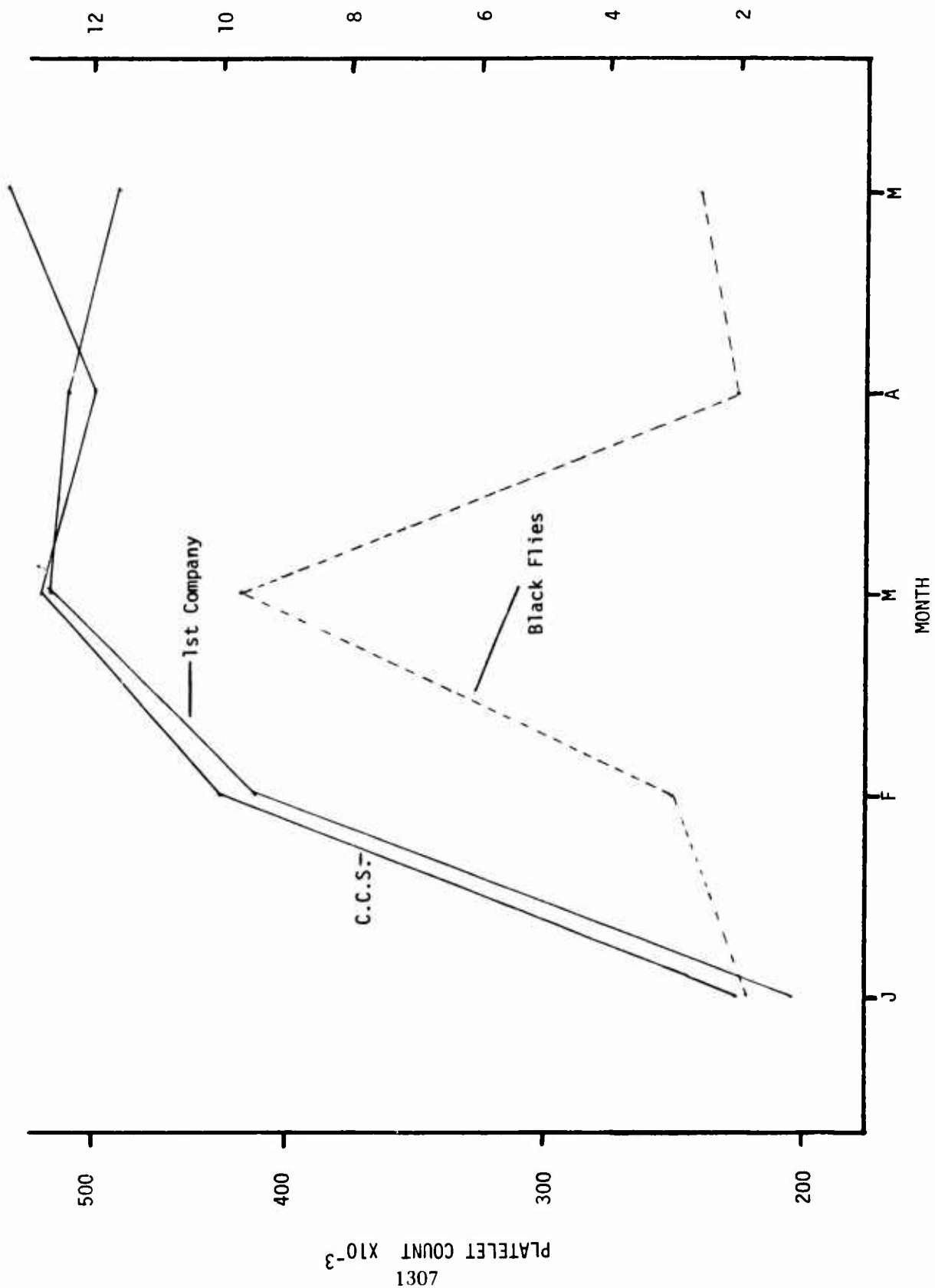
TABLE 3. Altamira Soldier Study - Platelets Counts

		<u>1a. Companhia</u>	<u>C.C.S.</u>
January	Mean S.D	202,000 + 47,000	224,000 + 50,000
February	Mean S.D	412,000 + 81,000	424,000 +116,000
March	Mean S.D	494,000 + 96,000	495,000 +101,000
April	Mean S.D	486,000 +121,000	481,000 +115,000
May	Mean S.D	467,000 + 94,000	522,000 +136,000
June	Mean S.D	448,000 + 89,000	480,000 +123,000

/zcm.

FIGURE 1. Comparison of Soldier Platelet Counts with Black Fly Population Density.

BLACK FLY POPULATIONS DENSITY



1307
PLATELET COUNT X10⁻³

V. ENTOMOLOGY

A. Surveillance for Medically Important Insects Along the Transamazon Highway

BACKGROUND: The surveillance program consists of a routine program of systematically collecting medically important insects along the highway in the Maraba and Altamira areas. The program was initiated, in part, in November, 1974, with specific objectives for providing:

1. Distributional information on medically important insect species by habitat, time, season of year and location along the highway.
2. Insect specimens for pathogen identification and/or isolation.

Routine surveillance is conducted at 12 sites from the Araguaia River, east of Maraba, to 160 Km west of Altamira. These sites are also included in the epidemiology and animal ecology surveillance programs. Two separate sites were established for malaria studies.

The collecting program is designed to sample a variety of habitats through much of the day and night using several collecting methods and is staffed with 3 teams of collectors (2 persons per team). Each lote is sampled during 2 days every 3 weeks. Collections of insects landing on man are emphasized but collections are also made with the Shannon trap and portable CDC light traps.

PROGRESS TO DATE: The program is in its 2nd years of collecting from the original 12 sites along the highway. The systematic nature of the collecting program has provided comparable data from sites which cover a large geographical area. Due to the time requirements for detailed analysis of data, only data from Gleba 18 lote 4 (Altamira jurisdiction) will be used to illustrate the type of entomological information available from this program. Future analyses will include correlation with environmental variables as well.

A total of 46 species of mosquitoes have been collected at Gleba 18 lote 4 from January, 1975, to May, 1976 (TABLE 1). During this time 554 collections have been made giving a total of 14,265 specimens. The landing collections (collections of insects landing on man or "man-biting collections") made in the forest during the day produced the largest number of species (TABLE 2). Species represented by largest numbers were Mansonia titillans (Walker), Psorophora albipes (Theobald), and Aedes serratus (Theobald). Collections conducted in the secondary scrub growth during the day produced a comparable number of species with fewer collections (TABLE 3). The 2 species represented by largest numbers were Ma. titillans (generic and subgeneric abbreviations are taken from Reinert, 1975), and Anopheles nuneztovari Galardon. The early evening collection (1830 hr) in the forest was very productive in numbers of species and specimens (TABLE 4). Mansonia titillans, An. nuneztovari, An. triannulatus (Neiva and Pinto), and An. oswaldoi (Peryassu) were well represented in these collections at sunset. The late evening collections were considerably less productive in species and specimens but did contribute a

large number of sandflies.

No mosquitoes were collected near the house during the 0930-0945 collections. Sunset and night collections at this locale netted 7 species and revealed the presence of large numbers of Ma. titillans biting near the house (TABLE 5). This species was present in most collections in every habitat, day and night (TABLE 7). Mansonia titillans is present throughout the year but seems least abundant during the late dry season (FIGURE 1). The pest value of this species is increased by its willingness to feed inside houses with an outdoor-indoor ratio ("O/I ratio") of 1.96 (see malaria).

The number of Ae. scapularis near the house was significant in respect to the relative absence of this species in all other collections in other habitats. Forattini (1961) stated that this species is becoming domesticated in Sao Paulo, Brazil.

The absence of An. darlingi Root and the relative absence of anopheline species in the peridomiliary environment indicate that near the house transmission of malaria is improbable (TABLE 7). However, the diurnal biting of several species in the forest and secondary scrub habitats present a possibility for secondary transmission.

Mansonia titillans, Haemagogus capricornii (Lutz), Sabethes chloropterus (Humboldt) and Sabethes glaucodaemon (Dyar and Shannon) were dominant in the canopy collections (TABLE 6). The scarcity of the latter 3 species in other habitats typify strong preference for the tree canopy. Sabethes chloropterus and Haemagogus leucocelaenus Dyar and Shannon are recognized vectors of sylvan yellow fever. Both species were collected at ground level in the forest and secondary scrub habitats. Equal numbers of Hg. leucocelaenus were collected at ground level and in the tree tower. The numbers are low but low population densities seem characteristic for most species at all sites.

Four species of anophelines and Ma. titillans were dominantly represented in the Shannon trap collections. One anopheline, Culex coronator Dyar and Knab and Ma. titillans were most abundant in the CDC light trap collections. These collections increase the spectrum of Culex and Uranotaenia species collected and produce large members of Psychodidae and Ceratopogonidae.

In total, almost equal numbers of Culicidae, Ceratopogonidae and Psychodidae were collected at Gleba 18 lote 4. The black flies (Simuliidae) were collected in all habitats but were represented by fewer numbers than the other 3 families. Detailed observations on the biting midges will be presented in another section.

COMMENTS: A variety of keys and descriptive literature is employed in identifying the field collected material. The specific identifications are valid only to the extent that the available literature is correct. Unquestionably, much taxonomic work is required for the mosquitoes of the Amazon region, as exemplified by such designations as Culex sp. B #19 etc. in this report.

Seasonal data are being compiled for several species and will be presented

when the data from several sites have been fully analyzed. The routine program is presently being evaluated and changes will be made as necessary. Some aspects of the program will become oriented to answering specific problems of vector behavior and disease ecology.

The 538 pools of sandflies and biting midges processed for virus isolation have been negative. Hopefully, the bulk of the remaining material will be processed for virus isolation in the near future.

TABLE 1. Species list of Culicidae Collected at Gleba 18 Lote 4, in the Altamira Jurisdiction. from January 1975 to May 1976.

<u>Aedes (Ochlerotatus)</u>	<u>Limatus</u>
<u>serratus</u>	<u>durhamii</u>
<u>fulvus</u>	<u>flavisetosus</u>
<u>scapularis</u>	
(Finlaya)	<u>Mansonia (Mansonia)</u>
<u>argyrothorax</u>	<u>titillans</u>
	<u>humeralis</u>
(Howardina)	<u>Psophora (Grabhamia)</u>
<u>arborealis</u>	<u>cingulata</u>
<u>septemstriatus</u>	
<u>fulvithorax</u>	(Janthinosoma)
	<u>albipes</u>
<u>Aedeomyia</u>	<u>ferox</u>
<u>squamipennis</u>	<u>lutzi</u>
<u>Anopheles (Nyssorhynchus)</u>	<u>Sabethes (Sabethes)</u>
<u>nuneztovari</u>	<u>cyaneus</u>
<u>oswaldoi</u>	<u>quasicyaneus</u>
<u>triannulatus</u>	(Sabethoides)
	<u>chloropterus</u>
(Anopheles)	<u>glaucodaemon</u>
<u>mediopunctatus</u>	<u>bipartipes</u>
<u>mattogrossensis</u>	<u>belisarioi</u>
(Stethomyia)	<u>Trichoprosopon (Trichoprosopon)</u>
<u>nimbus</u>	<u>digitatum</u>
<u>Coquillettidia</u>	(Runchomyia)
<u>lynchi</u>	<u>longipes</u>
<u>arribalzagia</u>	
<u>venezuelensis</u>	
<u>Culex (Culex)</u>	<u>Uranotaenia</u>
<u>corniger</u>	<u>geometrica</u>
<u>coronator</u>	<u>calosomata</u>
<u>declarator</u>	
<u>pipiens quinquefasciatus</u>	<u>Wyeomyia (Dendromyia)</u>
	<u>apronoma</u>
(Lutzia)	
<u>bigoti</u>	
(Melanoconion)	
<u>spissipes</u>	
<u>taeniopus</u>	
<u>vormifer</u>	
Culex sp B # 19	
sp B # 18	
<u>Haemagogus (Stegoconops)</u>	
<u>capricornii</u>	
<u>leucocelaenus</u>	

/zcm.

TABLE 2. Insects from 15 min. Landing Collections Made in the Forest at Gleba 18 Lote 04 on the Transamazon Highway Altamira, Brazil, January 1975-May 1976. A Total of 90 Collections were Made.

	0900-	1230-	1600-	TOTAL
Culicidae				
<u>Ae. (Fin.) argyrothorax</u>	1	0	0	1
<u>Ae. (How.) fulvitorax</u>	0	1	2	3
<u>Ae. (How.) septemstriatus</u>	0	1	1	2
<u>Ae. (Och.) scapularis</u>	0	5	0	5
<u>Ae. (Och.) serratus</u>	2	19	30	51
<u>An. (Ano.) mediopunctatus</u>	1	0	1	2
<u>An. (Nys.) nuneztovari</u>	3	8	3	14
<u>An. (Nys.) oswaldoi</u>	1	6	13	20
<u>An. (Nys.) triannulatus</u>	3	2	8	13
<u>An. (Ste.) nimbus</u>	2	1	4	7
<u>Cq. lynchi</u>	0	1	1	2
<u>Hg. Teucocelaenus</u>	1	6	0	7
<u>Li. durhami</u>	0	4	0	4
<u>Ma. titillans</u>	5	39	21	65
<u>Ps. albipes</u>	8	16	26	50
<u>Ps. ferox</u>	2	6	7	15
<u>Ps. Tutzii</u>	0	2	0	2
<u>Sa. bipartipes</u>	0	1	0	1
<u>Sa. chloropterus</u>	0	2	0	2
<u>Sa. cyaneus</u>	1	2	0	3
<u>Sa. glaucodaemon</u>	0	0	1	1
<u>Sa. quasicyaneus</u>	0	3	0	3
<u>Tr. digitatum</u>	1	3	1	5
<u>Wy. aporonoma</u>	4	7	9	20
<u>Anopheles spp.</u>	7	18	12	37
<u>Aedes spp.</u>	0	0	2	2
<u>Culex spp.</u>	2	12	91	105
<u>Haemagogus spp.</u>	1	7	6	14
<u>Chagasia spp.</u>	0	0	1	1
<u>Limiatu s p p .</u>	2	5	4	11
<u>Sabethes spp.</u>	0	1	2	3
<u>Trichoprosopon spp.</u>	0	0	3	3
<u>Wyeomyia spp.</u>	8	16	16	40
TOTAL	55	194	137	374
Simuliidae	9	56	241	306
Ceratopogonidae	46	138	111	295
Psychodidae (<u>Phlebotomus spp.</u>)	13	2	14	29
TOTAL	68	196	366	630
GRAND TOTAL	123	390	503	1004
NUMBER OF COLLECTIONS	17	37	36	
X PER COLLECTIONS	7.23	10.54	11.19	

/zcm.

TABLE 3. Insects from 68 15-min Landing Collections Conducted in an Area of Scrub Growth (Secondary Vegetation) at Gleba 18 Lote 4 on the Transamazon Highway in the Altamira Jurisdiction, Brazil, January 1975-May 1976.

	1200	1530	TOTALS
Culicidae			
<u>Ae. (How.) arborealis</u>	0	1	1
<u>Ae. (Och.) scapularis</u>	9	1	10
<u>Ae. (Och.) serratus</u>	0	3	3
<u>An. (Nys.) nuneztovari</u>	4	11	15
<u>An. (Nys.) oswaldoi</u>	1	5	6
<u>An. (Nys.) triannulatus</u>	3	8	11
<u>Culex sp. B # 18</u>	0	1	1
<u>Li. durhamii</u>	6	4	10
<u>Li. flavisetosus</u>	0	2	2
<u>Hg. leucocelaenus</u>	0	1	1
<u>Ma. titillans</u>	2	24	26
<u>Ps. (Jan.) albipes</u>	1	4	5
<u>Ps. (Jan.) ferox</u>	1	0	1
<u>Ps. (Jan.) lutzii</u>	1	0	1
<u>Sa. bipartipes</u>	1	1	2
<u>Sa. chloropterus</u>	1	4	5
<u>Sa. cyaneus</u>	0	1	1
<u>Sa. glaucodaemon</u>	3	1	4
<u>Tr. digitatum</u>	0	2	2
<u>Tr. longipes</u>	0	2	2
<u>Wy. aporonoma</u>	4	3	7
<u>Anopheles spp.</u>	20	11	31
<u>Culex spp.</u>	31	57	92
<u>Haemagogus spp.</u>	9	5	14
<u>Limatus spp.</u>	2	11	13
<u>Sabethes spp.</u>	12	8	20
<u>Wyeomyia spp.</u>	3	20	23
TOTALS	118	191	309
Simuliidae	151	315	466
Ceratopogonidae	1	3	4
TOTALS	152	318	470
GRAND TOTALS	270	509	779
\bar{X} PER COLLECTION	8.4	14.1	11.5

/zcm.

TABLE 4. Insects from 106 15-min Landing Collections Conducted in the Forest at Gleba 18 Lote 4 on the Transamazon Highway in the Altamira Jurisdiction, Brazil, January 1975-May 1976.

	1830	1925	2145	TOTALS
<i>Culicidae</i>				
<i>Ae.</i> (<i>Och.</i>) <i>fulvus</i>	1	1	0	2
<i>Ae.</i> (<i>Och.</i>) <i>scapularis</i>	1	0	0	1
<i>Ae.</i> (<i>Och.</i>) <i>serratus</i>	13	0	0	13
<i>An.</i> (<i>Ano.</i>) <i>mattogrossensis</i>	2	5	1	8
<i>An.</i> (<i>Ano.</i>) <i>mediopunctatus</i>	5	2	1	8
<i>An.</i> (<i>Nys.</i>) <i>nuneztovari</i>	30	16	7	53
<i>An.</i> (<i>Nys.</i>) <i>oswaldoi</i>	57	0	3	60
<i>An.</i> (<i>Nys.</i>) <i>triannulatus</i>	32	54	11	67
<i>Cq.</i> <i>arribalzagaglia</i>	0	1	1	2
<i>Cq.</i> <i>lynchi</i>	3	0	0	3
<i>Cx.</i> (<i>Cux.</i>) <i>coronator</i>	7	5	0	12
<i>Cx.</i> (<i>Cux.</i>) <i>declarator</i>	1	0	0	1
<i>Cx.</i> (<i>MeI.</i>) <i>spissipes</i>	3	0	0	3
<i>Cx.</i> (<i>MeI.</i>) <i>taeniopus</i>	1	0	0	1
<i>Cx.</i> (<i>MeI.</i>) <i>vomerifer</i>	1	0	0	1
<i>Cx.</i> sp B # 22	1	0	0	1
<i>Ma.</i> <i>titillans</i>	178	32	10	120
<i>Ps.</i> (<i>Gra.</i>) <i>cingulata</i>	8	1	0	9
<i>Ps.</i> (<i>Jan.</i>) <i>albipes</i>	6	1	1	8
<i>Tr.</i> <i>digitatum</i>	6	0	0	6
<i>Anopheles</i> sp.	64	24	5	93
<i>Culex</i> sp.	44	1	0	45
<i>Mansonia</i> sp.	0	1	0	1
<i>Wyeomyia</i> sp.	1	0	0	1
TOTALS	465	144	40	649
<i>Simuliidae</i>	90	1	0	91
<i>Ceratopogonidae</i>	271	256	606	1133
<i>Psychodidae</i>	73	242	495	810
TOTALS	434	499	1101	2034
GRAND TOTALS	899	649	1141	2683
\bar{X} PER COLLECTION	11.73	14.7	31.45	19.2

/zcm.

TABLE 5. Insects from 125 15-min Landing Collections Conducted Within 10 m of the House at Gleba 18 Lote 04 on the Transamazon Highway Near Altamira, Brazil, January 1975-May 1976.

	0930- 0945	1855- 1910	1955- 2010	2215- 2230	TOTALS
<u>Culicidae</u>					
<u>Ae. (Och.) scapularis</u>	0	21	0	0	21
<u>An. (Ano.) mattogrossensis</u>	0	2	5	0	7
<u>An. (Nys.) nuneztovari</u>	0	4	2	0	6
<u>An. (Nys.) triannulatus</u>	0	8	0	0	8
<u>Cx. (Cux.) coronator</u>	0	1	0	0	1
<u>Cx. (MeI.) spissipes</u>	0	1	0	0	1
<u>Ma. titillans</u>	0	1846	224	94	2164
<u>Anopheles spp.</u>	0	37	1	0	38
TOTALS	0	1920	232	94	2246
<u>Simuliidae</u>	118	38	49	0	205
<u>Ceratopogonidae</u>	0	0	5	7	12
<u>Psychodidae</u>	0	0	0	1	1
TOTALS	118	38	54	8	218
GRAND TOTALS	118	1958	286	102	2464
\bar{X} PER COLLECTION	6.94	52.9	7.73	2.76	19.25

/zcm.

TABLE 6. Insects from 53 1-hr Landing Collections Conducted in the Forest Canopy at Gleba 18 Lote 4 on the Transamazon Highway in the Altamira Jurisdiction, Brazil, January 1975-May 1976.

	1000- 1100	1400- 1500	TOTALS
<u>Culicidae</u>			
<u>An. (Nys.) oswaldoi</u>	0	2	2
<u>An. (Nys.) triannulatus</u>	0	1	1
<u>Hg. capricornii</u>	2	33	35
<u>Hg. leucocelaenus</u>	4	3	7
<u>Ma. titillans</u>	18	54	72
<u>Ps. albipes</u>	3	3	6
<u>Sa. belisarioi</u>	2	9	11
<u>Sa. chloropterus</u>	1	24	25
<u>Sa. cyaneus</u>	0	1	1
<u>Sa. glaucodaemon</u>	8	14	22
<u>Wy. aporonoma</u>	0	1	1
<u>Haemagogus spp.</u>	11	85	96
<u>Chagasia spp.</u>	0	1	1
<u>Sabethes spp.</u>	0	1	1
<u>Wyeomyia spp.</u>	2	1	3
TOTALS	51	233	284
<u>Simuliidae</u>	216	482	698
<u>Ceratopogonidae</u>	3	16	19
<u>Psychodidae</u>	0	1	1
TOTALS	219	499	718
GRAND TOTALS	270	732	1002
\bar{X} PER COLLECTION	15.9	20.3	18.9

/zcm.

TABLE 7. Mean Numbers Collected per 15 min. Landing Collection, by Time and Habitat, for 4 Species.^a The Collections were Conducted at Gleba 18. Lote 4 on the Transamazon Highway in the Altamira Jurisdiction, Para January 1975-May 1976.

TIME AND PLACE OF COLLECTIONS	NUMBER OF COLLECTIONS	NUMBERS COLLECTED PER COLLECTION			
		An. (Nys.) <u>triannulatus</u>	An. (Nys.) <u>nuneztovari</u>	An. (Nys.) <u>oswaldoi</u>	Ma. <u>titillans</u>
Tree Canopy					
1000-1100	17	0	0	0	1.06
1400-1500	36	0	0	0.12	1.5
Secondary Shrub					
1200-1215	32	0.09	0.13	0.03	0.06
1530-1545	36	0.22	0.31	0.14	0.67
Peridomiciliary					
0930-0945	17	0	0	0	0
1855-1910	37	0.22	0.11	0	49.9
1955-2010	37	0	0	0.05	6.05
2215-2230	37	0	0	0	2.54
Forest-ground level					
0900-0915	17	0.17	0.17	0.05	0.29
1230-1245	37	0.05	0.22	0.16	1.05
1600-1615	36	0.22	0.08	0.36	0.58
1830-1845	37	0.86	0.81	1.54	4.81
1925-1940	34	1.59	0.47	0	0.94
2145-2200	35	0.31	0.20	0.09	0.29

^a These data represent a total of 222.5 man-hr of collecting time.

/zcm.

TABLE 8. Species and Family Representation in Shannon Trap, and CDC Light Trap Collections. All Collections were Made in the Forest of Gleba 18 Lote 4 in the Altamira Jurisdiction, Para, Brazil from January 1975-May 1976.

CULICIDAE	SHANNON ^a	CDC ^b
Ae. (Och.) <u>fulvus</u>	2	0
Ae. (Och.) <u>scapularis</u>	0	1
Ae. (Och.) <u>serratus</u>	3	1
An. (Ano.) <u>mattogrossensis</u>	23	25
An. (Ano.) <u>mediopunctatus</u>	3	1
An. (Nys.) <u>nuneztovari</u>	38	7
An. (Nys.) <u>oswaldoi</u>	20	2
An. (Nys.) <u>triannulatus</u>	52	5
An. (Ste.) <u>nimbus</u>	0	1
Cq. <u>arribalzagala</u>	0	0
Cq. <u>venezuelensis</u>	1	0
Cx. (Cux.) <u>coronator</u>	4	24
Cx. (Cux.) <u>declarator</u>	0	3
Cx. (Lut.) <u>bigoti</u>	1	0
Cx. (Mel.) <u>spissipes</u>	0	8
Cx. (Mel.) <u>taenifopus</u>	0	15
Cx. (Mel.) <u>vomerifer</u>	0	1
Cx. sp B#19	0	2
Cx. sp B#22	0	1
Ma. <u>titillans</u>	23	18
Ps. (Jan.) <u>albipes</u>	6	2
Ps. (Jan.) <u>ferox</u>	0	1
Ur. <u>calosomata</u>	1	11
Ur. <u>geometrica</u>	0	1
Anopheles spp.	39	9
Culex spp.	17	70
Uranotaenia spp.	1	12
Trichoprosopon spp.		
TOTALS	238	221
Simuliidae	0	0
Ceratopogonidae	2758	319
	female 372	female 112
Psychodidae	male 2600	male 362
TOTALS	5730	793
GRAND TOTALS	5968	1014
NUMBER GENERA	8	6
NUMBER SPECIES	13	20
		73 trap nights

^a Each value is taken from 36 trap hours of collecting.

^b Each value is taken from 73 trap nights of operation.

B. Culicoides Taxonomy and Behavior

Culicoides are a cosmopolitan pest in the Amazon basin, breeding in a diversity of habitats, i.e., tree holes, decaying plant material, impoundments, and river edges. Large numbers of culicoides have been collected in all study sites along the Transamazon highway in the Maraba and Altamira areas (TABLE 1). In the developmental stages of the entomology surveillance program, culicoides were only being processed for virus isolation test; however, within recent months taxonomic capabilities have been established for this group of insects. As with any disease-vector program, taxonomic capabilities are an essential part of any epidemiological investigation where the disease agent may be vector borne.

Routine slide preparations and preliminary identification efforts are performed in the Belem laboratory. Representative specimens of the various species are also forwarded to authorities at the Smithsonian Institute in Washington, D.C. for confirmation of identified species and for identifying species beyond the taxonomic capabilities of this unit. A preliminary list of species identified to date is listed in TABLE 2.

The enumeration of culicoide species will be increased as the taxonomic efforts of this group are expanded.

In addition to identifying the species present during the routine capture program, considerable progress is being made in monitoring the daily and seasonal biting activities of biting midges.

As with most species of insects in the Amazon basin, the population of culicoides is greatly influenced by seasonal variations. The data in FIGURES 1 and 2 is an example of the seasonal activities as determined by numerous man-biting and Shannon trap collections. Even though only evening time biting collection are shown in FIGURES 1 and 2, certain species of culicoides are also abundant and very active during the day. Their seasonal prevalence varies with the locality, but it is usually highest from January to May with a peak in the months of January, February and March. The rainy season for our study areas normally starts in December and terminates in April.

The breeding sites for the common species found in our study sites have not been studied; however, it would seem logical that the majority of culicoides species are breeding in those areas which require a high moisture content (decaying plant material) or frequent replenishment of water (tree holes, plant axils).

COMMENTS: Emphasis will continue to be placed on collecting adult culicoides along the Transamazon highway and in the municipalities. Particular areas of interest will be those of daily and seasonal activities. Taxonomic capabilities of the unit will be further developed and emphasis will be placed on recognizing morphological characteristics which may lead to the recognition of new species. A reference collection of known species is being established at the USAMRU-Belem laboratory for future training and research efforts. Since this group of insects is thought to be important in transmission of human and animal pathogens, e.g., viruses and filariasis, the monitoring of culicoides for virus isolations will be continued.

MILITARY IMPORTANCE: Culicoides are a cosmopolitan group of insects found in a diversity of habitats throughout the world. In some geographical areas, these minute insects exist in such high densities that they may render an area uninhabitable. Although individuals vary considerably in their reactions to insect bites, this group of insects normally causes considerable irritation and itching. Debilitating allergic reaction to the toxic feeding secretion of culicoides is not uncommon. In addition to their annoyance capabilities, several species of culicoides are involved in the transmission of human filarial diseases, Acanthocheilonema perstans, Acanthocheilonema streptocerca, Mansonella ozzardi, and viruses. The medical importance of this group of insects is continuously being renewed as new diseases transmitted by culicoides are discovered.

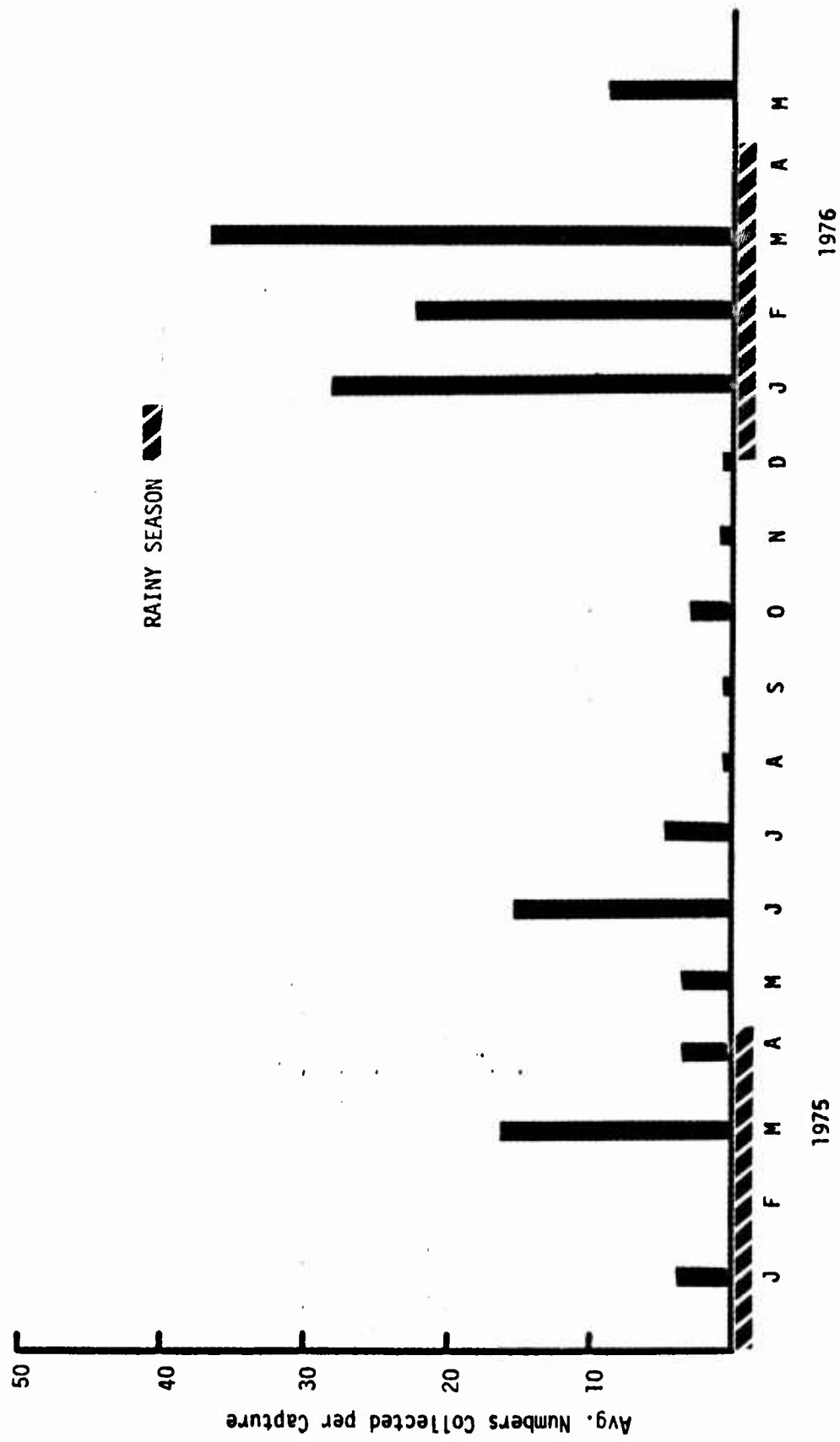


FIGURE 1. Seasonal distribution of *Culicoides* at Gleba 18 Lote 4 in Altamira, Para, Brazil, 1975-1976. Data obtained from man-biting collections in the forest during the evening hrs.

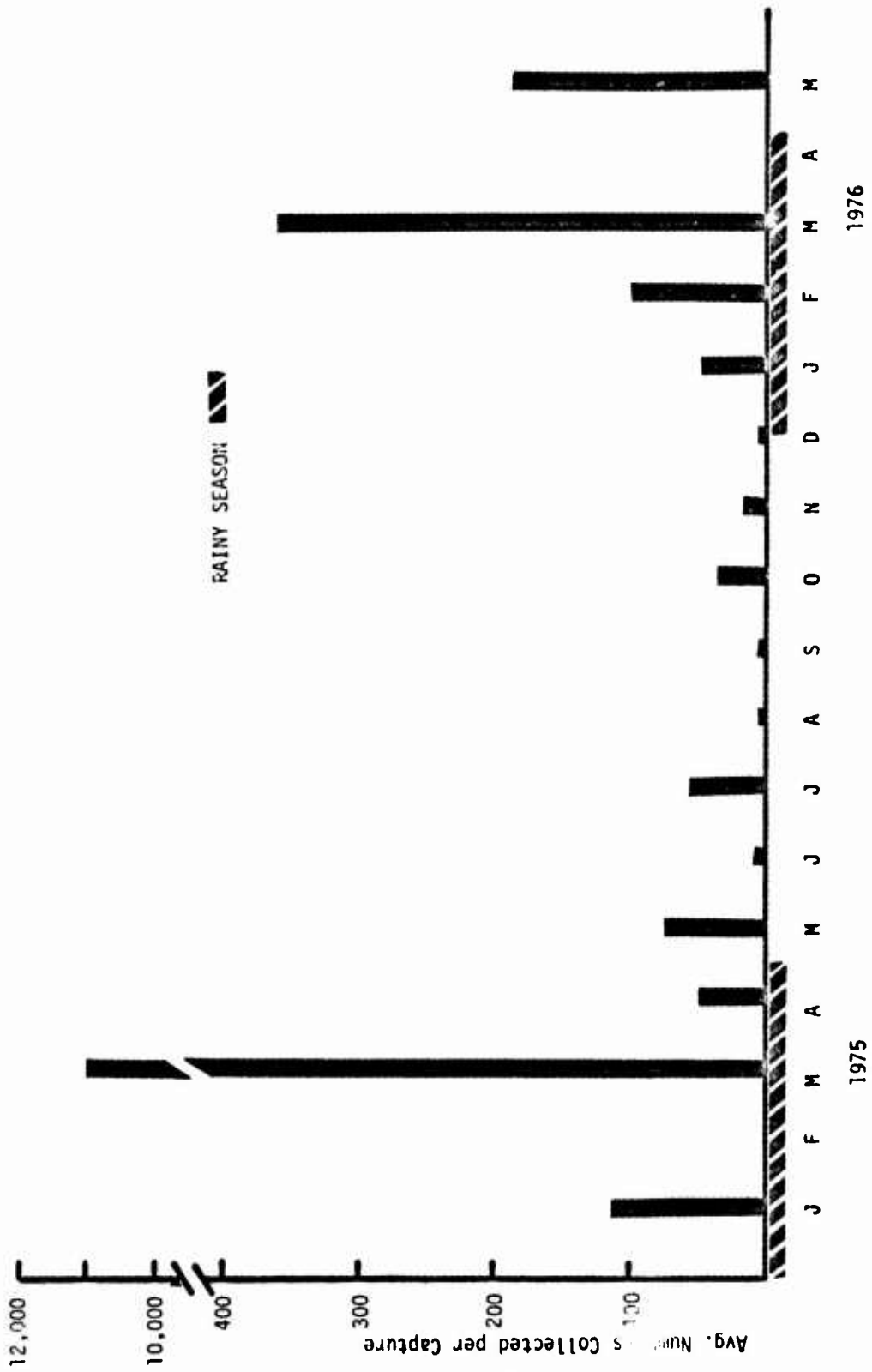


FIGURE 2. Seasonal distribution of *Culicoides* at Gleba 18 Lote 4 in Altamira, Para, Brazil, 1975-1976. Data obtained from Shannon trap collections in the forest during the evening hrs.

TABLE 1. Preliminary Observations on the Distribution of Ceratopogonidae Species (Culicoides) by Study Area in the Amazon Basin, Para, Brazil, 1975-1976.

SPECIES	ALTAMIRA AREA								MARABA AREA				MUNICIPALITIES			
	6 18 L 04	6 10 L 01	6 31 L 01	6 36 L 07	6 62 L 02	6 35 L 01	6 51 L 03	6 18 L 06	6 66 L 09	6 38 L 06	6 29 L 03	6 05 L 05	Belém	Palästina	Santarem	Itupiranga
<i>C. foxi</i>					X	X	X		X		X	X	X			
<i>C. fusipalpis</i>								X								
<i>C. paucienfuscatus</i>		X				X										
<i>C. flavivenula</i>																
<i>C. batesi</i>																
<i>C. filariferus</i>					X		X		X							
<i>C. insignis</i>																
<i>C. ignacioi</i>			X		X								X			
<i>C. lutzi</i>																
<i>C. paraensis</i>																
<i>C. insinuatus</i>																
<i>C. pseudodiabolicus</i>																
<i>C. hylas</i>	X															
<i>C. pusillus</i>																
<i>C. palpalis</i>																
<i>C. debilpalpis</i>																
<i>Forcipomyia</i> sp		X														

/zcm.

TABLE 2. List of Ceratopogonidae Species Collected by Trap Methods in the Amazon Basin, Para, Brazil:
1975-1976.

SPECIES	COLLECTING METHODS				
	SHANNON	GROUND COLLECTION	MAN COLLECTION	CDC LIGHT TRAP	MAN TREE CANOPY
<i>C. fusalpalpis</i>	X		X	X	
<i>C. foxi</i>	X		X	X	
<i>C. paucienfuscatus</i>	X				
<i>C. flavivenula</i>	X				
<i>C. batesi</i>	X		X		
<i>C. filariferus</i>	X		X	X	
<i>C. insignis</i>	X		X	X	X
<i>C. ignacioi</i>	X		X	X	
<i>C. lutzi</i>	X		X		
<i>C. paraensis</i>	X			X	
<i>C. insinuatus</i>	X		X		
<i>C. pseudodiaboli</i>	X			X	
<i>C. hylas</i>				X	
<i>C. pusillus</i>				X	
<i>C. palpalis</i>				X	
<i>C. debilpalpis</i>			X		
<i>Forcipomyia</i> sp	X		X	X	X

/zcm.

C. Black Flies

BACKGROUND: Seasonally, the recurrent black fly population occurs in the Amazon basin shortly following the beginning of the rainy season in December-April. In numerous areas throughout the Amazon basin, the black fly population attains sufficient numbers to be considered a major public health problem. As more people migrate to the interior of the Amazon basin, there undoubtedly will be more health problems related to this hematophagous insect. Symptomatic reactions of persons exposed to the voracious biting attacks of the black fly varies due to the number of bites received and immunologic response of the victim. Some people appear to be only slightly annoyed by this insect's bite, while other persons exhibit debilitating reactions. The initial biting process itself may be painless; however, after a short interim of feeding, there is usually pain, swelling and general local discomfort. Intense discomfort of the afflicted areas of the body often occurs for days after the initial bite(s). Lesions may develop resulting in secondary bacterial infections. The more serious infections usually result in permanent scarring and discoloration of the tissue. Sometimes, persons who are exposed to repeated bites develop allergic manifestations referred to as the "hemorrhagic syndrome of Altamira". In addition to the physiological reaction of the host to black fly toxic feeding secretions, the black fly is also noted as an important vector of Mansonella ozzardi and Onchocerca volvulus in some areas of the Amazon basin.

GENERAL REMARKS ABOUT THE BLACK FLY: The various members of the black fly taxonomic family (Simuliidae) are small (2-3 mm in length), robust, and dark in coloration with short piercing mouth parts. They are popularly referred to as black flies, turkey gnats or buffalo gnats. The females normally only seek their host during the day, particularly in the morning and evening hours. However, the peak feeding activity of black flies may vary according to species and weather conditions. The preferred breeding grounds range small streams to large rivers with rapid to a moderate current. Although the black fly population usually abounds near its breeding sites, a large population may exist at a considerable distance from these areas.

PROGRESS TO DATE: Field evaluation of repellent impregnated mesh jackets and aerosol spray skin repellents was conducted at the "Ponta da Terra" ranch approximately 38 Km northwest of Maraba, Para, Brazil. Testing was conducted from 18 May to 26 May 1976. The cotton-nylon mesh jackets were provided by S.C. Johnson and Son, Inc. and the U.S. Navy.

Two pre-treated jackets used in the test were impregnated with deet (N-N-diethyl-meta-toluamide) and "R69" (3-Acetyl-2-(2,6-dimethyl-5-heptenyl)-oxazolidine) at 23% by weight of active ingredients or 0.302 gm. of actives per gram of fabric "test material provided by S.C. Johnson and Son, Inc."

Additional repellents evaluated were "R-11" (2,3:4, 5-Bis (2 butylene) tetrahydro-2-furaldehyde) and "MGK264" (N-octyl bicycloheptene dicarboximide) which were impregnated into one jacket. These repellents were formulated at 0.5 gr of 50% "R-11" and 50% MGK264"/gm weight of jacket.

The fourth test jacket contained chemicals "R-326 (Di-n-propyl isocin-

chomerone) and "MGK264" (N-octyl bicyc heptane dicarboximide) formulated at 0.5 gm of 50% "R-11" and 50% "MGK-264"/gm weight of jacket.

Non-pretreated cotton-nylon mesh jackets were prepared for testing in the following manner. Test repellents were formulated on a ratio of chemical weight to weight of jacket basis. The repellent mixtures were then added to a 2000 ml beaker containing 250 ml of absolute ethanol. Test jackets were then placed in containers and sealed for 24 hours. After the jackets were impregnated they were allowed to dry for 6 hours and resealed in a plastic sack to await testing. After each test trial, the jackets were resealed in their original plastic sacks.

The repellent mesh jackets were tested and evaluated in conjunction with two types of controls: 1) non-treated control mesh jacket and 2) one test subject without a mesh jacket. The second control method was selected to determine the influence of a control mesh jacket on the landing and biting rates of black flies.

Field evaluation of the test repellent mesh jackets were conducted as follows: 1) Each test subject was required to wear protective boots and trousers 2) The test jacket was the only clothing item worn on the torso and 3) Test subjects were randomly spaced (10-15 meters) apart. Each test was divided into 3 time periods with a 2-5 minute rest interim between each testing period. The test subjects wearing repellent impregnated jackets would count all black fly landings and/or bites received during each 10 (ten) minute test period. During the 2-5 minute rest interim, the test subjects would leave the test area and then return to continue the testing sequence until three 10 minute (30 minute testing time) test periods were completed.

The same testing method was also performed with the controls; however, the actual testing time was reduced to 5 minutes per test period (15 minutes total testing time) due to the discomfort caused by the biting black flies. After each complete test, the test subjects would wear a different repellent mesh jacket to negate differential host attractions. Biting rates were recorded for 3 areas of the body: 1) the number of bites received on the face 2) the number of bites received on the hands, and 3) the number of bites received through the mesh jacket. An accountable bite for this field test was described as one in which the black fly makes contact with the skin or the black fly entered the mesh of the jacket or the black fly was actually feeding. Those black flies landing on the outside of the jacket were not recorded.

The temperature and relative humidity were monitored for each test.

Skin repellents were also tested to determine their effectiveness as a personal protective measure against black flies. Three aerosol skin repellents and control were evaluated for 1, 2, 3, hours of testing time. The repellents tested were 1) deet (N,N-Diethyl-Meta-toluamide), 71,25%, Standard Army Stock Number 6840-864-5432 2) Twenty and Five (N,N-Diethyl-meta-toluamide, 20%, and 3-Acetyl-2-(2,6-dimethyl-5-heptenyl-oxozolidine, 5%), and 3) Ten and Five (N,N-Diethyl-meta-toluamide), 10% and 3-Acetyl-2-(2,6-dimethyl-5-heptenyl-oxozolidine, 5%). The last two chemicals were provided by S.C. Johnson and Son, Inc.

The arms of each test subject were sprayed with one of the candidate repellents from the elbow to the finger tips. A different compound was applied to each arm of a test subject. All persons performing the test were wearing protective clothing and head nets to restrict biting activity to the exposed body areas. Only those bites received between the elbow and wrist were recorded. For this test a bite was defined as one in which the black fly's mouth parts were penetrating into the skin. In order to confirm a bite for a particular repellent, the subject must receive two bites on the same arm within a 30 minute time period. The protection time of a repellent is calculated from the treatment time until the first bite of a confirmed bite pair. The biting pressure of the black flies was monitored by the control by making periodic 5 minute biting counts. Each arm tested was considered to be 1 replication. The control did not receive a repellent on either arm. Assuming the black fly population was randomly distributed, the test subjects were positioned in a circular pattern approximately 10 meters apart.

RESULTS: TABLE 1 shows the results obtained with 2 types of controls and 4 candidate repellents which were replicated 8 times. The reason for having two types of controls was to compare the biting pressures received by a subject not wearing a jacket with one in which the person has a non-treated mesh jacket.

The results show that there was an approximate 50% reduction in total bites/min when a non-treated jacket was worn. However, when comparing biting rates for similar areas of the body (BITES/MIN, JACKET) for the two control treatments, there was generally a 3-fold decrease in the biting rate for the control mesh jacket treatment. This reduction in biting rate is believed to be due to a reduction in exposed body area and behavioral characteristics of the black flies rather than a repellent factors of the non-treated cotton-nylon mesh fabrics.

The results in TABLE 1 and TABLE 2 show that deet and R-11 plus MGK 264 were the most effective repellents. When comparing the total number of bites received by those subjects wearing the deet jacket and R-11 plus MGK 264 jacket, the number of bites received were the same (16 bites), however; deet offered more protection to the exposed areas of the body (hands and face) than R-11 plus MGK 264 treated jackets.

Repellents R-326 plus MGK 264 and R-69 gave a 96% and 90% reduction of black fly bites respectively when compared to the control jacket but failed to give above an 80% reduction in total cumulative bites (TABLE 2).

The data in TABLE 3 shows the total number of bites received per body area. As the data indicates, a large portion of the biting activity occurred on the exposed areas of the face for both the repellent and control groups. The large number of bites received in this area tends to support the hypothesis that hematophagous insects are attracted by CO₂ expelled by their host animals.

The results of the skin repellents for black flies are listed in TABLE 4. Some formulation of deet was incorporated into all of the repellents used in the test. Each repellent exhibited the same protection time except for 1 replication with Ten and Five, which had a confirmed bite after 2:20 hrs of protection time. It was felt that longer testing periods will be

necessary to demonstrate a difference in protection time offered by the skin repellents.

MILITARY IMPORTANCE: Due to the ubiquitous breeding areas and flight range capabilities of black flies in the Amazon basin, chemical control measures would normally prove to be ineffective and economically unfeasible. When personnel are required to operate in areas where disease-vector control techniques are prohibitive or impractical, effective personal protective chemicals would provide the most flexible and economical system available.

Previous studies performed by other researchers in the laboratory and under field conditions have shown that personal skin repellents and chemically-treated jackets are effective in reducing the number of bites from black flies and other blood feeding pest. However, extensive research still needs to be conducted to evaluate the effectiveness of black fly repellents under different geographical and climatological conditions. Other factors which must be evaluated are comparative test with different species groups and population densities.

Preliminary observation in the field by some workers observed that deet failed to give satisfactory personal protection when working in the Altamira area. It is this same area where black flies are known to cause the "hemorrhagic syndrome of Altamira". Future test are planned in this area to determine the effectiveness of new repellents. Candidate test repellents will be routinely evaluated for black flies and other medically important insect groups.

TABLE 1. Results of Controls and Repellent Mesh Jackets for Determining the Biting Rates of Black Flies in the Amazon Basin, Para, Brazil, 1976.

REPELLENT	TEST TIME (MINUTES)	Nº BITES/FACE	Nº BITES/HANDS	Nº BITES/JACKET	TOTAL BITES	BITES/MIN FACE	BITES/MIN HANDS	BITES/MIN JACKET	TOTAL BITES/MIN.
TEST Nº 1									
Without Jacket	15	55	13	130	198	3.70	.90	8.70	13.20
Control Jacket	15	34	8	51	93	2.30	.50	3.40	6.20
deet	30	0	0	0	0	0	0	0	0
R-69	30	11	1	2	14	.37	.03	.07	.07
R-326 & MGK 264	30	6	1	1	8	.20	.03	.03	.26
R-11 & MGK 264	30	1	4	5	10	.03	.13	.17	.33
TEST Nº 2									
Without Jacket	15	19	2	163	184	1.30	.07	10.90	12.30
Control Jacket	15	51	16	58	125	3.40	1.07	3.87	8.33
deet	30	8	8	16	32	.26	.26	.53	1.06
R-69	30	34	12	9	55	1.13	.40	.30	5.50
R-326 & MGK 264	30	81	37	19	137	2.70	1.23	.63	4.57
R-11 & MGK 264	30	0	6	3	9	0	.20	.10	.30
TEST Nº 3									
Without Jacket	15	68	25	143	236	4.53	1.67	9.53	15.73
Control Jacket	15	100	5	133	238	6.67	.33	8.86	15.87
deet	30	1	1	0	2	.03	.03	0	.07
R-69	30	108	46	102	256	3.60	1.53	3.40	8.53
R-326 & MGK 264	30	108	13	33	154	3.60	.43	1.10	5.13
R-11 & MGK 264	30	20	0	0	20	.66	0	0	.66

TABLE 1. Results of Controls and Repellent Mesh Jackets for Determining the Biting Rates of Black Flies in the Amazon Basin, Para, Brazil, 1976. (Cont.)

REPELLENT	TEST TIME (MINUTES)	NO BITES/FACE	NO BITES/HANDS	NO BITES/JACKET	TOTAL BITES	BITES/MIN FACE	BITES/MIN HANDS	BITES/MIN JACKET	TOTAL BITES/MIN.
TEST NO 7									
Without Jacket	15	122	8	382	512	8.13	.53	25.46	28.44
Control Jacket	15	49	8	96	153	3.26	.53	6.40	10.20
deet	30	1	0	0	1	.33	0	0	.33
R-69	30	19	0	3	23	.63	0	.10	.77
R-326 & MGK 264	30	50	4	0	54	1.67	.13	0	1.80
R-11 & MGK 264	30	24	1	1	26	.80	.03	.03	.87
TEST NO 8									
Without Jacket	15	145	2	422	569	9.67	.13	28.13	37.93
Control Jacket	15	67	63	143	273	4.46	4.20	9.53	18.20
deet	30	0	3	0	3	0	.10	0	.10
R-69	30	15	5	6	26	.50	.17	.20	.87
R-326 & MGK 264	30	20	3	5	28	.67	.10	.17	.93
R-11 & MGK 264	30	0	0	0	0	0	0	0	0

/zcm.

TABLE 1. Results of Control and Repellent Mesh Jackets for Determining the Biting Rates of Black Flies in the Amazon Basin, Para, Brazil, 1976. (Cont.)

REPELLENT	TEST TIME (MINUTES)	NO BITES/FACE	NO BITES/HANDS	NO BITES/JACKET	TOTAL BITES	BITES/MIN FACE	BITES/MIN HANDS	BITES/MIN JACKET	TOTAL BITES/MIN
TEST NO 4									
Without Jacket	15	48	5	308	361	3.20	.33	20.53	24.07
Control Jacket	15	41	5	97	143	2.73	.33	6.46	9.53
deet	30	2	1	0	3	.07	.03	0	.10
K-69	30	30	11	0	41	1.00	.37	0	1.37
R-326 & MGK 264	30	96	1	4	101	3.20	.03	.13	3.37
R-11 & MGK 264	30	33	6	4	43	1.10	.20	.13	1.43
TEST NO 5									
Without Jacket	15	78	29	338	445	5.20	1.93	22.53	29.67
Control Jacket	15	45	27	129	201	3.00	1.80	8.60	13.40
deet	30	2	0	0	2	.07	0	0	.07
R-69	30	77	7	0	84	2.57	.23	0	2.80
R-326 & MGK 264	30	66	6	1	73	2.20	1.20	.03	2.43
R-11 & MGK 264	30	57	10	2	69	1.90	.33	.07	2.30
TEST NO 6									
Without Jacket	15	81	6	384	471	5.40	.40	25.60	31.40
Control Jacket	15	49	7	70	126	3.27	.47	4.67	8.40
deet	30	4	0	0	4	.13	0	0	.13
R-69	30	44	10	33	87	1.47	.33	1.10	2.90
R-326 & MGK 264	30	79	10	2	91	2.63	.33	.07	3.03
R-11 & MGK 264	30	13	1	1	15	.43	.03	.03	.50

TABLE 2. Results of the Black Fly Repellent Efficacy for the Different Jacket Treatment Types, Para, Brazil, 1976.

TREATMENT	TOTAL BITES/ CUMULATIVE	% BITE/ REDUCTIONS	TOTAL BITES/ JACKET	% BITE/ REDUCTIONS
Control Jacket	2,704 ^b		1,554 ^b	
deet	47	98	16	99
R-11 + MGK 264	192	93	16	99
R-326 + MGK 264	646	76	65	96
R-69	586	78	155	90

^a Represents all bite recorded for face, hands and mesh jackets.

^b These figures represents 2X the actual number recorded for the control jacket so that the treatments could be compared on an equal time factor.

/zcm.

TABLE 3. Results of the Number of Bites Received for Three Body Areas, Para, Brazil, 1976

TREATMENT	TOTAL BITES/ FACE	TOTAL BITES/ HANDS	TOTAL BITES/ JACKET	TOTAL CUMULATIVE BITES
Without Jacket	616 (21%) [@]	90 (3%)	2,270 (76%)	2,976
Control Jacket	436 (32%)	139 (10%)	777 (58%)	1,352
deet	18 (38%)	13 (28%)	16 (34%)	47
R-11 & MGK 364	148 (77%)	28 (15%)	15 (8%)	192
R-326 & MGK 364	506 (78%)	75 (12%)	65 (10%)	646
R-69	338 (58%)	92 (16%)	155 (26%)	586

@ () represents % of Total Cumulative bites received per body area

/zcm.

TABLE 4. Results of Selected Skin Repellents for Determining the Protection Time Against Black Fly Species in the Amazon Basin, Para, Brazil, 1976.

TREATMENT	TREATMENT ^a REPLICATION	TEST TIME (Hrs.)	PROTECTION TIME (Hrs.)	CONTROL NO BITES/MIN	MEAN TEMP (Co)	AVERAGE RH (%)
			TEST NO 1			
Control	2	1	-	1.5	32.5	68
Deet	2	1	1			
Ten & Five	4	1	1			
Twenty & Five	4	1	1			
			TEST NO 2			
Control	2	2	-	3	28	75
Deet	2	2	2			
Ten & Five	4	2	2			
Twenty & Five	4	2	2			
			TEST NO 3			
Control	2	2	-	3.5	29	75
Deet	2	2	2			
Ten & Five	4	2	2			
Twenty & Five	4	2	2			
			TEST NO 4			
Control	2	3	-	20.2	30	55
Deet	2	3	3			
Ten & Five	4 ^b	3	3			
Twenty & Five	4	3	3			

^aEach arm treated is considered to be 1 replication.

^bProtection time for one replication was 2:40 hrs.

/zcm.

Skin repellent No. _____

Field species: Black flies
 Location: Yanaba, Brazil

Date: 12 May 76

Repellent	Sub- ject	Arm L/R	TIME			Protection Time
			Treat- ment	1st bite	2nd bite	
A	1	L	0800	0830	0845	30 min.
B		R	0802	0802	0820	60 min.
A	2	L				
B		R	Example of a confirmed bite within 30 min.			
A	1	L				
B		R				
A	4	L	0800	0830	0910	
B		R				
A	2		Example of a nonconfirmed bite within 30 min.			
B						
A	3					
B						
B	4					
A						
B						
A	1					
B						
A						
B						

(FIELD FORM: REPELLENT-JACKET)

Repellent _____

	START	FINISH	DATE _____
TEMP	_____	_____	TEST NO. _____
RH	_____	_____	
LOCATION	_____	_____	

TRIAL NO. 1 (5-10 Min.)

_____ No. bites/min.

TRIAL NO. 2 (5-10 Min.)

_____ No. bites/min.

TRIAL NO. 3 (5-10 Min.)

_____ No. bites/min.

VI. MAMMALIAN ECOLOGY

A. Surveillance Program

BACKGROUND: Reported mammalian studies in the Amazon region have been mainly taxonomic and conducted in riverine ecology. Little work has been reported on the influence of seasonal changes in climate and vegetation on animal populations. The opening of the Transamazon highway has permitted access to a variety of ecologically different areas. The great diversity of tropical habitats and mammalian species will require that studies be made in a variety of sites. The extremely rapid ecological changes caused by human perturbation of forests in the American tropics also necessitate studies over a sufficient period of time to understand corresponding changes in mammal communities and abundance. The mammalian ecology program has been developed to monitor the changes in animal populations along the Transamazon as man changes the habitat from forest to cropland and pasture.

Many arboviruses and leishmaniasis have been described in the Transamazon area. Brucellosis, schistosomiasis, leptospirosis and Chagas' disease have been described elsewhere in Brazil with both sylvatic and domestic mammals implicated in their transmission cycles. Information concerning the kinds of mammalian species present and their relative abundance in various habitats throughout the year when compared with simultaneous epidemiological and entomological data from the same areas assists in better understanding vector reservoir interactions and the present and potential disease risk to humans from zoonoses.

PROGRAM DESCRIPTION: The trapping program compares the relative abundance of various animals in different habitats over time. As the mammals in the Transamazon region are highly diversified, small changes in the habitats may cause changes in the types and relative numbers of species found in each. Seasonal, climatic, and vegetation changes play an important role in the reproductive cycles and numbers of mammals present in a population during any one period of the year.

Trapping in the two sites in the Maraba area and the two in the Altamira area which were selected for intensive surveillance last year has been continued. These sites were selected on the basis of obtaining a representative sample of mammals from as many habitat sites as possible. Epidemiological and entomological data have been collected from the same areas in order to observe the disease agents present in humans and insects as well as in mammals. Because of the importance of different habitats, traps were dispersed in cropland, orchards, and in as many forest types as possible. The edge between forest and cleared areas was similarly covered. Trapping was carried out for two weeks of every month in each of four sites in the Maraba and Altamira study areas. Wooden live traps (155 x 175 x 450 mm) and Rinker live traps (80 x 80 x 255 mm) were placed 30 m apart along grids both in the forest and agricultural areas. Because some mammals are essentially arboreal, the Rinker traps were also placed in trees and on lianas. Traps were also placed in colonists houses to sample the mammals living there. A mixture of corn and bananas was the most common trap bait used. Larger mammals were hunted to obtain necessary specimens for evidence of disease agents. The general forest type in this area ranges from a tropical moist to a tropical wet forest formation as described by Holdridge (1947). Richards (1952) considers almost all of the Amazon basin as a tropical rain forest,

disagreeing with Beard (1944) whose classification ranges from evergreen seasonal to semi-evergreen seasonal tropical forests.

The trapping site in Gleba 5 lote 5 (FIGURE 1) was located 26 Km north and 30 Km west of Maraba near Itupiranga (5° 06' S, 49° 24' W). Three types of forest were sampled on this site: lowland sidehill, low flatland, and upland forest. A detailed description of this trapping site was included in the 1975 annual report. Traps were also placed in secondary scrub; and corn, rice, and cassava fields.

The trapping site in Gleba 29 lote 06 (FIGURE 2) was located 73 Km north and 45 Km west of Maraba near Jatobal (4° 41' S, and 49° 32' W). Three types of forest were sampled in this site: lowland, lowland but not inundated, and upland forest. See the 1975 annual report for a detailed description. Traps were also placed in secondary scrub and agricultural areas.

The trapping site in Agrovila da União (FIGURE 3) was located 19 Km south and 18 Km west of Altamira (3° 22' S, 52° 23' W). Four types of forest were sampled in this site; acaí swamp, lowland, sidehill, and upland forest. The 1975 annual report also contains a detailed description of this site. Traps were also placed in a tall secondary scrub forest which had been cleared 4 years earlier, and in agricultural areas.

The trapping site in Gleba 61 lote 02 (FIGURE 4) was located 54 Km south and 150 Km west of Altamira (3° 41' S, 53° 45' W). Two types of terrain were sampled, lowland and sidehill, although both types of forest appeared similar. This site is also described in detail in the 1975 report. Traps were also placed in secondary scrub and in agricultural areas.

Every trap location in each trapping site has a permanent number enabling the exact location of where the mammal was trapped to be noted. Thus, the exact location where a potential zoonoses may exist and also the preferred habitat type of a certain species can be determined. This information is invaluable in combatting future disease problems in which animals from a similar habitat are involved.

The traps were checked early in the morning to reduce the number of animals dying. The captured mammals were transferred to cloth bags, and taken to the field laboratory for processing. At the field laboratory, located near the trapping site, each mammal was given a collection number which corresponded to the date and area (example: M-010675-01, which is decoded as M-Maraba, 010675- 1 June 1975, 01 - first specimen processed on this date). All specimens taken from one animal were labelled with the same number. The mammal was exsanguinated, 1cc whole blood preserved in liquid nitrogen for virus isolation attempts, a thick and a thin smear for blood parasite examination, and the remainder centrifuged and serum preserved in liquid nitrogen. After the animal was exsanguinated, it was placed in a paper bag with chloroform, and the ectoparasites removed. A representative sample of these was preserved for later identification, and the remainder placed in liquid nitrogen to be examined for pathogens. The standard measurements (total length, tail length, hind foot length, ear length, and weight) were recorded. Organs were removed and preserved both in liquid nitrogen for isolation attempts, and in 10% formalin for pathological studies. Endoparasites were preserved in 10% formalin also. The preserved specimens were sent to the base laboratory in Belem for processing or transshipment to

WRAIR. Each mammal specimen was preserved either as skin and skull, skull only, or in formalin and shipped to Belem for tentative identification, and later to taxonomists specializing in South American mammals for confirmation. All the information was recorded on field forms which were described in the 1975 annual report.

PROGRESS TO DATE: From November 1974 through June 1976 2,249 mammals had been collected by trapping and hunting. A panel of sera from 291 mammals collected along the Transamazon highway were sent to Walter Reed Army Institute of Research and have been tested for plague and tularemia antibodies. Sera from 372 mammals have been screened for Chagas' disease, toxoplasmosis, leptospirosis, brucellosis, and schistosomiasis in the Belem laboratory. Blood smears from 1,213 mammals have been examined for anthrax, microfilariae, trypanosomes, and malaria in Belem. The results of the laboratory examination will be discussed in the following section of this report. The changes in habitat preferences and reproduction of sylvatic mammals during the year are reported in the respective sections of this report also.

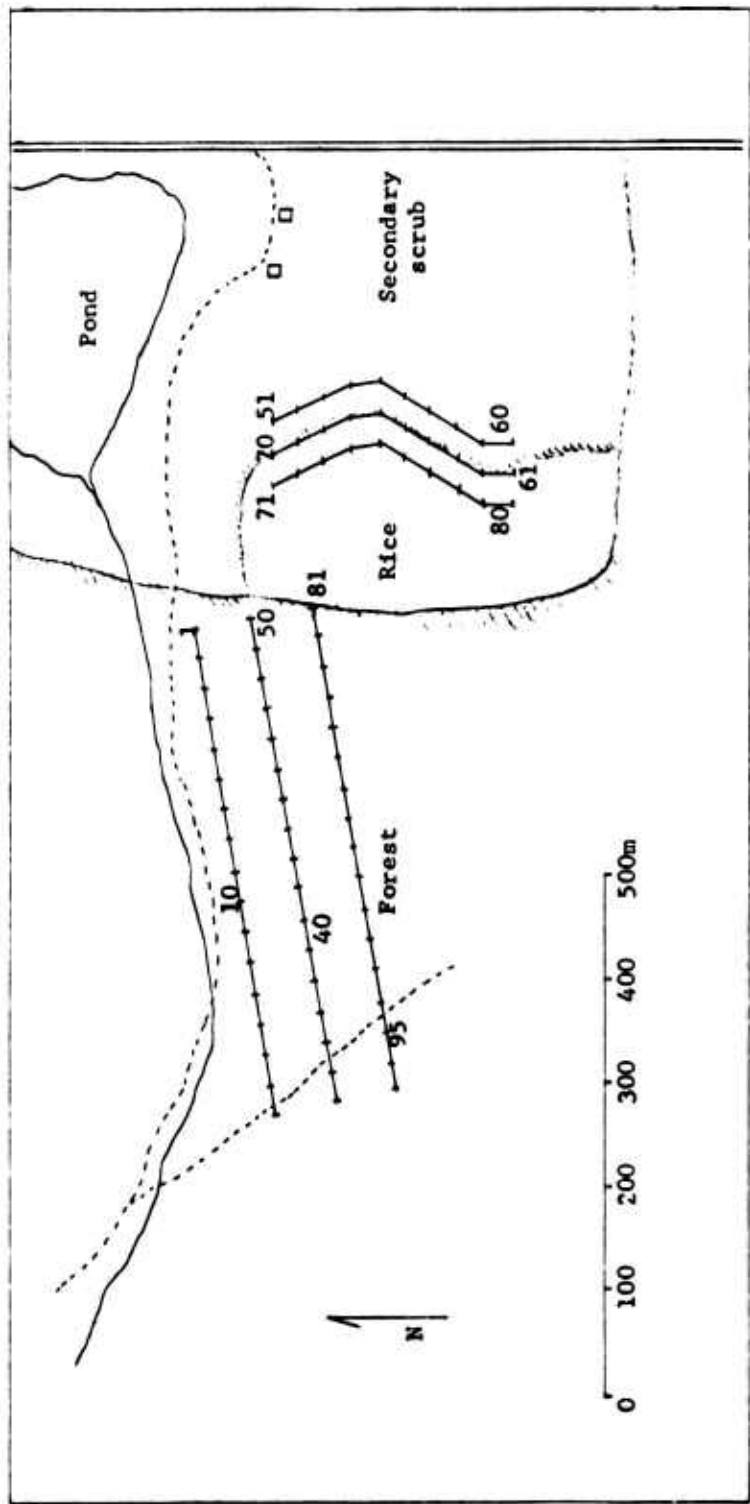


Figure 1. Trapping sites in Gleba 05 Lote 05 west of Maraba. Located Brazil: Para; 26 km north and 30 km west of Maraba (near Itupiranga). Latitude 5° 06' S and longitude 49° 24' W.

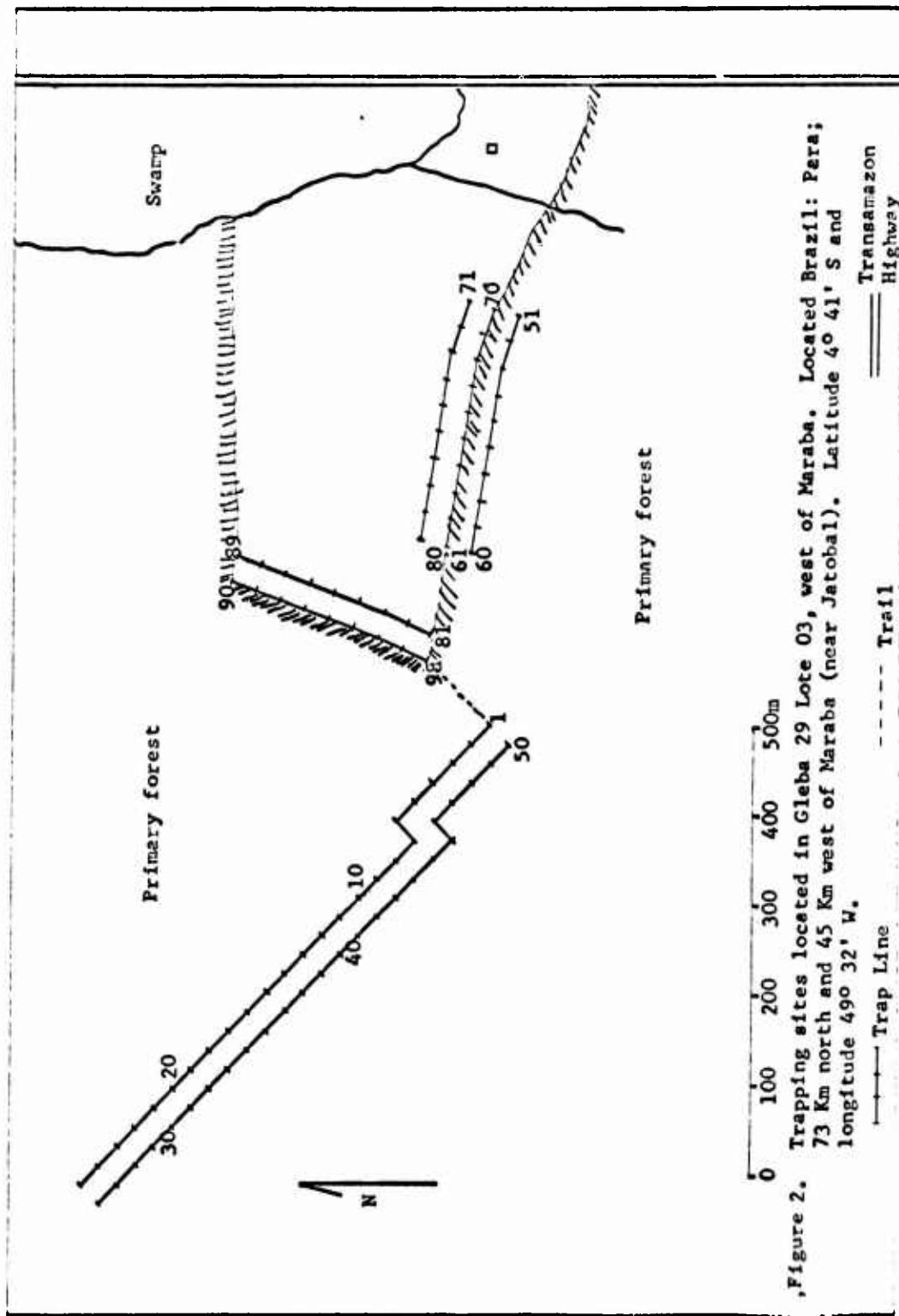


Figure 2. Trapping sites located in Gleba 29 Lote 03, west of Maraba. Located Brazil: Para; 73 Km north and 45 Km west of Maraba (near Jatobal). Latitude 4° 41' S and longitude 49° 32' W.

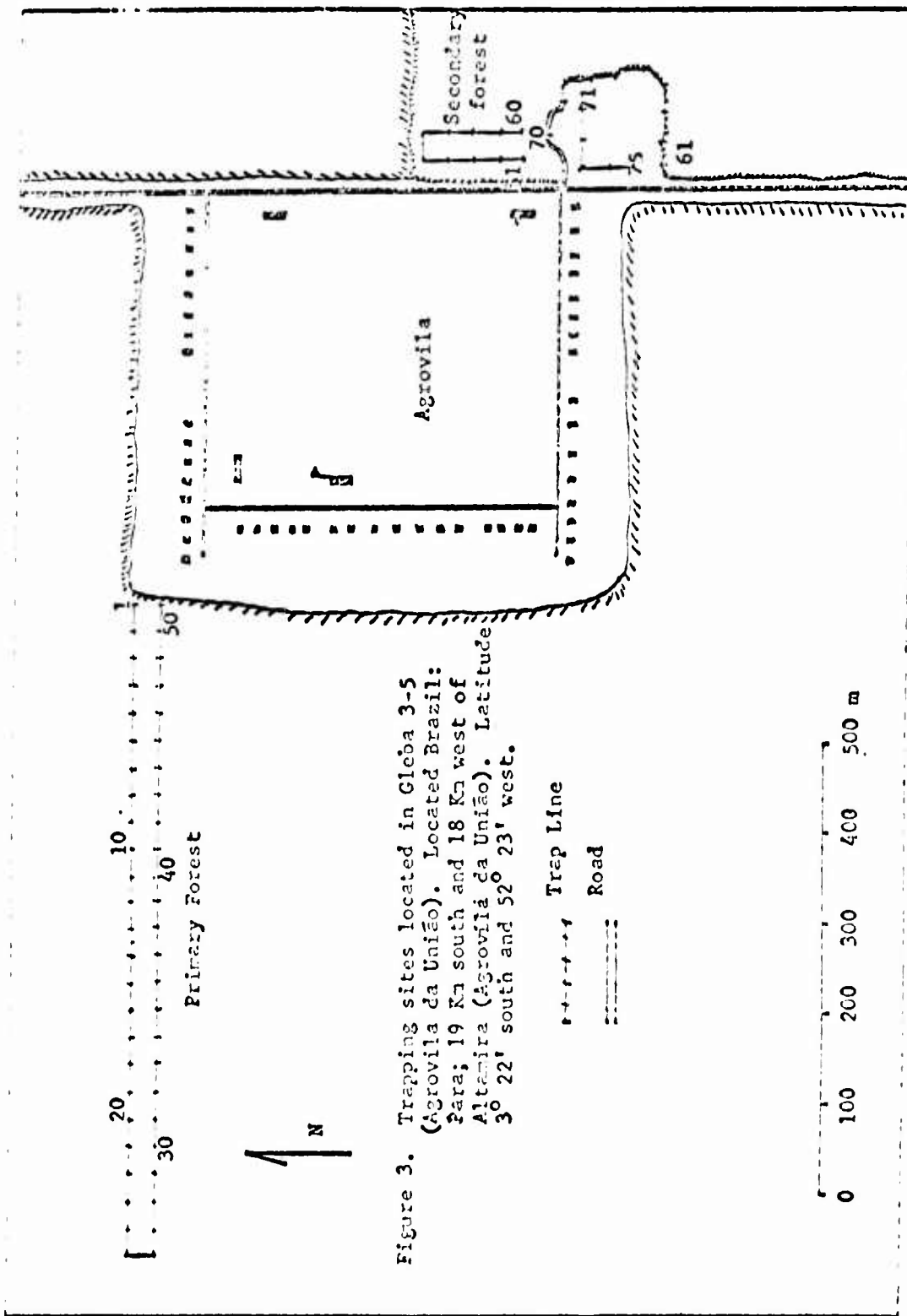
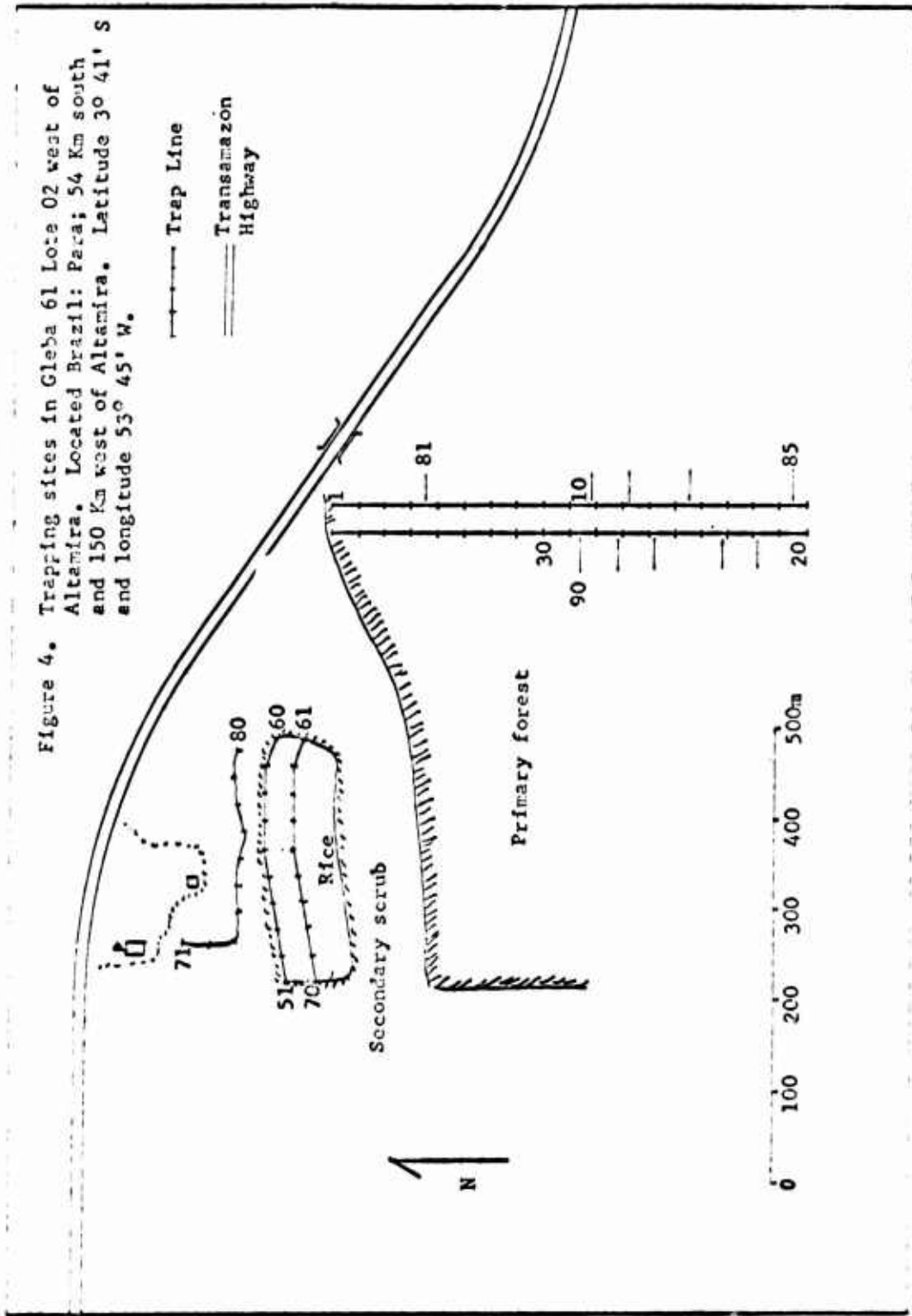


Figure 3. Trapping sites located in Globo 3-5 (Agrovila da União). Located Brazil: Para; 19 Km south and 18 Km west of Altamira (Agrovila da União). Latitude 3° 22' south and 52° 23' west.



B. Laboratory Test Results for Mammal Blood and Tissue

BACKGROUND: The wild mammals collected either by hunting or by live trapping in the four trapping sites, described in the surveillance section of this report, were taken to a field laboratory to be processed. The animals were exsanguinated, whole blood, sera, blood slides, and organ specimens were preserved and shipped to the main laboratory in Belem. The sera were tested for Chagas disease, toxoplasmosis, leptospirosis, brucellosis, and schistosomiasis antibodies in Belem and for plague and tularemia antibodies at the Division of Hazardous Microorganisms, WRAIR. The blood slides were examined microscopically for evidence of anthrax, microfilariae, trypanosomes, and malaria. The organs preserved in 10% formalin were sent to the Department of Veterinary Pathology, WRAIR for examination.

RESULTS: Sufficient blood serum for testing was collected from 372 mammals (TABLE 1). The rodents with 299 serum specimens accounted for most. The results of the agglutination test for the determination of antibodies against Trypanosoma cruzi (TABLE 2) indicated 45% of the rodent sera tested in Gleba 61 Lote 02 West of Altamira were positive. This is significantly ($\chi^2 p < 0.99$) more than the rodent sera of the other trapping sites. All mammal groups tested from each trapping site included at least some sera which reacted positively to T. cruzi antigen using this test. Antibodies to Toxoplasma gondii were found in the sera of only five of the 308 mammals tested (TABLE 3). A titer of 1:256 and higher was considered positive for infection. Leptospirosis was found in the sera of 10 of the 80 rodents from trapping area 29-03 (TABLE 4). Remaining sera were rarely positive. The slide agglutination test for brucellosis indicated four of 59 sera in trapping site 05-05 and four of 69 sera in 29-03 were positive (TABLE 5). Both of these sites are located in the Maraba area. The IHA test for schistosomiasis produced low titers in a number of mammal sera, but only one rodent sera produced a titer as high as 1:256 (TABLE 6).

Blood specimens from 1,213 mammals were examined microscopically for presence of anthrax, microfilariae, trypanosomes, and malaria. Microfilariae were found in 11 slides, and seven slides contained trypanosomes (TABLE 7). Neither anthrax nor malaria were observed.

A panel of sera from 291 mammals was sent to the Division of Hazardous Microorganisms, WRAIR, to be tested for plague, tularemia, and rickettsia. No plague antibodies were found using the indirect hemagglutination test. The test for opsonizing antibody indicated one rodent sera was positive for tularemia. The results have not been received on the rickettial antibody test.

Pathological specimens of lung, liver, heart, kidney, spleen, stomach, large intestine, and small intestine preserved in 10% formalin were sent to the Department of Veterinary Pathology, WRAIR to be examined, but the results from the 600 cases are not available as yet.

Few mammal sera of blood slides were positive for pathogenic organisms. The high percentage of rodent sera containing antibodies which reacted in the Trypanosoma cruzi agglutination test was significantly higher in trapping site 61-02 than in the other three. More tests will be conducted next year to determine if T. cruzi is actually present, or if other antibodies are cross reacting with this antigen. There are very few livestock along the

Transamazon highway at the present time, but cattle are being brought from the south. Brucellosis may become more common in the future. Continued surveillance will be necessary to discover what changes in the disease picture will take place in the future as more livestock are introduced and more stable agricultural practices are utilized.

TABLE 1. Numbers of Blood Sera Collected from Mammals.

SPECIES	ALTAMIRA		MARABA		TOTAL
	3/5	61-02	05-05	29-03	
Rodents	102	86	42	69	299
Marsupials	9	23	18	13	63
Monkeys	5	--	--	--	5
Edentates	2	--	--	--	2
Carnivores	--	1	--	--	1
T O T A L	120	110	60	82	372

/zcm.

TABLE 2. Results of the Agglutination Test for the Determination of Antibodies Against Trypanosoma cruzi.

SPECIES	3/5		61-02		05-05		29-03		TOTAL	
	POS	TOT	POS	TOT	POS	TOT	POS	TOT	POS	TOT
Marsupials	2	5	5	22	9	17	2	9	18	53
Monkeys	3	5	-	-	-	-	-	-	3	5
Edentates	1	2	-	-	-	-	-	-	1	2
Rodents	8	45	30	67	6	34	6	53	50	199
Carnivores	-	-	1	1	-	-	-	-	1	1
T O T A L	14	57	36	90	15	51	8	62	73	260

/zcm.

TABLE 3. Results of the Indirect Hemagglutination Test for Antibodies to Toxoplasma gondii

SPECIES	3/5		61-02		05-05		29-03		TOTAL	
	POS	TOT	POS	TOT	POS	TOT	POS	TOT	POS	TOT
Marsupials	0	6	164	23	14096	17	0	10	1*	56
Monkeys	0	5	-	-	-	-	-	-	0	5
Edentates	0	2	-	-	-	-	-	-	0	2
Rodents	0	61	1512	80	164 1512 1:1024	42	1*	61	4*	244
Carnivores	-	-	0	1	-	-	-	-	0	1
TOTAL	0	74	1*	104	3*	59	1*	71	5*	308

* A titer of 1:256 was considered positive.

/zcm.

TABLE 4. Results of the Agglutination Test for Leptospirosis.

SPECIES	3/5		61-02		05-05		29-03		TOTAL	
	POS	TOT	POS	TOT	POS	TOT	POS	TOT	POS	TOT
Marsupials	0	2	0	19	1	19	0	10	1	50
Monkeys	0	5	-	-	-	-	-	-	0	5
Edentates	0	2	-	-	-	-	-	-	0	2
Rodents	0	53	1	57	2	47	10	80	13	237
Carnivores	-	-	0	2	-	-	-	-	0	2
T O T A L	0	62	1	78	3	66	10	90	14	296

/zcm.

TABLE 6. Results of the IHA Test for Shistosomiasis. The Numbers in Parenthesis Indicate the Number of Sera Positive at the Specific Titer.

SPECIES	3/5		61-02		05-05		29-03		TOTAL	
	POS	TOT	POS	TOT	POS	TOT	POS	TOT	POS	TOT
Marsupials	0	5	18(5) 1:16(5)	23	18(7) 1:16(3) 1:32(3)	18	18(2) 1:16(2)	10	0*	56
Monkeys	18(1) 1:128(1)	5	--	--	--	--	--	--	0	5
Edentates	0	2	--	--	--	--	--	--	0	2
Rodents	18(4) 1:32(3) 1:256(1)	52	18(3) 1:16(6) 1:32(4) 1:64(5) 1:128(3)	73	18(4) 1:16(4) 1:32(1) 1:64(1)	40	18(6) 1:16(2) 1:32(3)	57	1*	222
Carnivores	--	--	0	1	--	--	--	--	0	1
TOTAL	1*	64	0	97	0	58	0	67	1*	286

* A titer of 1:256 was considered positive

/zcin.

TABLE 5. Results of the Agglutination Test for Brucella

SPECIES	3/5		61-02		05-05		29-03		TOTAL	
	POS	TOT	POS	TOT	POS	TOT	POS	TOT	POS	TOT
Marsupials	0	2	0	18	1	17	0	10	1	47
Monkeys	0	5	-	-	-	-	-	-	0	5
Edentates	0	2	-	-	-	-	-	-	0	2
Rodents	0	52	0	56	3	42	4	59	7	209
Carnivores	-	-	0	1	-	-	-	-	0	1
T O T A L	0	61	0	75	4	59	4	69	8	264

/zcm.

TABLE 7. Results of Microscopic Examination of Blood Slides from Mammals.

AREA	ANTHRAX		MICROFILARIA		TRYPANASOMA		MALARIA	
	POS	TOT	POS	TOT	POS	TOT	POS	TOT
3/5	0	286	0	286	1	286	0	6
61-02	0	334	3	334	0	334	0	334
05-05	0	244	3	244	2	244	0	244
29-03	0	349	5	349	4	349	0	349
TOTAL	0	1213	11	1213	7	1213	0	1213

/zcm.

C. Habitat Preference and Relative Abundance of Mammals

BACKGROUND: As most studies of neotropical mammals have mainly been concerned with taxonomy, few data are available on population and habitat preference. A certain species may exist in a given habitat in insufficient numbers to be considered an economic or a medical problem. As man enters and alters this habitat, clearing the forest and planting crops for example, the population density of this species may rise drastically, providing an efficient host for a number of pathogens which were never a problem in the area before.

OBSERVATIONS: Traps were placed at intervals of 30 m in the forest, secondary scrub, and cropland in the four trapping sites described in the mammalian surveillance section of this report. Monkeys, large rodents and other mammals which do not readily enter traps were hunted both during the day and the night. As the mammals were captured, the type of habitat in which each was collected was recorded. The total number of trap nights (one trap set out for one night equals one trap night) for each habitat type was also recorded.

During the mammal surveillance program, a total of 2,243 mammals were collected. Forty one were collected by hunting, and the remaining 2,202 were trapped in a total of 31,722 trap nights. This gave a trapping success of 6.94 mammals per 100 trap nights (TABLE 1). The highest trapping success was in the secondary scrub with 11.86 mammals per 100 trap nights. The cropland with 5.18 mammals per 100 trap nights had the lowest success. The trapping success in the four trapping sites ranged from 4.74 to 9.81 trap nights per mammal. The number and species of mammals collected by hunting are presented in TABLE 2. All except the rabbit (*Sylvilagus brasiliensis*), which was shot in a grass clearing, were collected in the forest.

The data demonstrate a preference of certain species to certain habitats (TABLE 3, TABLE 4). Of the marsupials, only *Monodelphis brevicaudata* preferred the secondary scrub and cropland to the forest. The other marsupials tended to prefer the forest habitat. Rodents such as *Oryzomys capito*, *O. macconnelli*, *Reacomys guianae* and *Proechimys* definitely preferred the forest habitat. *Oryzomys delicatus*, *Zygodontomys lasiurus* and *Oxymycterus* sp. preferred the secondary scrub and cropland habitats over the forest. Most of the species were found in all four trapping sites (TABLE 4), although *Oryzomys bicolor*, *O. delicatus* and *Holochilus brasiliensis* were only collected in the Maraba sites. *Zygodontomys lasiurus* was collected only in the forest in small numbers in one Altamira site while it was definitely most plentiful in the secondary scrub and cropland in the Maraba sites. *Oxymycterus* sp. was one of the most commonly collected species in the cropland and secondary scrub in the Altamira sites, but was rarely taken in the Maraba sites. The three species which appeared to undergo a distinct population increase when the forest was cleared, crops planted, and the land allowed to revert to secondary scrub must be observed closely. The possibility of it becoming a host to pathogens which may lead to an enzootic and later to an epidemic when the human population becomes involved is always present when a mammalian population increases rapidly.

The relative abundance of mammals during the various months of the year plays an important role in some disease cycles. The monthly mammalian density in the forest appears to be more stable than either the secondary scrub or the

cropland (FIGS. 1-3). In the Altamira trapping sites (FIG. 1) the trapping success in the secondary scrub was lower when the trapping success in the cropland was higher, and was higher when the trapping success in the cropland was lower. This suggests a migration of the small mammals between the secondary scrub and cropland which depends on the food availability in each. Very little correlation between the trapping success in the secondary scrub and cropland in trapping sites 05-05 (FIG. 2) and 29-03 (FIG. 3) in the Maraba area is evident. The monthly fluctuations are much higher in the Maraba area than in the Altamira area. The species of mammals which prefer the secondary scrub are also common in the cropland habitat (TABLE 4). The forest preferring species tended to remain in the forest and were infrequently found in the other two habitats.

TABLE 1. Total Mammals Trapped per Habitat in the Four Trapping Sites per 100 Trap Nights.

TRAP SITE	FOREST	SCRUB	CROP	TOTAL
3/5	4.56	6.83	4.06	4.74
61-02	5.86	10.41	5.70	6.67
05-05	5.43	17.92	3.46	6.71
29-03	10.06	10.90	8.10	9.81
TOTAL	6.32	11.86	5.18	6.94

/zcm.

TABLE 2. Mammals Collected per Area by Hunting.

SPECIES	3/5	61-02	05-05	29-03	TOTAL
Primates					
<u>Callicebus</u>	2	1	-	2	5
<u>Alouatta</u>	2	-	-	1	3
<u>Cebus</u>	2	-	-	-	2
<u>Callithrix</u>	2	-	-	-	2
<u>Saguinus</u>	-	-	-	-	1
Edentata					
<u>Dasybus</u>	2	-	3	-	5
Lagomorpha					
<u>Sylvilagus brasiliensis</u>	1*	-	-	-	1*
Rodentia					
<u>Agouti paca</u>	1	2	-	3	6
<u>Dasyprocta</u>	9	-	-	-	9
Carnivora					
<u>Nasua nasua</u>	1	-	2	-	3
<u>Eira barbara</u>	-	1	-	-	1
Artiodactyla					
<u>Mazama</u>	2	1	-	-	3
TOTAL	24	5	5	7	41

* All the Mammals Except Sylvilagus brasiliensis Which was Collected in a Clearing were Collected in the Forest.

/zcm

TABLE 3. Species Collected per 10,000 Trap Nights in Three Habitat Types.

SPECIES	FOREST	SCRUB	CROPLAND	TOTAL
Marsupialia				
<u>Caluromys philander</u>	3.5	--	1.5	2.5
<u>Monodelphis brevicaudata</u>	5.5	62.8	44.2	22.7
<u>Marmosa cinerea</u>	13.0	--	--	8.2
<u>M. murina</u>	3.0	2.0	1.5	2.5
<u>M. parvidens</u>	1.0	--	--	0.6
<u>Philander opossum</u>	28.5	--	--	18.0
<u>Metachirus nudicaudatus</u>	7.0	2.0	--	4.7
<u>Didelphis marsupialis</u>	36.5	--	--	23.0
Rodentia				
<u>Oryzomys bicolor</u>	9.0	6.1	2.9	7.2
<u>O. capito</u>	209.6	44.5	63.3	152.6
<u>O. concolor</u>	19.0	14.2	7.4	15.8
<u>O. delicatus</u>	1.5	116.0	73.6	42.6
<u>O. macconnelli</u>	26.0	6.1	4.4	18.3
<u>Neacomys guianae</u>	39.5	8.1	8.8	28.0
<u>N. spinosus</u>	12.5	34.4	20.6	17.6
<u>Nectomys squamipes</u>	12.0	14.2	19.2	13.9
<u>Zygodontomys lasiurus</u>	3.5	502.0	135.5	109.4
<u>Oxymycterus sp.</u>	0.5	220.6	72.2	50.1
<u>Holochilus brasiliensis</u>	0.5	4.0	--	0.9
<u>Rattus rattus</u>	2.0	--	5.9	2.5
<u>Proechimys spp.</u>	194.0	99.2	56.0	149.7
<u>Mesomys hispidus</u>	4.0	--	--	2.5

/zcm.

TABLE 4. Species Collected per 10,000 Trap Nights in the Four Trapping Sites.

SPECIES	ALTAMIRA						MARABA					
	SITE 3/5			SITE 61-02			SITE 05-05			SITE 29-03		
	FOREST	SCRUB	CROP	FOREST	SCRUB	CROP	FOREST	SCRUB	CROP	FOREST	SCRUB	CROP
Marsupialia												
<i>Caluromys philander</i>	1.7	--	--	4.8	--	--	3.6	--	--	4.4	--	--
<i>Monodelphis brevicaudata</i>	8.6	52.6	90.2	--	156.6	113.9	3.6	7.7	4.2	8.8	28.1	6.0
<i>Marmosa cinerea</i>	10.3	--	--	9.6	7.9	--	21.9	--	--	8.8	--	--
<i>M. murina</i>	3.4	--	--	2.4	--	7.1	--	--	--	6.6	--	--
<i>M. parvidens</i>	1.7	--	--	--	--	--	1.8	--	--	--	--	--
<i>Philander opossum</i>	6.9	--	--	60.0	--	--	5.5	--	--	55.2	--	--
<i>Metachirus nudicaudatus</i>	6.9	--	--	12.0	--	--	3.6	--	--	6.6	7.0	--
<i>Didelphis marsupialis</i>	31.0	--	--	45.6	--	--	47.4	--	--	22.1	--	--
Rodentia												
<i>Oryzomys bicolor</i>	--	--	--	--	--	--	9.1	--	--	28.7	21.1	12.1
<i>O. capito</i>	184.1	10.5	7.5	76.8	7.9	--	220.6	23.0	20.8	351.1	119.5	223.6
<i>O. concolor</i>	10.3	21.0	7.5	26.4	31.5	21.4	21.9	--	--	19.9	7.0	6.0
<i>O. delicatus</i>	--	--	--	--	--	--	--	130.8	20.8	6.6	457.1	271.9
<i>O. macconnelli</i>	8.6	--	--	7.2	--	7.1	5.5	--	--	90.5	21.1	12.1
<i>Neacomys guianae</i>	15.5	10.5	15.0	21.6	--	21.4	49.2	15.4	--	75.1	7.0	6.0
<i>N. spinosus</i>	15.5	42.1	30.1	38.4	102.5	71.2	--	--	--	--	--	--
<i>Nectomys squamipes</i>	3.4	10.5	--	16.8	15.8	7.1	1.8	--	--	30.9	28.1	72.5
<i>Zygodontomys lasiurus</i>	5.2	--	--	--	--	--	7.3	584.6	300.2	--	295.4	120.8
<i>Oxymycterus</i> sp.	1.7	378.9	195.5	--	528.4	163.7	--	23.1	--	--	21.1	--
<i>Holochilus brasiliensis</i>	--	--	--	--	--	--	--	7.7	--	2.2	7.0	--
<i>Rattus rattus</i>	--	--	7.5	2.4	--	21.4	1.8	--	--	4.4	70.3	--
<i>Proechimys</i> sp.	132.5	157.9	52.6	256.8	181.4	135.2	138.5	--	--	282.7	--	72.5
<i>Mesomys hispidus</i>	8.6	--	--	4.8	--	--	--	--	--	2.2	--	--
TOTAL	455.9	683.9	405.9	585.6	1041.0	569.5	543.1	792.3	346.0	1006.8	1089.8	809.5

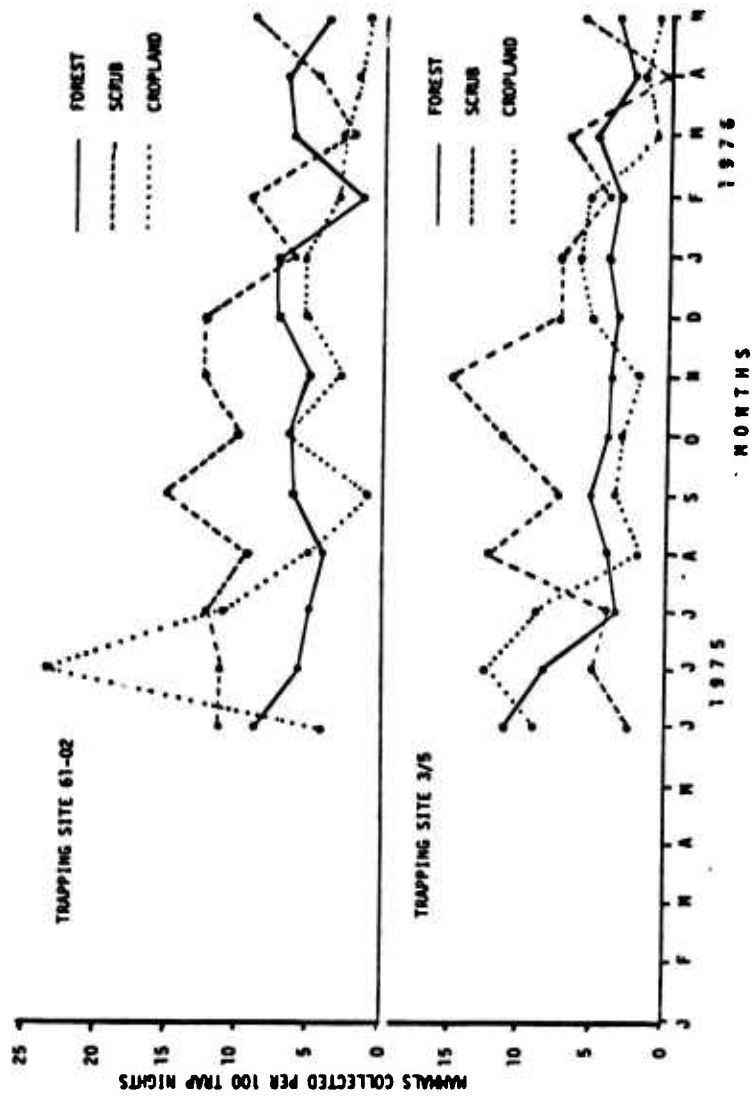


FIGURE 1. The number of mammals collected per 100 trap nights in trapping sites 3/S and 61-02 per month in the forest, secondary scrub, and cropland habitats.

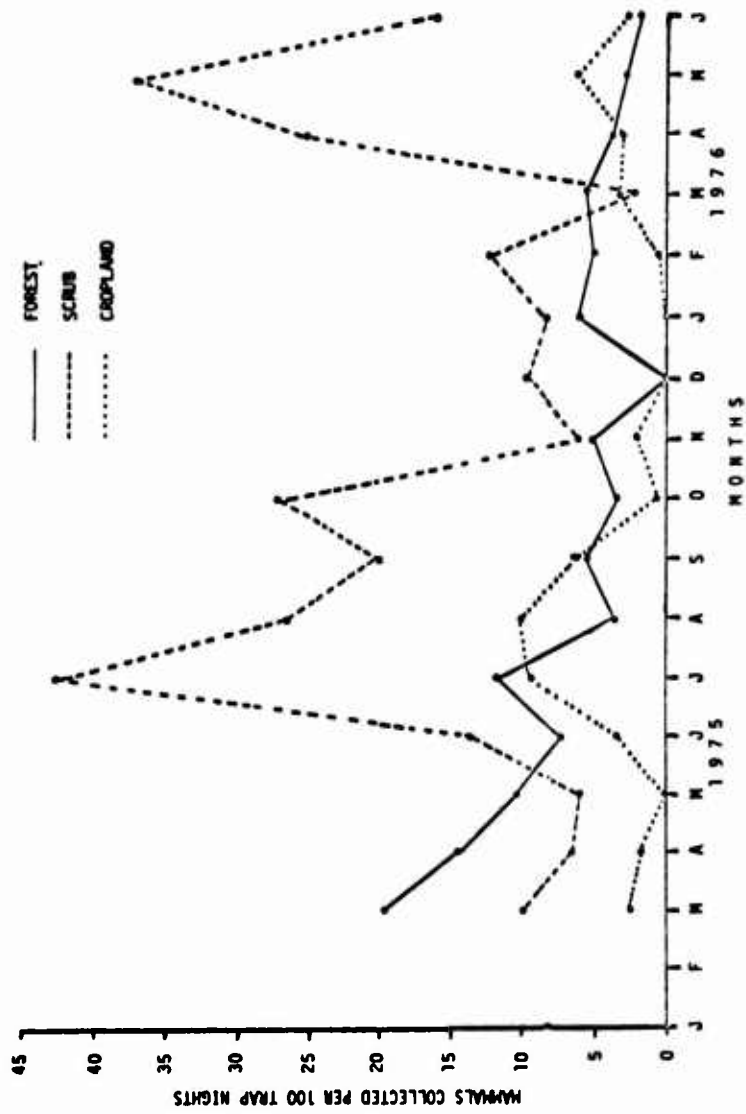


FIGURE 2. The numbers of mammals collected per 100 trap nights in trapping site 05-05 per month in the forest, secondary scrub, and cropland habitats.

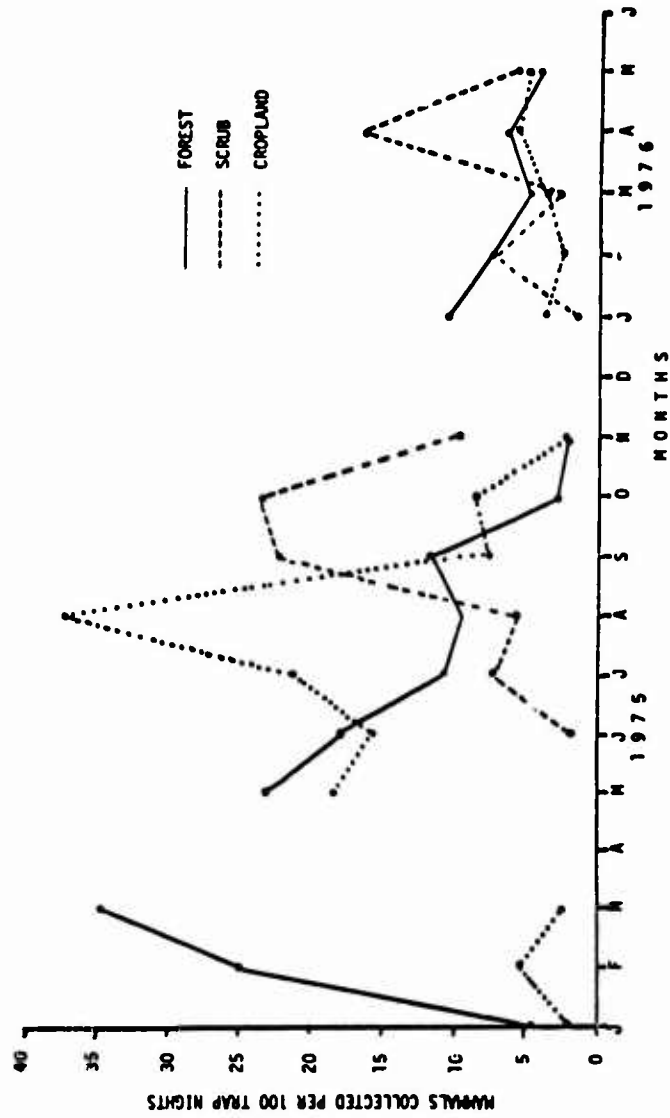


FIGURE 3. The number of mammals collected per 100 trap nights in trapping site 29-03 per month in the forest, secondary scrub, and cropland habitats.

D. Annual Reproduction in Five Common Rodents

BACKGROUND: Information on reproduction in neotropical mammals is scant, and the few published studies are from localities north of the equator. Litter sizes and seasons during which the mammals produce young are two important factors in the epidemiologic studies of zoonotic diseases. Transmission cycles of pathogens require susceptible individuals, and young mammals are able to fill this need. Studies in Panama (Fleming, 1970, Tesh, 1970) indicated that most rodents bred year-round, but the sample sizes were too small to show meaningful differences between seasons. In northern Colombia, although breeding was observed throughout the year, the highest percentage of reproductively active females was found from January through March (unpublished manuscript).

OBSERVATIONS: During the present study the mammals collected in the mammal surveillance program in the two trapping sites in Maraba and the two trapping sites in Altamira were examined for evidence of being reproductively active. Temperature and rainfall data compiled during this same period indicated that 73% of the total annual precipitation fell during the 5 month rainy season from December through April, and the remaining 27% fell during the 7 month dry season. The wet season was somewhat cooler than the dry season. The monthly average high temperature ranged from 29°C to 32°C and the monthly average low ranged from 24°C to 25°C throughout the year. Although the rainfall varied greatly between the wet season and dry season, the temperature varied slightly.

Females of Oryzomys capito, Neacomys, Zygodontomys, Oxymycterus and Proechimys were collected during this period. The pregnant or lactating individuals were considered reproductively active.

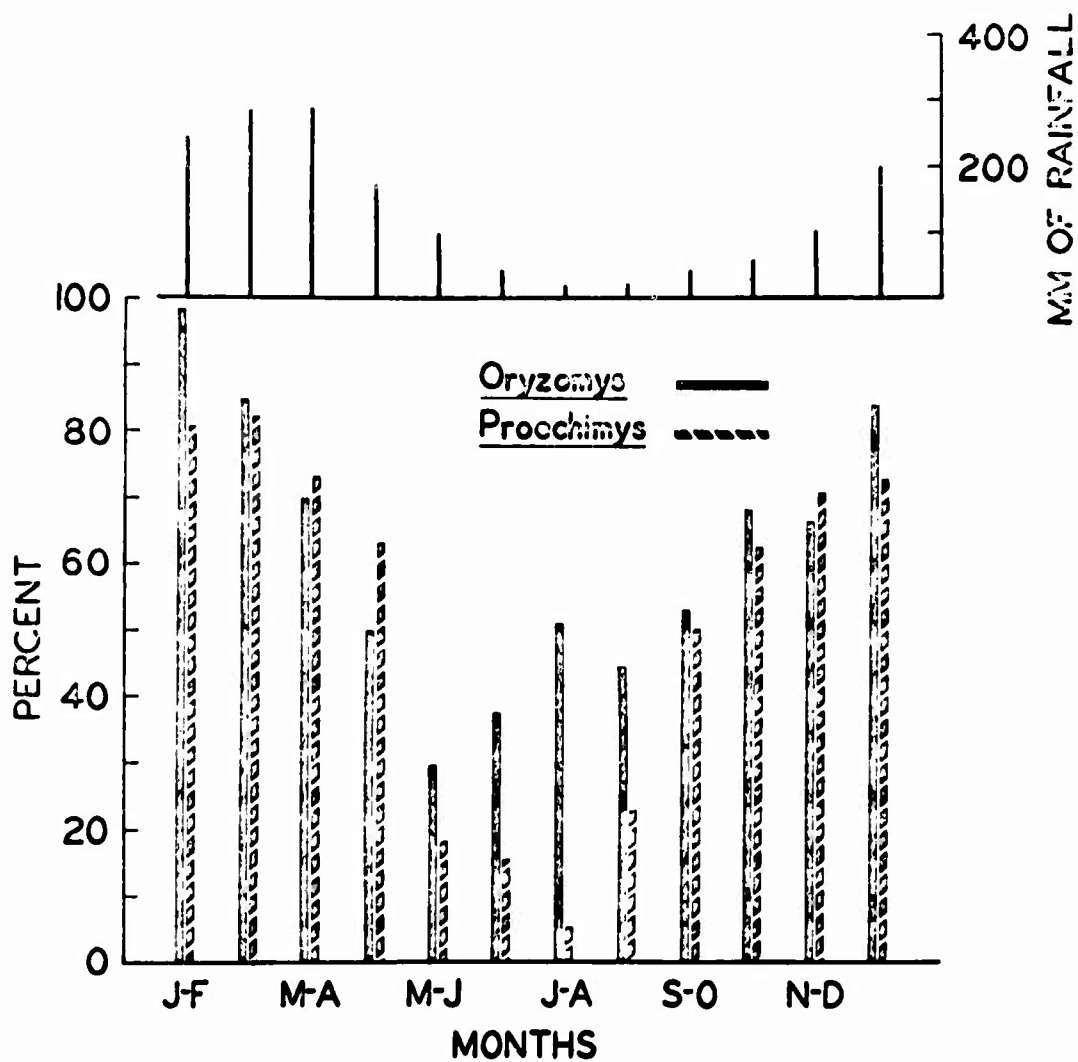
FIGURE 1 shows the annual reproductive activity of Oryzomys capito and Proechimys per 2 month running means. Females of Oryzomys capito and Proechimys were reproductively active during all months of the year. A much higher proportion of Oryzomys capito was reproductively active just before and during the wet season, and least active during the first part of the dry season. Proechimys females tended to follow the seasons more closely showing very little reproductive evidence during the dry season, and a high percentage during the rainy season.

FIGURE 2 shows the annual reproductive activity of Neacomys and Oxymycterus per two month running means. The females of Neacomys were reproductively active during all the months of the year, although more evidence of reproduction occurred during the rainy season than during the dry season. They became more active shortly before the start of the dry season. The Oxymycterus females were reproductively active during the rainy season, but during the driest months of July through October, the 22 adult females collected showed no signs of being pregnant or lactating.

FIGURE 3 shows the annual reproductive activity of adult Zygodontomys females per 2 month running means. Female Zygodontomys were reproductively active throughout the year and at least 50% of the females were active during most months of the year. During the rainy season months of March, April and May, 36 of the 37 adult females collected were either pregnant or lactating.

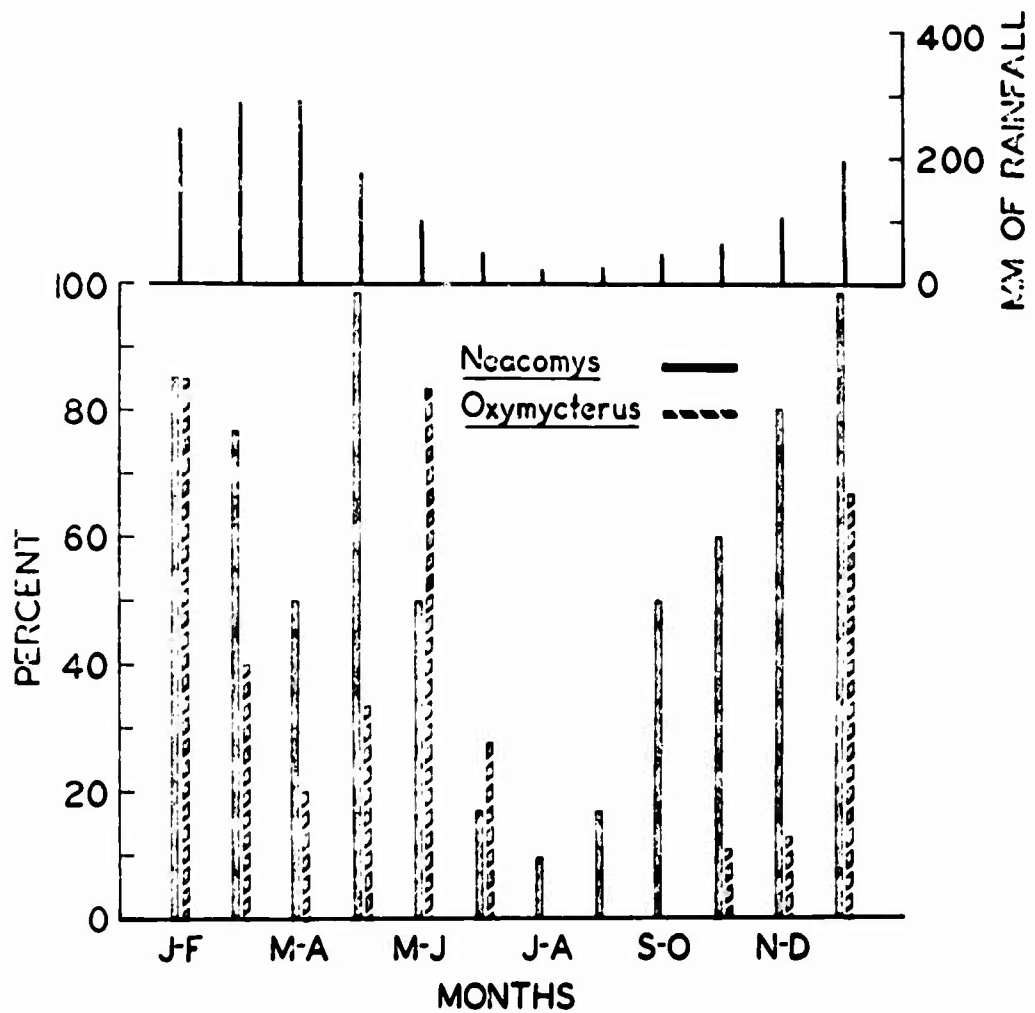
Of the five rodents, all except Oxymycterus females showed some reproductive

activity throughout the year. The Oryzomys, Proechimys, Neacomys and Oxymycterus females were much more active during the rainy season than during the dry season. Only Zygodontomys reproduced in high numbers throughout the year. Allowing sufficient time for the young mammals to terminate nursing and to lose any maternal antibodies they may have, the highest numbers of new potential hosts would be available during the second half of the rainy season and the first part of the dry season. Data compiled from the insect surveillance program conducted in the same sites in which the mammals were captured indicated the insect populations were high during the rainy season and the first half of the dry season (see Entomology). A high density of new susceptible hosts during the same period when the insect vectors are also at a high density is an ideal situation for the transmission of pathogens.



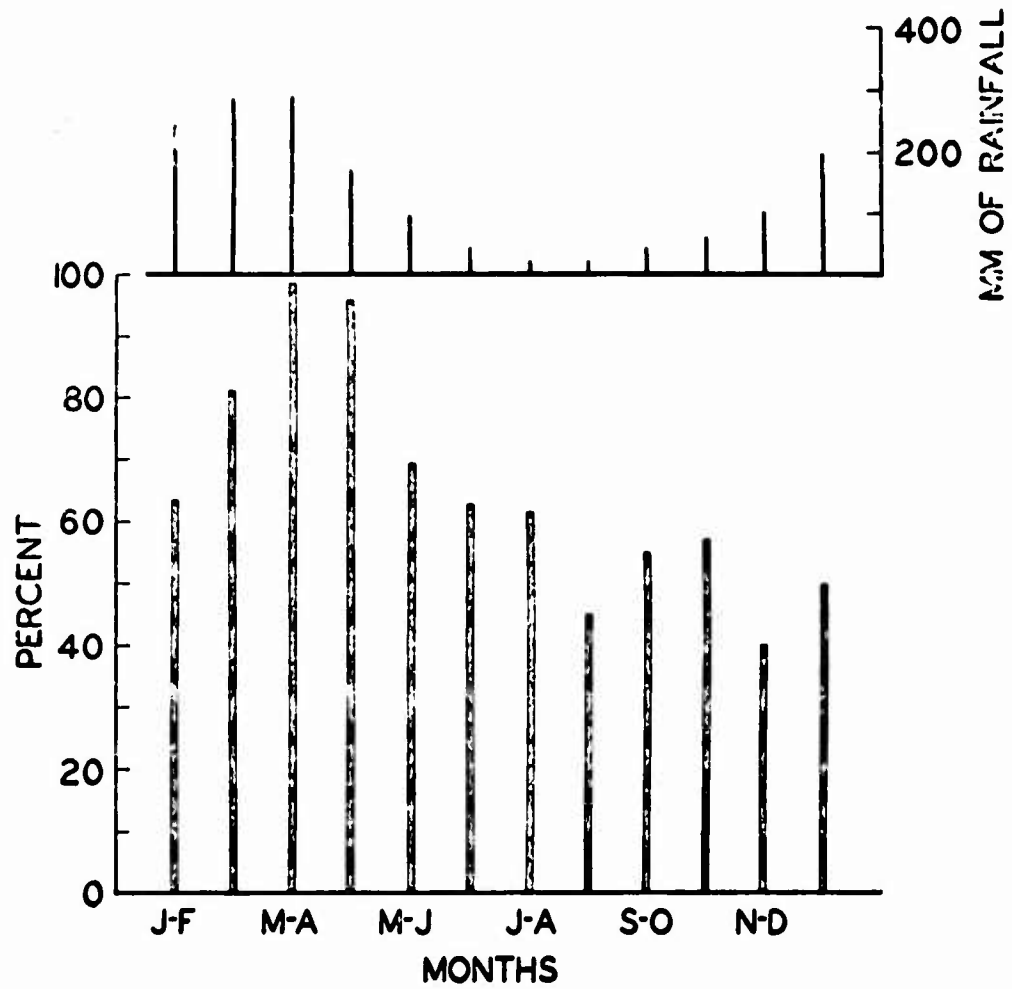
<u>Oryzomys</u>	18	34	27	26	34	40	41	25	15	19	15	6
<u>Proechimys</u>	21	17	15	11	22	33	20	13	8	8	7	11

FIGURE 1. The annual reproductive activity of female *Oryzomys capito* and *Proechimys*. The upper graph shows the average rainfall per 2 month running means, the lower graph demonstrates the percent of adult females either pregnant or lactating per 2 month running means, and the numbers below indicate the sample size of adult females.



<u>Neacomys</u>	14	13	2	3	8	12	11	6	8	10	5	4
<u>Oxymycterus</u>	7	5	5	3	6	18	15	7	7	9	8	6

FIGURE 2. The annual reproductive activity of female Neacomys and Oxymycterus. The upper graph shows the average rainfall per 2 month running means, the lower graph demonstrates the percent of adult females either pregnant or lactating per 2 month running means, and the numbers below indicate the sample size of adult females.



Zygodontomys 11 16 28 30 13 22 29 20 18 14 5 2

FIGURE 3. The annual reproductive activity of female Zygodontomys. The upper graph shows the average rainfall per 2 month running means, the lower graph demonstrates the percent of adult females either pregnant or lactating per 2 month running means, and the numbers below indicate the sample size of adult females.

E. Photographic Documentation Program

BACKGROUND: Little information is presently available on the ecology of Amazonian plant formations. The effects of the wet and dry seasons on leaf fall, flowering, and fruiting are largely unknown. The length of time for agricultural crops to grow and produce fruit in the many different soil types, and how many crops may be planted and harvested before the soil becomes exhausted has not been recorded. After the agricultural plots are abandoned the secondary scrub takes over. The rate of this secondary succession and the make up of plant species play an important role in the mammalian and insect populations. Through photographic documentation the types of forest, secondary scrub and cropland in which wild mammal surveillance, entomological and epidemiological programs are being conducted are being recorded. The seasonal changes in vegetation and water levels throughout the year are also documented.

PROGRAM DESCRIPTIONS: A series of photographic stations are located along the traplines in trapping area 3/5, west of Altamira, and also in trapping area 29-03, west of Maraba. Photographs are taken at each area every 2 months. Each photographic station contains a permanent mount for the camera, and a target at which the camera is aimed. The photograph number is recorded on a form (FIGS. 1-4) for future reference to compare the series of photographs from each photographic station for monthly changes in vegetation. The photographic stations are located in agricultural areas as well as in the forest to document the influx of mammals as the crops produce fruit. The seasonal changes in the water level of swamps, and the inundation of lowland forests are also recorded in this manner. These photographs will be shown to botanists and tropical plant ecologists for identification of specific plants and vegetation types.

OBSERVATIONS: The changes in the vegetation between the wet season and dry season vary slightly in the forest habitat. Even though the water levels change drastically in the streams flowing through the lowland forests, the overall vegetation changes little. The secondary scrub and cropland habitats undergo a very noticeable change between the wet and dry seasons as they have no overhead canopy to limit the effects of the sun and wind. Corn and rice grow quickly; they may be harvested 3 months or less after planting. Corn, rice, and cassava are normally planted together. The corn is harvested first, the rice shortly after, and the cassava much later. When the land is abandoned to secondary succession it grows quickly and becomes impenetrable within a few months.

/zcm.

Photographic Stations at Cleba 3/5, Lote 48, West of Altamira

Date: _____ Roll numbers: _____

Station number

1	185° _____ _____	_____	260° _____ _____	_____	355° _____ _____	_____
2	165° _____ _____	_____	332° _____ _____	_____		
3	155° _____ _____	_____	335° _____ _____	_____		
4	20° _____ _____	_____	150° _____ _____	_____		
5	155° _____ _____	_____	345° _____ _____	_____		
6	240° _____ _____	_____	322° _____ _____	_____		
7	135° _____ _____	_____	270° _____ _____	_____		
8	95° _____ _____	_____	315° _____ _____	_____		
9	125° _____ _____	_____	265° _____ _____	_____		
10	160° _____ _____	_____	350° _____ _____	_____		
11	130° _____ _____	_____	260° _____ _____	_____		
12	73° _____ _____	_____	140° _____ _____	_____	(Palm tree nr rd in field)	

FIGURE 1. Front of Photographic Form Used for Trapping Site 3/5.

FIGURE 2. Back of Photographic Form Used for Trapping Site 3/5.

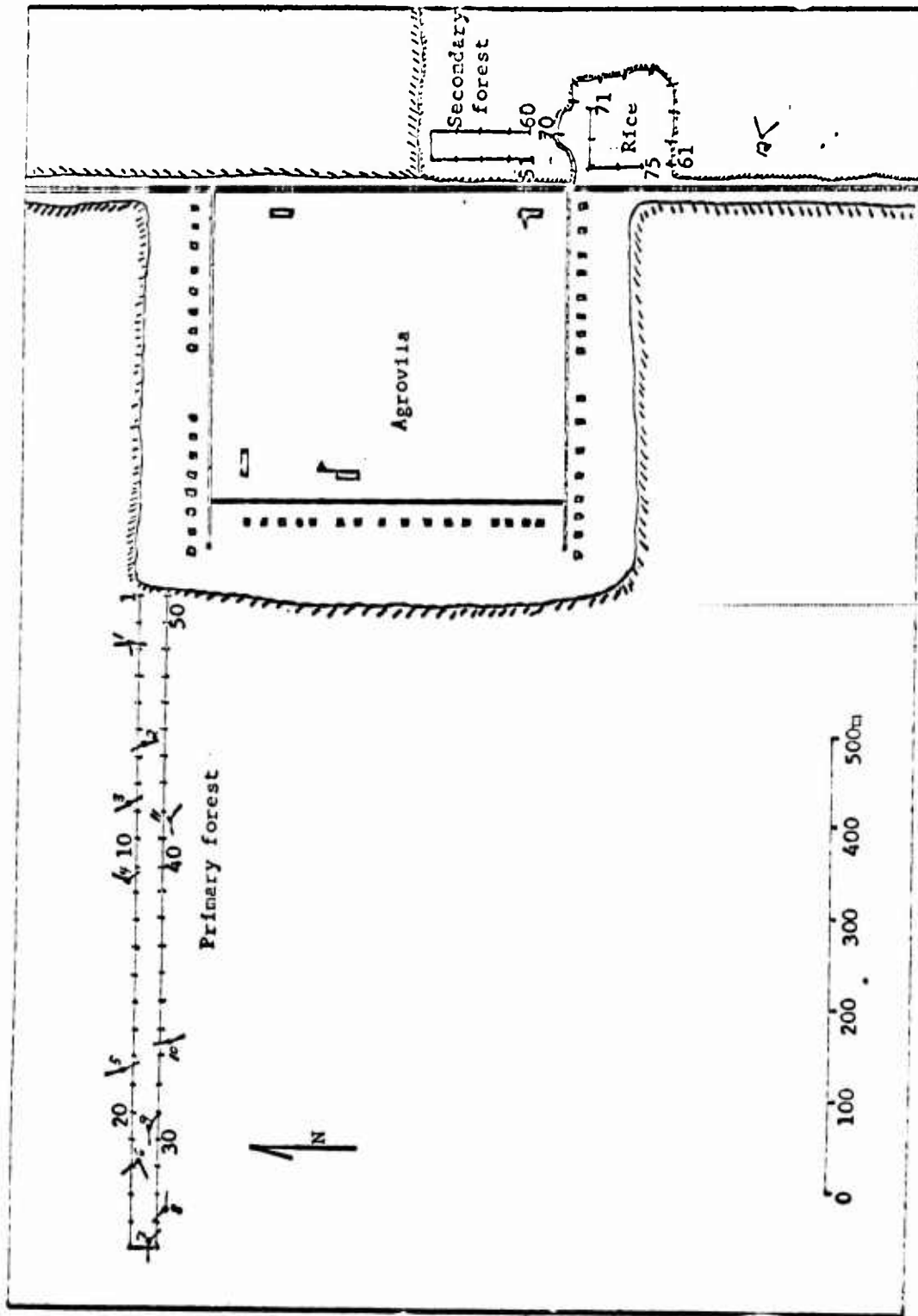


FIGURE 3. Front of photographic Form Used for Trapping Site 29-02

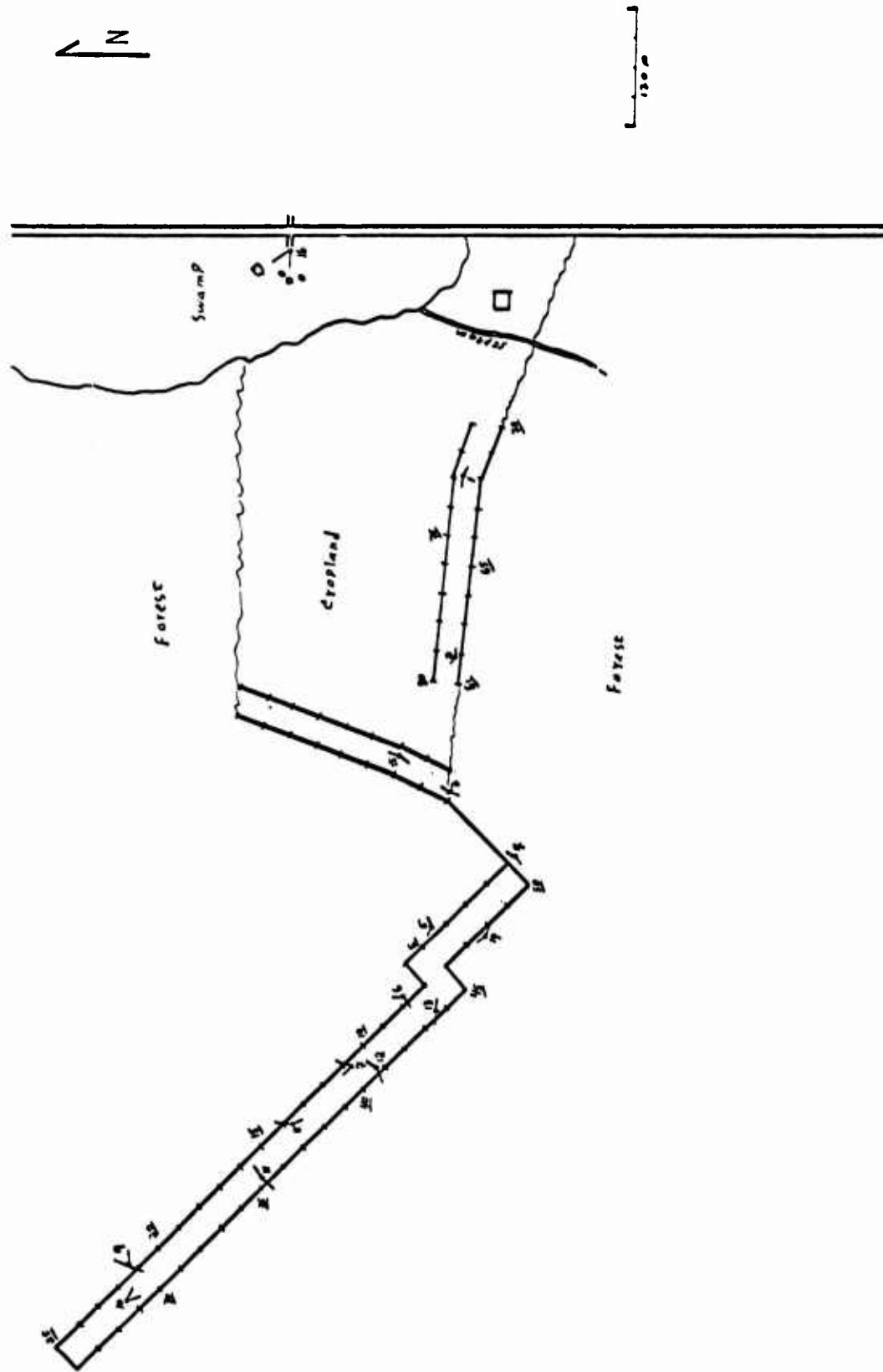
Photographic Stations at Gleba 29, Lote 03, West of Maraba

Date: _____ Roll numbers: _____

Station number

1 (tree)	122° _____ _____	275° _____ _____		
2 (stump)	90° _____ _____	310° _____ _____		
3 (palm)	31° _____ _____	205° _____ _____		
4 (palm)	30° _____ _____	228° _____ _____		
5 (tree)	65° _____ _____	295° _____ _____		
6 (tree)	230° _____ _____	360° _____ _____		
7 (tree)	19° _____ _____	317° _____ _____		
8 (tree)	5° _____ _____	159° _____ _____		
9 (tree)	26° _____ _____	82° _____ _____	198° _____ _____	_____
10 (tree)	118° _____ _____	163° _____ _____		
11 (tree)	552° _____ _____	222° _____ _____		
12 (palm)	30° _____ _____	245° _____ _____		
13 (tree)	96° _____ _____	275° _____ _____		
14 (tree)	112° _____ _____	326° _____ _____		
15 (palm)	19° _____ _____	215° _____ _____		
16 (culvert)	270° _____ _____	328° _____ _____		
17 T-rd east to Jatobal, aimed at emergent on ridge at			300° _____	_____

FIGURE 4. Back of Photographic Form Used for Trapping Area 29-02.



PROJECT 3A762759A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 073 Disease transmission in tropical populations

Literature Cited.

References:

1. Beard, J.S.: Climax vegetation in tropical America. *Ecology* 25: 127-158, 1944.
2. Cuadrado, R. R. and I. G. Kagan: The prevalence of antibodies to parasitic diseases in sera of young army recruits from the United States and Brazil. *Am. J. Epid.* 86: 330-340, 1967.
3. Fleming, T. H.: Notes on the rodent faunas of two Panamanian forests. *J. Mammal.* 51: 473-490, 1970.
4. Forattini, O. P.: Some data on the domesticity of *Aedes scapularis* (Rondani) in Sao Paulo, Brazil. *Mosquito News* 21: 295-296, 1961.
5. Healy, G. R., I. G. Kagan, and N. N. Gleason: Use of the indirect hemagglutination test in some studies of seroepidemiology in the western hemisphere. *Health Lab. Science* 7: 109-116, 1970.
6. Holdridge, L. R.: Determination of world plant formations from simple climatic data. *Science* 105: 367-368, 1947.
7. Lacaz C. da Silva, R. G. Baruzzi, and W. Siqueira, Jr.: Introdução a Geografia Médica do Brasil. Editora Blucher Ltda., Sao Paulo, 1972.
8. Reinert, J. F.: Mosquito generic and subgeneric abbreviations (Diptera: Culicidae). *Mosquito Systematics* 7: 105-110, 1975.
9. Richards, P. W.: The Tropical Rain Forest. University Press, Cambridge, 1952.
10. Tesh, R. B.: Notes on the reproduction, growth and development of echimyid rodents in Panama. *J. Mammal.* 51: 199-202, 1970.

/zcm.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OC 6465	76 07 01	DD-DR&E/AR/636	
3. DATE PREV. SUMMRY	4. KIND OF SUMMARY	5. SUMMARY SCY ^b	6. WORK SECURITY ^b	7. REGRADING ^c	8A. DISB ^d INSTR ⁿ	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 ^e	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	62759A	3A762759A831		00	074		
B. CONTRIBUTING							
C. XREF/REF/INTX	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ^f							
(U) Tropical and Subtropical Diseases in Military Medicine							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^g							
010100 Microbiology 002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 08		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: NA				PRECEDING		B. FUNDS (In thousands)	
B. NUMBER: NA				FISCAL YEAR		76	
C. TYPE:				77		10.0	
D. KIND OF AWARD:				AMOUNT:		1,100	
E. CUM. AMT.				9		566	
20. RESPONSIBLE OOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: US Army Medical Component, SEATO			
ADDRESS: Washington, DC 20012				ADDRESS: Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME if U.S. Academic Institution)			
NAME: JOY, R. J. T. COL				NAME: BEACH, D.J., COL			
TELEPHONE: 202-576-3551				TELEPHONE: 2817776			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				[REDACTED]			
22. ASSOCIATE INVESTIGATORS				NAME: BANCROFT, W. H., LTC			
				NAME: SCOTT, R. M., LTC			
23. KEYWORDS (Precede EACH with Security Classification Code) (U)Infectious Diseases; (U)Virus Diseases; (U)Bacterial Diseases; (U)Parasitic Diseases; (U)Veterinary Medicine; (U)Hepatitis; (U)Dengue fever							
23. (U) To define the ecology and basic biology of causal agents of tropical diseases, and to study environmental variables that may affect the performance of U.S. servicemen in tropical areas.							
24. (U) Routine diagnostic, epidemiological, serological, biochemical, microbiological, and entomological methods are being utilized. Field studies are emphasized and are supplemented by appropriate laboratory investigations.							
25. (U) 75 07 - 76 06 Dengue and hepatitis virus infections in man were investigated with emphasis on epidemiological and clinical aspects. Primary dengue infections with shock were clearly documented but occurred rarely in Bangkok. Hepatitis B virus was found to be transmitted during the first six months of life to 60% of infants of mothers who were hepatitis B carriers. Mothers whose older children were hepatitis B carriers, or who had e-antigen and/or high complement fixation titers of hepatitis B surface antigen in their blood were at the highest risk of transmitting hepatitis B to their offspring. Only a minority of adult carriers in the Thai population could be accounted for by mother-child transmission. Saliva was shown to be a vehicle of hepatitis B transmission. Studies of mosquito parasites in Thailand have identified 25 host-parasite associations. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76. Support in the amount of \$290,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

PII Redacted

DD FORM 1498

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

Project 3A762759A931 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 074 Tropical and Subtropical Disease in military
medicine

Investigators

Principals: COL Douglas J. Beach, MSC; LTC William H. Bancroft, MC; LTC David E. Davidson, VC; LTC Eliot J. Pearlman, MC; LTC Robert M. Scott, MC; Douglas J. Gould, Ph.D.; Joe T. Marshall, Ph.D.; MAJ John L. Brown, VC; MAJ Garrett S. Dill, Jr., VC; MAJ William T. Watson, VC; MAJ Stephen C. Hembree, MSC; CPT Richard G. Andre, MSC; CPT Charles L. Bailey, MSC; CPT Gary D. Phillips, MSC; CPT Robert J. Schneider, MSC; Chiraphun Duangmani, M.D.; Ananda Nisalak, M.D.; Kwanyuen Lawhaswadi, DVM; Santi Haoupara, M.D.; Rapin Snitbhan, M.D.; Pramool Sukwatana, M.D.; Prayot Tanticharoenyos, DVM; Markpol Tingpalapong, DVM; MSGT Robert E. Weaver, Jr.; SFC William K. Andrews; SP6 Jerome Karwacki;

Associate: Aree Boriharnvanakett, M.T.; Chalam Chantarasri, B.Sc.; Sitipantu Chaiyanunta, B.Sc.; LTC Edward B. Deberstyn, MC; Pairatana Gunakasem, M.D.; MAJ Gilbert R. Irwin, MC; Suchart Jatanasen, M.D.; Yeepu Keokarn, R.N.; Nonglak Khananuraksa, B.Sc.; Kenneth L. Knight, Ph.D.; LTC Pralong La-or Nguen, DVM, RTA; Chumpun Manomuth, B.Sc.; Kol Mongkolpanya; Sumitda Narupiti, B.Sc.; Suchitra Nimmanitya, M.D.; Naowayubul Nutkumhang, B.Sc.; Nathada Plavuth, R.N.; COL Suchai Premabutra, DVM, RTA; Rampa Rattanarithikul; Miodrag Ristic, Ph.D.; LTC Edward H. Stephenson, VC; Chumduen Satavuthi, B.Sc.; Surapee Siridhoranakul, M.D.; Pinit Simasathien, M.D.; Suleela Srimachaibovorn, B.Sc.; Sommai Sringarm, M.D.; Suranga Srimarut; Panor Srisongkram, B.Sc.; MAJ Vanich Vanapruks, MC, RTA; Suwana Vitanomsat, B.Sc.; Suwatana Vongpradist

I. VIRUS DISEASES OF MAN AND ANIMALS

A. DENGUE HEMORRHAGIC FEVER

1. Shock Syndrome in Primary Dengue Infections

OBJECTIVE: To document by intensive laboratory investigation primary dengue infection associated with dengue shock syndrome (DSS).

BACKGROUND: During the year 1974 five patients presented at the Bangkok Children's Hospital (BCH) with shock and preliminary laboratory findings suggestive of primary dengue infections. This report summarizes the clinical histories of these patients and reviews laboratory findings, establishing them as primary dengue infections with shock.

DESCRIPTION: The detection of patients for this study has been previously described, (SEATO Medical Research Laboratory Annual Report 1974-1975). Briefly, patients with a hospital admission diagnosis compatible with dengue infection were studied. Clinical diagnosis of dengue hemorrhagic fever and grading of the severity of disease followed criteria previously reported (SEATO Medical Research Laboratory Annual Report 1974-1975). Virus isolation used a direct and delayed plaque technique on LLC-MK₂ cells. Isolates were identified by plaque reduction neutralization tests. Serum obtained from each individual was tested for antibodies by hemagglutination inhibition (HI) and plaque reduction neutralization (PRNT). Primary dengue infections were tentatively identified as patients with convalescent HI titers of less than 1:640.

Final classification of primary infections was based on classical characteristics, i.e., absent or low titered antibodies in the acute serum and the development of IgM during convalescence. IgM was separated from serum proteins by sucrose gradient ultracentrifugation. Fractions were tested for content of IgM by radial immuno-diffusion and for antibody activity by HI. Antibody activity was proved to be associated with IgM by a significant reduction in HI titer following treatment of the fraction with 2-mercaptoethanol.

Previous investigation of shock associated with secondary dengue infections have indicated a fall in B1c/B1a concentrations in acute serum related to the severity of disease. B1c/B1a levels were determined in primary patients using commercially prepared radial diffusion plates (Hyland Laboratories).

Three patients with shock met the preliminary criteria for primary dengue. Clinical and laboratory findings on these patients are presented.

Patient 77, a four year old Thai female, had a five day history of fever, anorexia and vomiting. On the day of admission, she had a cough, a rash on both arms, and became increasingly lethargic. Physical examination revealed an axillary temperature of 38.5°C, a pulse of 116 and a blood pressure (BP) of 120/80 mm Hg (pulse pressure 40 mm Hg). There were petechiae on both arms. The tourniquet test was positive. Her posterior cervical lymph nodes were enlarged and her liver was palpated at the right costal margin. Her hematocrit was 42%, WBC, 6,900 per mm³; and platelet count, 186,000 per mm³.

Despite the administration of intravenous fluids, vascular collapse developed (Figure 1 a). Plasma was administered intravenously with satisfactory improvement and the patient was discharged from the hospital on the ninth day of disease.

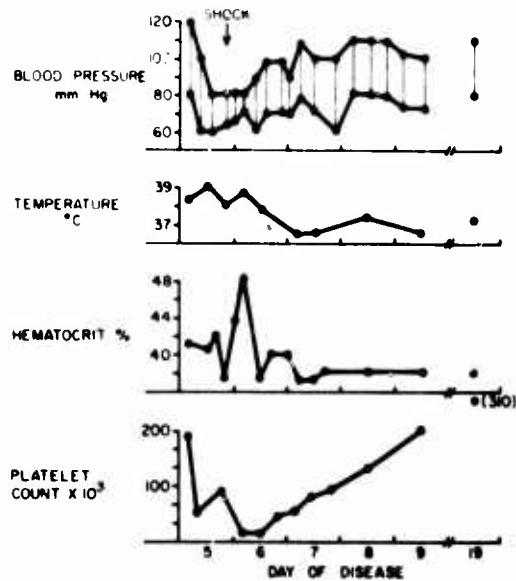
Patient 91, a 12 year old Thai female, had a five day history of fever, headache and abdominal pain. On the day of admission a rash developed and the child became increasingly obtunded. Physical examination revealed mild dehydration, an axillary temperature of 37°C, and a pulse of 100. A light macular rash was present over her entire body and petechiae were on her face and arms. The tourniquet test was positive. Her liver was palpable below the right costal margin. The patient was in shock with cold, clammy extremities; a weak, thready pulse; and a BP of 110/90 mm Hg (pulse pressure 20 mm Hg). Her hematocrit was 47%; WBC, 6,400 per mm³; and platelet count, 42,000 per mm³.

Intravenous fluids led to improvement (Figure 1 b). During the first 12 hours in hospital, the patient passed a black tarry stool and the petechiae became more widespread. The patient was discharged on the eighth day of disease.

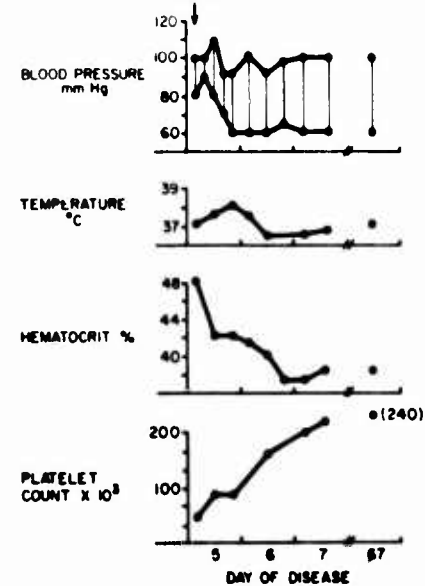
Patient 103, an eight year old Thai female, had a four day history of fever and vomiting. On admission, she had a temperature of 38°C; a pulse of 124 and a BP of 90/80 mm Hg (pulse pressure 10 mm Hg). Her tonsils were injected and her liver was palpable 3 cm below the right costal margin. No petechiae were noted; however, the tourniquet test was positive. Her hematocrit was 47%, WBC, 3,300 cells per mm³; and platelet count, 146,000 per mm³.

Intravenous fluid was administered and the blood pressure stabilized at 100/60 mm Hg (Figure 1 c). The patient was discharged on the seventh day of disease.

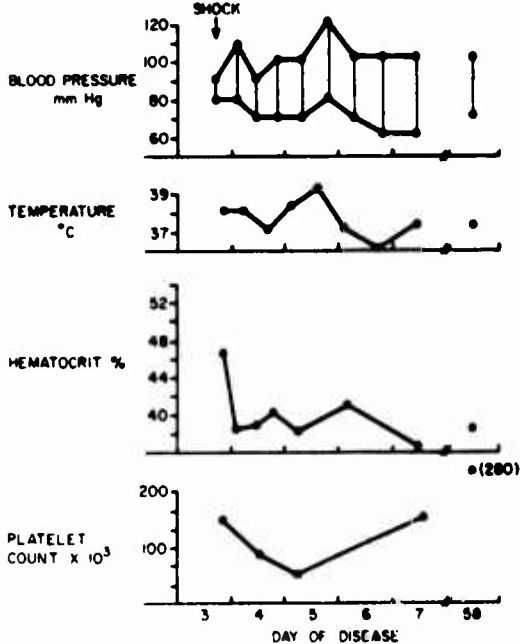
PATIENT 77 (a)



PATIENT 91 (b)



PATIENT 103 (c)



PATIENT 6 (d)

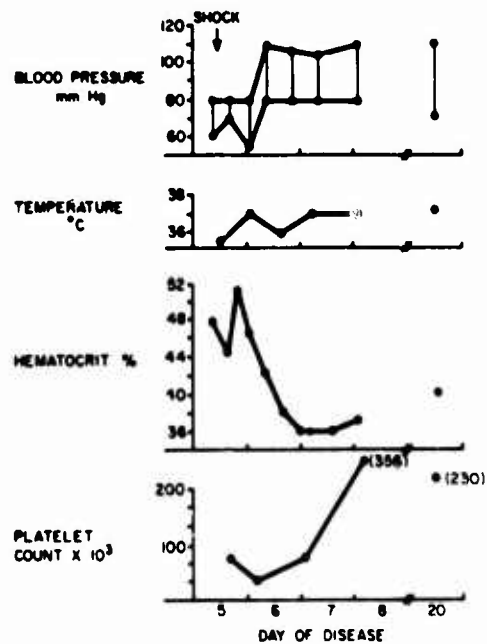


Figure 1 Diagrams of the clinical courses of primary dengue patients (a) 77, (b) 91 and (c) 103, showing the relationship of several clinical and laboratory variables to the onset of shock. A secondary dengue patient, (d) 6 is diagramed for comparison

The above three primary infections may be compared to the following patient with a secondary infection and shock.

Patient 6, a seven year old Thai female, was admitted with a five day history of fever, drowsiness and abdominal pain. On the day of admission she became increasingly lethargic and manifested clinical signs of shock. Her temperature was 35.5°C, her pulse was 120 and her BP was 80/60 mm Hg (pulse pressure 20 mm Hg). Her liver was palpable 1 cm below the right costal margin. Petechiae were noted on both upper extremities; the tourniquet test was positive. Her hematocrit was 48%; WBC, 5,600 per mm³; and platelet count, 85,000 per mm³.

During the first six hours in the hospital, the pulse pressure fell to 10 mm Hg (BP 80/70 mm Hg), the pulse rate rose to 130, and the hematocrit rose to 52%. Administration of intravenous fluid led to recovery and the patient was discharged on the eighth day of illness (Figure 1 d).

Laboratory Studies:

A strain of D3 virus was isolated from the acute serum of Patient 103. The other two patients with primary dengue infections did not yield virus isolates; however, D1 virus was isolated from two siblings of Patient 77 with undifferentiated fevers.

HI tests of sequential serum specimens showed a broadly reactive antibody response (Table 1). PRNT antibody gave the best indication of the infecting virus type, since they were nearly monospecific; Patients 77 and 91 had D1 and Patient 103 had D3.

Low titers of PRNT antibody were found in the convalescent sera of Patient 77 to D2, D3, D4 and chikungunya. Unlike the others, Patient 77 had received plasma infusions. The plasma administered was not tested for antibodies; however, nearly 100% of adults in Bangkok have antibodies to dengue and chikungunya viruses. The low titers of antibodies found in this patient may have been acquired from the plasma transfused. The HI and PRNT titers of Patients 77, 91 and 103 may be compared with those of Patient 6 in whom high titered cross reacting antibodies were found.

Using sucrose gradient ultracentrifugation, immunoglobulin separations were performed on sera collected early and late in the course of infection (Table 2). IgM was found in fractions two to five while IgG was found in fractions six to twelve. In the early sera, the IgM fractions contained higher HI titers of anti-dengue immunoglobulin than did the IgG fractions. Convalescent sera

Table 1. Antibody Titers in Patient with Dengue Infections

Patient Number	Day of Disease	Reciprocal HI Antibody Titer				Reciprocal PRNT Antibody Titer				
		D1	D2	D3	D4	D1	D2	D3	D4	Chik
77	5	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
	17	80	40	160	160	1280	40	120	40	160
	36	40	20	40	40	≥1280	40	230	40	120
91	5	40	< 20	< 20	< 20	20	< 20	< 20	< 20	NT
	11	160	40	80	160	NT ^a	NT	NT	NT	NT
	67	160	80	80	160	500	< 20	< 20	< 20	< 20
103 ^b	3	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
	7	< 20	20	40	< 20	< 20	< 20	40	< 20	< 20
	58	20	20	160	80	20	< 20	70	< 20	< 20
6	6	10240	10240	10240	≥20480	≥5120	≥5120	≥5120	≥5120	< 10
	11	≥20480	10240	10240	≥20480	NT	NT	NT	NT	NT
	20	≥20480	10240	≥20480	≥20480	≥5120	≥5120	≥5120	≥5120	< 10

^a NT = Not tested

^b Dengue 3 virus isolated from this patient

Table 2. Hemagglutination Inhibition Antibody Titers of Serum IgM and IgG Fractions in Patients with Dengue Infections

Patient Number	Day of Disease	Immuno-a globulin	Reciprocal HI Antibody Titer											
			D1		D2		D3		D4					
			Not Treated	ZME Treated	Not Treated	ZME Treated	Not Treated	ZME Treated	Not Treated	ZME Treated				
77	5	IgM	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	
		IgG	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	
	9	IgM	64	8	32	8	16	16	64	16	16	16	16	
		IgG	16	16	32	8	16	16	32	16	32	32	32	
91	36	IgM	8	8	<4	4	8	16	<4	16	16	<4	8	
		IgG	8	16	<4	4	8	16	<4	16	16	<4	8	
	5	IgM	4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	
		IgG	4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	
103	8	IgM	64	8	4	4	<4	8	4	4	4	8	8	
		IgG	64	8	4	4	<4	8	4	4	4	8	8	
	37	IgM	8	128	<4	32	32	64	<4	128	128	<4	64	
		IgG	8	128	<4	32	32	64	<4	128	128	<4	64	
6	3	IgM	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	
		IgG	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	
	7	IgM	<4	<4	<4	<4	<4	32	<4	4	<4	<4	<4	
		IgG	<4	<4	<4	<4	<4	32	<4	4	<4	<4	<4	
6	58	IgM	<4	32	<4	16	16	128	<4	256	64	<4	64	
		IgG	<4	32	<4	16	16	128	<4	256	64	<4	64	
	6	IgM	4	4	4	4	4	4	4	4	4	4	4	
		IgG	4	4	4	4	4	4	4	4	4	4	4	
1.	1.	IgM	8	4096	4	8	4096	4	4096	4	4096	4	4096	
		IgG	8	4096	4	8	4096	4	4096	4	4096	4	4096	
	20	IgM	<4	4096	4	4	4096	4	4096	4	4096	4	4096	
		IgG	<4	4096	4	4	4096	4	4096	4	4096	4	4096	

a. The sucrose gradient fraction with the highest concentration of IgM or IgG is shown.

contained predominantly IgG antibody. Although IgM antibody was often present against more than one type of dengue, the titer to the infecting virus type appeared to be higher and to last longer. Immunoglobulin separations on early convalescent sera from Patient 77 showed only IgG antibodies to chikungunya. This strengthened the impression that antibodies to this agent were passively acquired through transfusion.

Concentrations of Blc/Bla were determined on sequential sera from the presumptive primary dengue patients (Figure 2). The concentrations of these proteins were found to be subnormal in the acute sera from each case. By the time convalescent sera were obtained, the levels had risen to the normal range. These findings were similar to those previously reported in secondary dengue infections and were also seen in Patient 6.

Fatal Cases:

There were five fatal cases studied in 1974. Of the fatal cases, two (Patients 16 and 112) did not have detectable HI antibody in their acute sera. Since convalescent sera were unavailable, sequential studies of antibody titers and complement levels could not be performed. These two patients are presented here as possible primary dengue infections.

Patient 16, a 12 year old Thai female, had a five day history of fever, myalgia and lethargy. Two days prior to admission she developed anorexia and abdominal distention. On admission, a physical examination revealed an obtunded child with an axillary temperature of 38.5°C. Petechiae were present in the axilla and subclavicular areas and the tourniquet test was positive. Her axillary and right submandibular lymph nodes were enlarged, her abdomen was distended and her liver was not palpable. The patient was in shock with pallor, cold extremities, restlessness and tachypnea. Her pulse was 120 and BP was 90/80 mm Hg (pulse pressure 10 mm Hg). Her hematocrit was 54%, WBC, 6,800 per mm³.

Rapid administration of plasma led to a fall in the hematocrit to 40%; however, neither the pulse pressure nor the blood pressure improved. Intravascular coagulation and acidosis developed. Despite treatment with plasma, sodium bicarbonate and heparin, the patient died eight hours after admission to the hospital.

Patient 112, a seven year old Chinese male, had a four day history of fever, anorexia and abdominal pain. One day prior to admission he developed diaphoresis, cold extremities and lethargy. A rash was noted on his arms and legs. On admission he was lethargic with

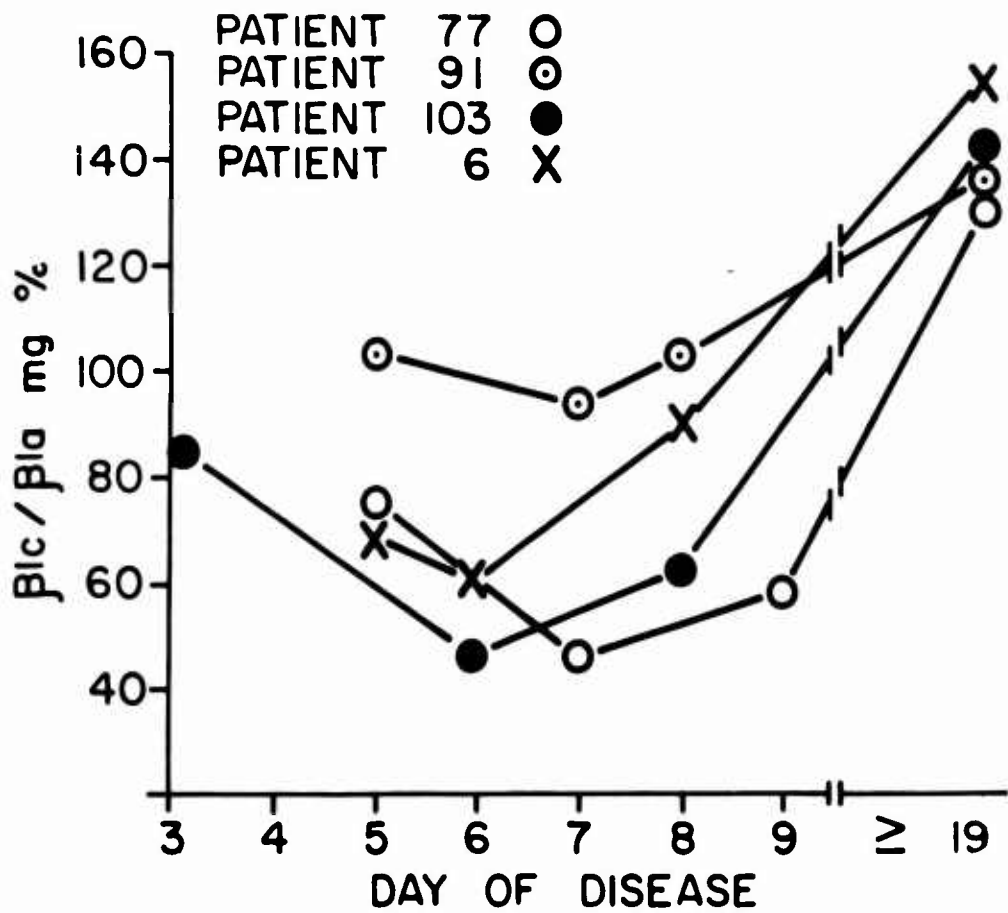


Figure 2. Sequential $\beta I_c / \beta I_a$ concentrations from patients with primary and secondary dengue infections.

an axillary temperature of 39.5°C. His pulse rate was 102 and BP was 110/90 mm Hg (pulse pressure 20 mm Hg). His hematocrit was 44%; WBC, 4,150 per mm³; and platelet count, 110,000 per mm³.

Intravenous fluid administration led to a transient improvement in clinical status and a rise in pulse pressure to 30 mm Hg. However, after three hours the child became restless and vomited copious amounts of fresh blood. The pulse pressure fell to 10 mm Hg (BP 110/100 mm Hg) and shock reappeared. Plasma and fresh whole blood were administered, but the hematemesis continued. Despite volume replacement, there was no improvement and the child died 12 hours after admission.

Laboratory Studies:

Dengue type 2 was isolated from the blood of Patient 16 and type DE from Patient 112. Although there were no detectable HI antibodies, the PRNT on the acute sera revealed low titers of antibody against the infecting virus in both patients. Immunoglobulin separations on the acute specimens were not informative as the concentrations of dengue antibodies were too low to be detected. From Patient 112 a second serum was obtained after death. HI antibody determinations on this serum showed low titer IgG antibodies against D1-4, JE and chikungunya. These antibodies were probably passively introduced by transfusion. The fatal cases also had markedly depressed concentrations of B1c/B1a.

DISCUSSION: The development of dengue shock syndrome in older children has been associated epidemiologically with secondary dengue infections (1). It was suggested that anamnestic antibody in the presence of dengue antigens triggered an immunologic mechanism (2). Three patients described here, however, had no evidence of prior dengue infections. They demonstrate that primary dengue infections can cause the dengue shock syndrome in older children. Furthermore, in two fatal dengue infections, the absence of HI antibodies and the low titered PRNT antibodies against the isolated virus types in acute sera are also consistent with primary infections. Although not substantiated by studies on convalescent sera, these data suggest that primary dengue may also be associated with fatalities.

DSS presenting in primary dengue infections had similar clinical and laboratory courses to those previously reported for secondary infections (3). The demonstration of a depletion in complement factor three in primary infections supports an immunological mechanism for shock. The development of immune complexes may be related to viral and host factors as yet unknown. Immune complexes may be more likely in secondary dengue infections because of the

anamnestic antibody response during the time when viral antigens are circulating. Conceivably, immune complexes may occur in primary dengue infections in patients who produce the appropriate amount and type of antibody at the appropriate time.

In Bangkok, primary dengue with shock is infrequently recognized. It occurred in less than 5% of the DHF patients admitted to the BCH in 1974. Dengue is endemic in Bangkok. The true incidence of primary dengue with shock could not be calculated as no information was available on the number of individuals infected or at risk. However, each secondary dengue infection must be preceded by a primary infection. If the severe manifestations of dengue occurred as commonly in primary infections as they do in secondary, the number of patients hospitalized with primary infections should be similar to the number hospitalized for secondary infections. Further studies are required to elucidate both the mechanism and epidemiology of DSS in primary and secondary infections.

SUMMARY: During 1974, 114 patients with dengue hemorrhagic fever were studied at the Bangkok Children's Hospital. Over 40% of the patients had dengue shock syndrome. Five fatal cases were included in the study. Primary dengue infections were identified by absent or low titered antibodies in acute sera and the development of IgM antibodies during convalescence. Three patients, age 4, 8 and 12 years had primary dengue infections with shock. Although no convalescent sera could be tested, two other patients with fatal disease, age 7 and 12 years, also appeared to have primary infections. At the time of shock, patients with primary infections had subnormal concentrations of complement factor 3. The data show that in older children dengue shock syndrome associated with complement depression can occur during primary as well as secondary infections.

2. Detection of Dengue Infected Mosquitoes by Direct Fluorescent Antibody Microscopy

OBJECTIVE: To detect the presence of dengue virus in human serum using the mosquito inoculation technique.

BACKGROUND: This is a continuation of work previously reported (4). For the identification of dengue antigens in mosquitoes a direct fluorescent antibody (DFA) technique was added to the plaque isolation technique. This report reviews the experience with both techniques for the identification of dengue infections in patients seen at the Bangkok Children's Hospital (BCH) in 1975.

DESCRIPTION: To the previously described standard plaque assay in LLC-MK₂ tissue culture (TC) and the mosquito amplification technique,

inoculation to *A. aegypti* followed by the standard plaque assay (MI/TC), was added an immunofluorescent technique similar to that used at the Pacific Research Section of the National Institute of Allergy and Infectious Disease, Hawaii (5).

Anti-dengue fluorescent antibody conjugate was made from immune high-titered human sera. The globulin in immune sera was precipitated using 50% ammonium sulfate. The precipitate which contained gamma globulin and traces of albumin was dissolved in 0.01 M phosphate buffer pH 7.5 (P.B.) to a final volume approximately equal to that of the original serum sample, and then dialyzed against PB overnight.

After determining the total amount of protein present, the globulin solution was diluted with PB to a final concentration of 10 mg protein/ml and chilled in an ice bath. A freshly made carbonate-bicarbonate buffer (0.5 M, pH 9.0), was added to the chilled globulin in an amount equal to 10% by volume of diluted globulin. To the chilled globulin 0.0125 mg FITC/mg protein was added slowly with constant stirring. The solution was stirred overnight in the cold (12-18 hrs.), then dialyzed against PB to remove any unbound fluorescein dye. Merthiolate 1:10,000 was added as a preservative and the conjugate was frozen in aliquots.

The heads of infected mosquitoes were squashed under a coverslip (16 heads per slide) and stained with fluorescein conjugated antibody by a direct method. In early experiments each individual mosquito body was ground and tested by micro plaque assay. Later, pooled mosquito bodies were tested by a standard plaque assay.

Control mosquitoes consisted of non-infected mosquitoes, and mosquitoes infected with D1, 2, 3 and 4 reference suckling mouse brain strains.

Each reading was made by two observers: S.V. and W.H.B. or S.V. and A.N.

PROGRESS: A comparison of the results of virus isolation using the standard plaque isolation technique with and without mosquito amplification is shown in Table 1. Using the mosquito isolation step five strains of dengue were identified beyond those found by plaque isolation alone. Only one strain was isolated using the plaque isolation technique without mosquito amplification. The five additional strains isolated using the mosquito amplification step came from sera with titers of 1:2560, 1:640, 1:40 and 1:20.

For the DFA technique, test sensitivity and specificity was determined after arraying the results in a "decision matrix" and

Table 1. Isolation of Dengue Viruses from Patients' Plasma by Standard and Mosquito Amplification Techniques

HI Titer	Number	Isolation	
		Standard TC	MI/TC
≤ 20	23	9 (39)*	10 (43)
40	4	0 (0)	1 (25)
80	5	2 (40)	1 (20)
160	19	1 (5)	1 (5)
320	20	2 (10)	2 (10)
640	27	0 (0)	2 (7)
1280	39	0 (0)	0 (0)
2560	32	0 (0)	1 (3)
5120	17	0 (0)	0 (0)
10240	7	0 (0)	0 (0)
Total	193	14 (7.2)	18 (9.3)

* Percent of number tested at each titer.

calculating the "conditional probability" of true positive results (sensitivity) and true negative results (specificity) (3). Each decision matrix was built by comparing DFA test results to some "either - or" characteristic of the test materials, e.g., successful isolation of virus from individual mosquitoes or from individual patients. Separate determinations were made for each observer, S.V., W.H.B. and A.N. Each matrix was constructed as follows:

	Infection +	Infection -
DFA+	a	c
DFA-	b	d

$$\text{True positive (TP)} = \frac{a}{a+b}$$

$$\text{True negative (TN)} = \frac{d}{c+d}$$

Each ratio gives the conditional probability.

DFA ability to detect dengue in control mosquitoes was shown in the following matrices.

S.V.

Dengue 1

	TC+	TC-
DFA+	0	5
DFA-	1	7

$$\text{TP} = 0.0$$

$$\text{TN} = 0.58$$

Dengue 2

	TC+	TC-
DFA+	10	3
DFA-	2	2

$$\text{TP} = 0.83$$

$$\text{TN} = 0.40$$

W.H.B.

Dengue 1

	TC+	TC-
DFA+	0	5
DFA-	1	7

$$\text{TP} = 0.0$$

$$\text{TN} = 0.58$$

Dengue 2

	TC+	TC-
DFA+	4	2
DFA-	6	3

$$\text{TP} = 0.40$$

$$\text{TN} = 0.60$$

Dengue 3

	TC+	TC-
DFA+	0	4
DFA-	0	12

TP = unknown
TN = 0.75

Dengue 3

	TC+	TC-
DFA+	0	0
DFA-	0	14

TP = unknown
TN = 1.00

Dengue 4

	TC+	TC-
DFA+	1	4
DFA-	6	6

TP = 0.14
TN = 0.60

Dengue 4

	TC+	TC-
DFA+	0	0
DFA-	6	8

TP = 0.0
TN = 1.0

All negative controls were arbitrarily considered to be DFA negative by both observers and none yielded a virus isolate.

The plaque isolation technique did not appear to be capable of isolating virus from all mosquitoes inoculated with suckling mouse brain strains. This suggests that either the tissue culture or the mosquito was not very sensitive to mouse adapted dengue strains.

The results of DFA for mosquitoes inoculated with plasma of DHF patients are summarized in Table 2. There was a correlation between the DFA readings of the pair observers. Separate determination of matrices were made for each observer, S.V., W.H.B. and A.N. as follows:

S.V.

and

W.H.B.

	TC+	TC-
DFA+	7	28
DFA-	3	62

n = 100
TP = 0.7
TN = 0.69

	TC+	TC-
DFA+	9	8
DFA-	1	82

n = 100
TP = 0.9
TN = 0.9

Table 2. Detection of Dengue Antigen in Mosquitoes
by Direct Fluorescent Antibody
(Performance of Two Observers, S.V. and W.H.B. or S.V. and A.N.)

HI D2 Titer	Observers		Observers		Isolation + FA + by both observers	Isolation + FA + by one observer only
	S.V.	W.H.B.	S.V.	A.N.		
<20	11/20	6/20	9/15	12/15	8	2
20	0/2	0/2	2/3	2/3	1	-
40	1/3	1/3	1/3	1/3	-	-
80	3/5	1/5	-	-	-	1
160	3/9	1/9	2/5	2/5	-	-
320	3/9	2/9	3/9	3/9	-	2
640	6/13	2/13	7/10	6/10	1	-
1280	2/17	3/17	5/13	4/13	-	-
2560	5/16	1/16	5/9	5/9	-	-
5120	1/6	0/6	4/8	4/8	-	-
> 10240	-	-	2/5	2/5	-	-
Total	35/100	17/100	40/80	41/80	-	-

	<u>S.V.</u>		and	<u>A.N.</u>	
	TC+	TC-		TC+	TC-
DFA+	7	33		4	34
DFA-	0	40		3	39
	n = 80			n = 80	
	TP = 1.0			TP = 0.6	
	TN = 0.55			TN = 0.56	

DISCUSSION AND SUMMARY: As has been found previously; the mosquito amplification step gave a greater number of virus isolations than the standard plaque assay alone. The DFA test as performed in this laboratory is difficult to interpret. Although it appears to be relatively sensitive in some cases it showed a very poor specificity. In the absence of a second test capable of detecting dengue antigens per se it is difficult to determine the usefulness of the DFA test.

3. Surveillance of Dengue Hemorrhagic Fever Cases in Thailand, 1975

OBJECTIVE: To provide laboratory confirmation of clinically diagnosed arbovirus infections reported to the Ministry of Health.

BACKGROUND: In the Kingdom of Thailand Dengue Hemorrhagic Fever (DHF) remains the greatest identifiable cause of hospitalization and death among children under the age of 15 years. Control of this mosquito borne virus infection will require a knowledge of its prevalence and distribution. In 1973 a laboratory based serological surveillance program for DHF was initiated by the Ministry of Health to confirm reported clinically diagnosed Hemorrhagic Fever (HF). Adequate collection of blood samples was achieved in 1974. An earlier report contains the results from laboratory surveillance of DHF in 1973 and 1974 (4). The present report compares the results of laboratory surveillance over the years 1974 and 1975. In addition to surveillance for dengue infections, the program allowed for the serological confirmation of other infections such as Japanese encephalitis (JE).

DESCRIPTION: The filter paper disc collection method and Hemagglutination Inhibition (HI) testing have been previously described (7). In 1973 acute and convalescent bloods from clinically diagnosed DHF patients were submitted for serological testing from sixty

provincial hospitals throughout the Kingdom. Blood was submitted from an additional ten hospitals in 1974 and 1975 (Figure 1). Although the blood samples collected were rather unsatisfactory in 1973, good blood samples were obtained from all hospitals in 1974 and 1975.

PROGRESS: In 1975, 4,682 pairs of acute and convalescent dried blood spots on filter paper discs were submitted from provincial hospitals for DHF serological evaluation (Table 1). This represents an increase of 164% over the 2,850 pairs submitted in 1974. During the same periods, however, the number of cases of clinically diagnosed HF reported to the Ministry of Health rose 215% from 8,160 cases in 1974 to 17,573 in 1975. The increased specimens received by the serological laboratory in 1975 probably reflected an increased incidence of disease and an increased participation in the surveillance program. Regional participation for 1975 is shown in Table 1.

Table 1. Cases Submitted for Laboratory Confirmation for Dengue Infection from Provincial Hospital, 1975

Regions	Clinical Diagnosis of Submitted Cases			Total Cases
	HF	PUO	No Diagnosis	
North	500	0	14	514
Northeast	1798	13	80	1891
Central	1502	39	112	1653
South	601	2	21	624
Thailand	4401	54	227	4682

Dengue virus infections were confirmed in 2,362 of the 4,682 patients or 55% of those studied in 1975. This is an increase from the 37% confirmation seen in 1974. The frequencies of confirmation are similar for the four regions of Thailand indicating similar clinical criteria for diagnosis of DHF in all areas (Table 2). The distribution of the cases submitted and confirmed throughout the year peaked during the epidemic season from May through September similar to that seen in 1974. The proportion of laboratory confirmed cases of DHF for each month was essentially similar, indicating that the clinical diagnosis was not influenced by the time of year (Figure 2).

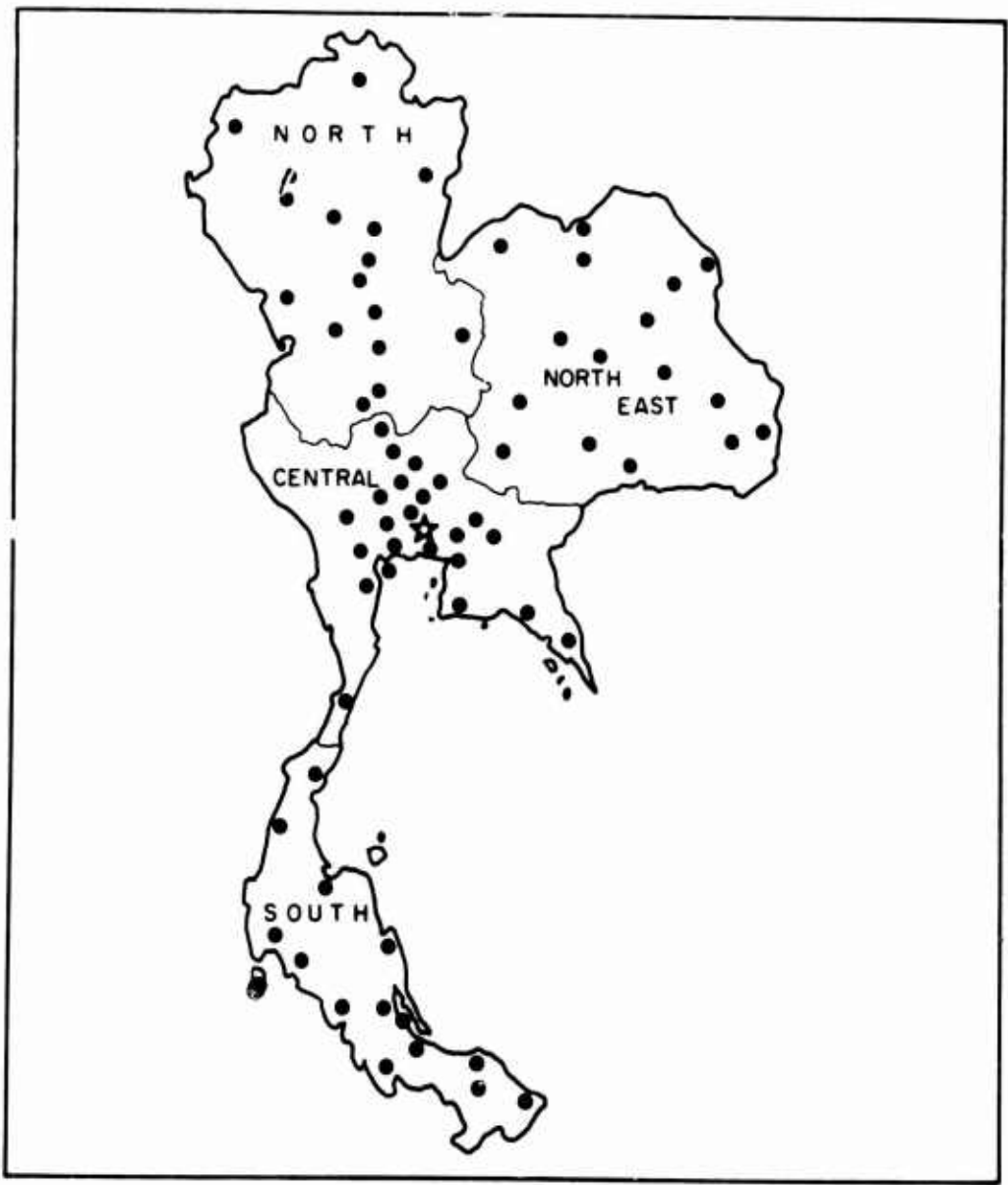


FIGURE 1. MAP DEMONSTRATING PROVINCES OR TOWNS OF STUDY (●)1975

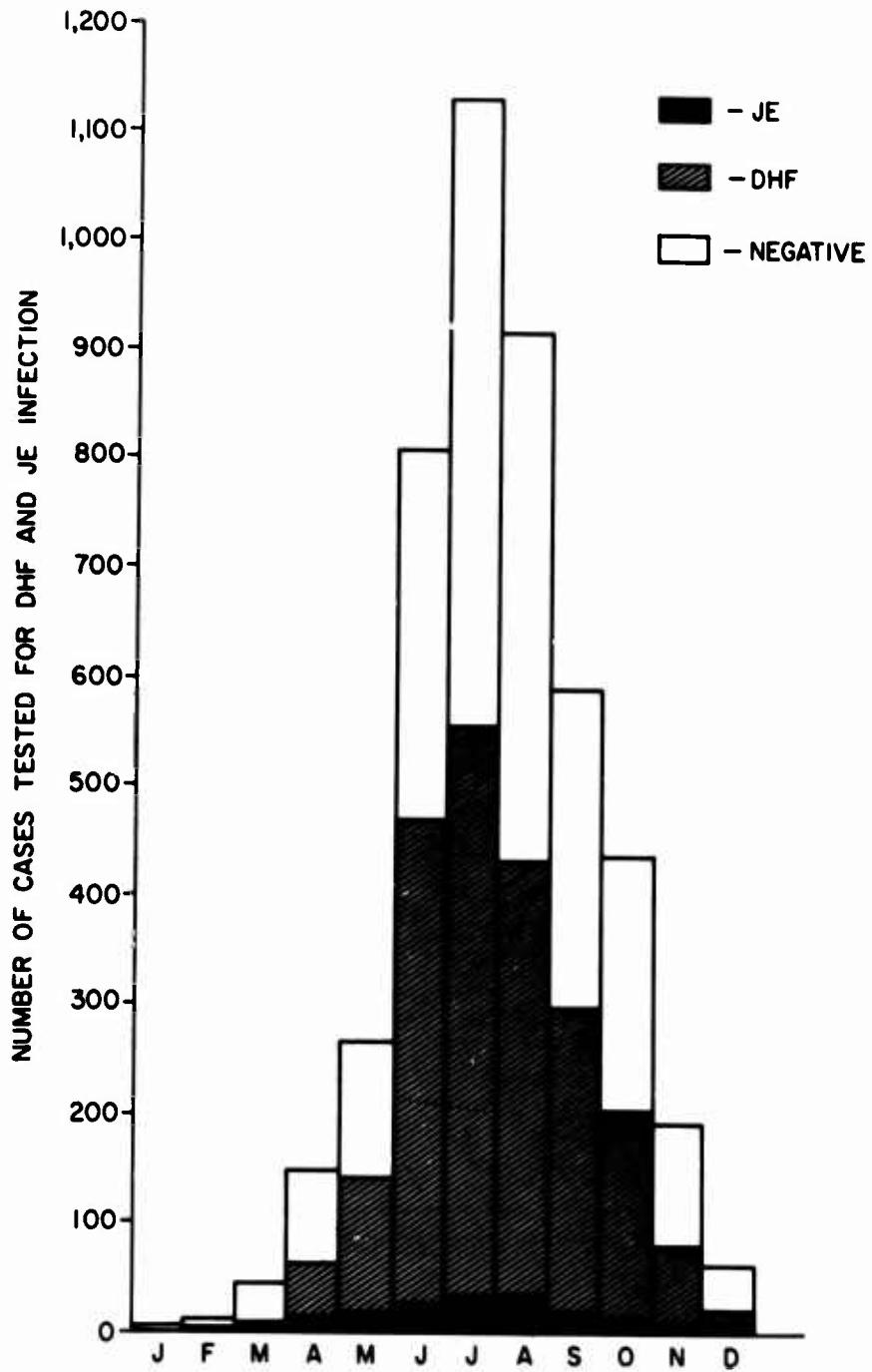


FIGURE 2. MONTHLY LABORATORY CASE CONFIRMATION FOR DHF AND JE INFECTION IN PROVINCES OF THAILAND, 1976

Table 2. Result of HI Tests for Dengue Infection in HF Patients Submitted from Provincial Hospitals, 1975 (By Region)

Region	Number of Cases Submitted for Blood Test	Cases with Recent Dengue Infection	
		Number	Percent
North	464	270	58
Northeast	1776	944	53
Central	1488	867	58
South	554	281	50
Thailand	4282	2862	55

Laboratory confirmed dengue infections were compared by age for the four regions of Thailand (Figure 3). The median age of infection for patients tested from the whole Kingdom was six years. There was little difference in the median age for the four regions. A similar median age of infection from 70 provincial hospitals was found in 1974 and for clinically diagnosed patients in Bangkok between 1971 and 1973 (8). This suggested that similar epidemiologic factors influence attack rates in Bangkok and in Thailand as a whole.

DHF was mis-diagnosed more often in the age group less than three years old (Figure 4). This may reflect the difficulty of precise diagnosis or perhaps a higher frequency of primary dengue infections in this age group. Primary dengue infections might not be identified by the testing method employed. Laboratory confirmation of clinical diagnosis for age groups greater than three years is significantly higher in 1975 than in 1974 indicating either a better understanding of the criteria for diagnosis of DHF by the hospital physician or possibly improved case selection because of increased numbers of patients.

The incidence of HF may be calculated using the figures reported to the Ministry of Health. The incidence of confirmed DHF may be

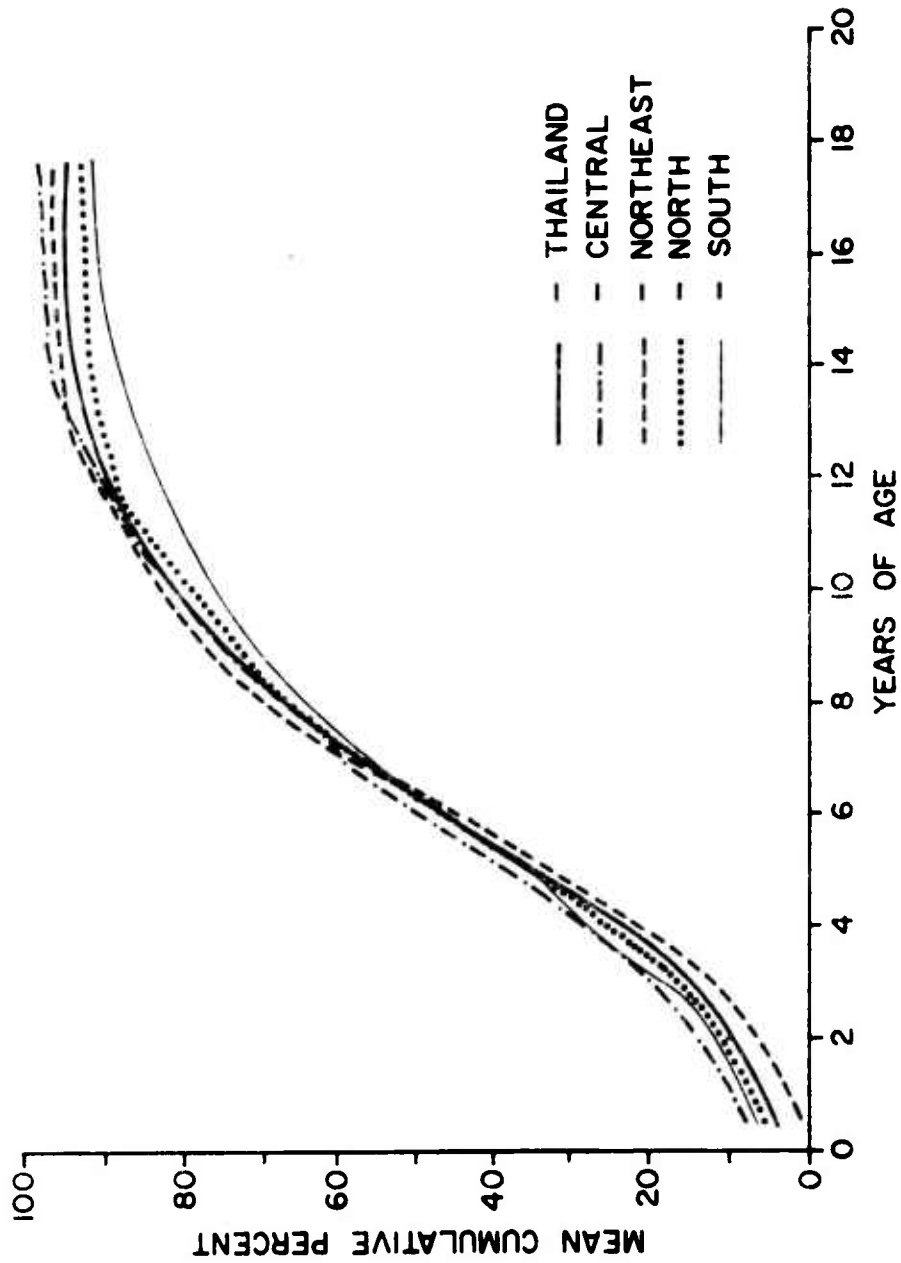


FIGURE 3. AGE DISTRIBUTION OF DHF SPECIFIC CASES (LABORATORY CONFIRMED) IN PATIENTS HOSPITALISED WITH HEMORRHAGIC FEVER IN PROVINCIAL HOSPITALS IN THAILAND, 1975.

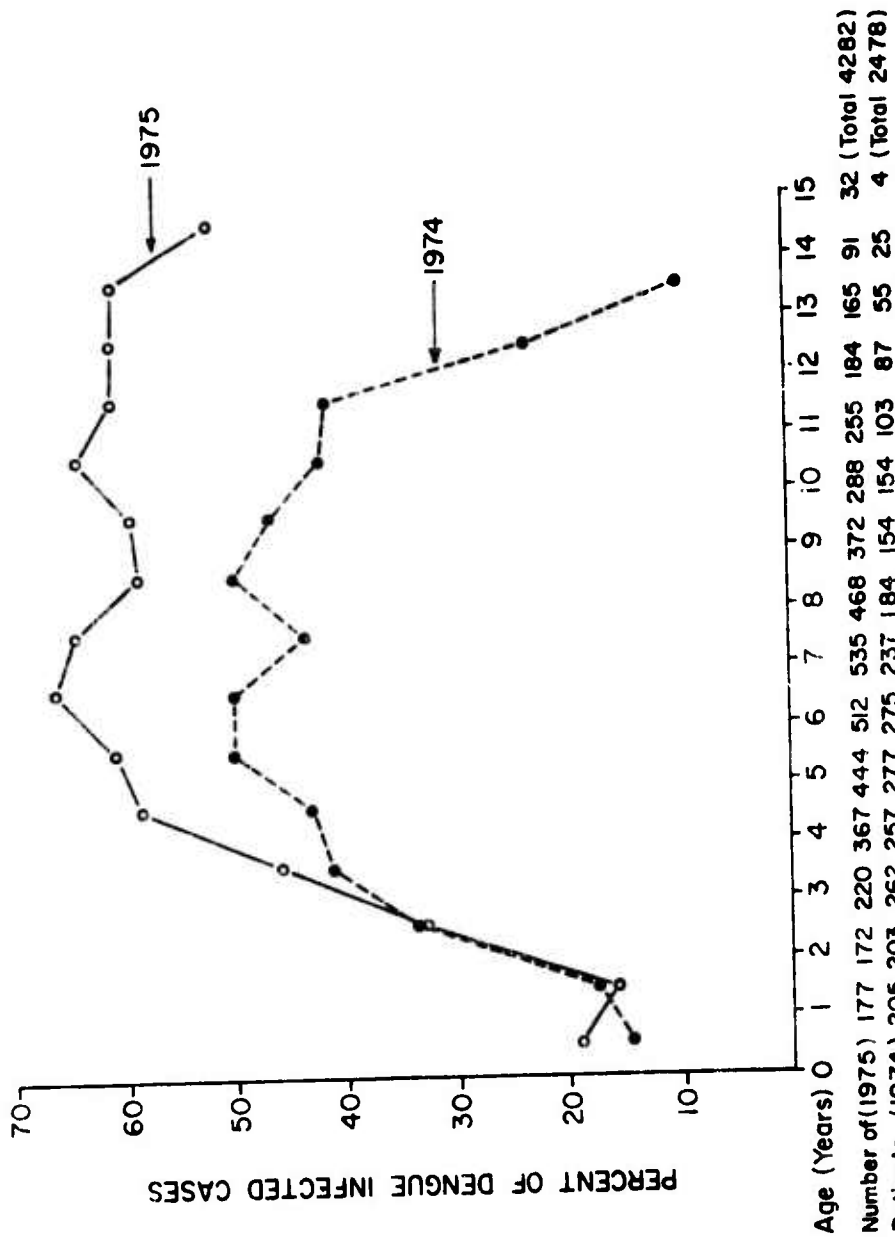


FIGURE 4. DENGUE INFECTED CASE RATIO IN EACH AGE GROUP AMONG CASES SUBMITTED FOR TEST FROM PROVINCIAL HOSPITALS IN THAILAND, 1974 AND 1975.

estimated using these figures and those developed by the laboratory surveillance program. The ratio of confirmed cases to clinically diagnosed cases rose in 1975 compared to 1974 (Table 3).

Table 3. Hemorrhagic Fever, Incidence Rate and DHF Specific Case Rate from Regions of Thailand, 1975

Region	Reported Incidence Case Rate Per 100,000 Age 15 Years	DHF Specific Case Rate Per 100,000 Age 15 Years	DHF Specific Case Rate Per Incidence Rate	
			1974	1975
North	66.3	38.5	1:2.2	1:1.7
Northeast	90.0	47.8	1:2.5	1:1.8
Central	141.0	82.2	1:2.4	1:1.7
South	45.3	23.0	1:2.4	1:1.9
Thailand	90.5	50.4	1:2.4	1:1.7

The methods employed for the laboratory confirmation of dengue infections were applied to the serological confirmation of JE cases. Acute and convalescent bloods were submitted on 349 clinically diagnosed cases of encephalitis seen in all regions of Thailand (Table 4). Forty percent or 129 cases were serologically confirmed as JE infections. The JE infections were found in all four regions of Thailand and occurred in all but the first two months of 1975.

DISCUSSION: A long-term surveillance of infection is one of the prerequisites for decision making in any control program. Such a program for DHF is presently active in Thailand and has confirmed approximately 50% of the reported cases of HF as due to dengue infection. Such a program will allow for the identification of the factors which influence epidemiologic patterns and allow for rapid implementation of control measures.

Table 4. Japanese Encephalitis Infection in Clinical Encephalitis Cases from 4 Regions of Thailand, 1975 (70 Provinces)

Region	Total Case Tested	Number of JE Infection	
		Number	Percent
North	116	27	23
Northeast	137	45	33
Central	92	57	62
South	4	0	0
Thailand	349	129	40

4. Radio-immuno Assays for the Rapid Detection of Dengue Antigens

OBJECTIVE: To develop a system to detect dengue antigen.

BACKGROUND: It has been hypothesized that antigen-antibody complexes form in the blood of Dengue Hemorrhagic Fever (DHF) patients. These are thought to trigger an immunologic mechanism which leads ultimately to the development of shock. This hypothesis is supported by a great deal of indirect evidence. Until recently, however, the technology has not been available to investigate this point directly.

Studies at the Walter Reed Army Institute of Research have indicated that antibody to structural and nonstructural dengue antigens can be measured independently by radio-immune assays (RIA). Using these techniques it may be possible to detect dengue antigens in patient's sera as well as in mosquitoes inoculated with patient's sera and in wild-caught mosquitoes.

DESCRIPTION: Immunoglobulin G was prepared from pooled human sera with high titered dengue antibody and without hepatitis B antigen or antibody. The globulin in pooled serum was precipitated with 50% ammonium sulfate. Adjustment of the pH of an aliquot of saturated $(\text{NH}_4)_2\text{SO}_4$ to about pH 7.8 was done by addition of 2N NaOH just prior to precipitation of the gamma globulin. The precipitate was dissolved in 0.01 M phosphate buffer pH 7.5, and

dialyzed overnight (approximately 18 hours) at 4°C against 0.01 M phosphate buffer, pH 7.5. The dialyzed protein was chromatographed on a diethylaminoethyl-cellulose column (0.9 x 15 cm) equilibrated with 0.01 M phosphate buffer pH 7.5. The first peak (IgG) was concentrated to original volume in an amicon filtration unit over a XM 50 membrane. The IgG preparations were stored at 4°C.

IgG was labeled with ^{125}I by a modification of the method of Hunter and Greenwood (9). The following reagents were added in order to a small beaker: 20 microliters of 0.25 M phosphate buffer, pH 7.3; 200 μCi of high-specific activity ^{125}I (in 1 to 2 microliters of a solution of chloramine T (3.5 $\mu\text{g}/\text{microliter}$); 20 microliters of a solution of sodium metabisulfate (4.8 $\mu\text{g}/\text{microliters}$); and 20 microliters of a solution of sucrose (22.5%), potassium iodide (2 mg/ml), and aqueous phenol red (0.025%). After the addition of chloramine T, the reaction was allowed to proceed for 15 seconds before being terminated by the addition of sodium metabisulfate. The mixture was applied to the top of a column (made from a 5 ml syringe) packed with sephadex G-50 and equilibrated with phosphate buffered saline, pH 7.4. The protein was eluted with the same buffer; fractions containing the first peak of radioactivity were pooled and diluted with an equal volume of calf serum. This stock mixture was stored at 4°C and diluted 1:2 with calf serum just before use.

Polyvinyl U-bottom microtiter plates (Cooke Engineering) served as the solid phase for the radio-immuno assay. The wells were coated with a 1:10 dilution of dengue antibody containing material (convalescent serum or purified IgG) in normal saline solution with 0.1% sodium azide. After four hours incubation at 4°C, the wells were washed and secondarily coated overnight with 1% bovine serum albumin in saline. Following a second wash with normal saline solution 25 microliters of the sample to be tested for the presence of antigen was put into the wells and the plates were incubated at 4°C for approximately 40 hours. After incubation the plates were washed and 50 microliters of ^{125}I labeled IgG were added to the wells. Plates were again incubated at 37°C for 4 to 6 hours, washed and cut apart with scissors. The wells were transferred to individual gamma counting tubes for quantification of residual radioactivity in a gamma spectrometer.

PROGRESS: Preliminary tests of this technique of radioimmune assay with antigen containing samples of 20% dengue 2 infected suckling mouse brain or dengue 2 infected tissue culture fluids failed to demonstrate specific antigens. Two areas of possible failure were identified. Questions of whether the IgG, used to identify the

presence of antigen, was actually labeled by the ^{125}I and/or whether the initial high titer dengue antibody was binding to the plastic plate, are being investigated further.

5. Predisposition to Dengue Hemorrhagic Fever: The Role of Glucose-6-Phosphate Dehydrogenase Deficiency and Abnormal Hemoglobins

BACKGROUND: Erythrocyte glucose 6 phosphate dehydrogenase (G-6-PD) deficiency affects an average of 14% of the Thai population. G-6-PD deficiency has been found more frequently in bacterial infections such as leprosy, typhoid or pneumococcal pneumonia than in non-infected controls (10). Of the viral diseases, hepatitis has been associated with G-6-PD deficiency (11). Both G-6-PD deficiency and dengue hemorrhagic fever occur frequently in Thailand. This report summarizes studies of the relationship of G-6-PD deficiency with the occurrence and the severity of dengue hemorrhagic fever. An additional investigation of the role of abnormal hemoglobins in dengue hemorrhagic fever is also reported.

MATERIALS AND METHODS: Children hospitalized at the Bangkok Children's Hospital (BCH) with laboratory diagnosed dengue infections were studied for G-6-PD deficiency and hemoglobin type. Two milliliters of blood were obtained using Acid Citrate Dextrose (ACD) solution as an anticoagulant. G-6-PD was examined using the methemoglobin technique of Gall (12) by which the genotype of the patient could be determined. Hemoglobin typing was performed using hemoglobin electrophoresis.

RESULTS AND DISCUSSION: Forty-seven patients with serologically proved dengue infections were studied and the frequency of G-6-PD deficiency in these patients was compared to those found in 131 control patients collected in the Well Baby Clinic of the BCH (Table 1).

Abnormal hemoglobins were also studied in 47 dengue infections and were compared to those found in 31 control patients collected in the Well Baby Clinic. (Table 2)

The dengue infections were graded for severity according to criteria previously established by one of us (S.N.).

Neither abnormal hemoglobin nor G-6-PD deficiency appeared to be associated with the severity of dengue infection (Tables 3 and 4).

Table 1. Glucose-6-Phosphate Dehydrogenase Deficiency in Dengue Hemorrhagic Fever Patients and Control Study

Patients	No.	Glucose-6-Phosphate Dehydrogenase Deficiency*	
		No.	Percent
DHF	47	8	17.02
Control**	131	13	9.9

Chi square = 1.6076 p > 0.157

*Includes all individuals, homozygous, hemizygous, or heterozygous for G-6-PD deficiency

**100 of the controls were previously reported by Lampe et al. (11)

Table 2. HB Type in Dengue Hemorrhagic Fever Patients

Patients	No.	HB Type		Percent
		AE	E	
DHF	47	8	-	17.02
Control Study	31	6	-	19.35

Chi square = 0.0594 p > 0.317

Table 3. Glucose 6 Phosphate Dehydrogenase Deficiency and the Severity of Dengue Infections

Dengue Patients	Total	Shock	
		No.	Percent
G-6-PD deficient*	8	6	75
G-6-PD normal	39	23	59
Total	47	29	61.5

Chi square = 0.2025 $p > 0.6$

Table 4. Abnormal Hemoglobin and the Severity of Dengue Infections

Dengue Patients	Total	Shock	
		No.	Percent
HbE trait (AE)	8	3	37.5
Normal Hb (A)	39	26	66.6
Total	47	29	61.7

Chi square = 1.3147 $p > 0.2$

SUMMARY: Forty-seven patients with serologically documented DHF were studied for G-6-PD deficiency and abnormal hemoglobin. The data reported shows no significant relationship between presence or the severity of dengue infections and the presence of either G-6-PD deficiency or abnormal hemoglobins.

B. HEPATITIS B VIRUS

1. Hepatitis B Virus in Bangkok Families. Study Sample and Analysis of Infections

OBJECTIVE: To determine when urban Thai children are first exposed to hepatitis B virus (HBV) and to search for the factors that influence transmission to infants during the first year of life.

BACKGROUND: This is a report of the long-term follow-up of the offspring of mothers who have Hepatitis B surface antigen (HB_sAg), antibody to HB_sAg (anti-HB_s), or are negative for both.

DESCRIPTION: The study design was described earlier (4). To the basic tests for HB_sAg and anti-HB_s was added an immunodiffusion test of serum for e-antigen and antibody (13). Antibody to Hepatitis B core Antigen (anti-HB_c) was determined on selected mothers' sera by radioimmune assay at WRAIR. The family follow-up period was extended to 24 months in order to detect HBV infections of infants during the second year of life.

PROGRESS: From 1 February 1974 to 31 January 1975, there were 2450 live births at Phra Mongkutklao Hospital; 1040 mothers (42.4%) were interviewed and bled. In order to describe the overall population of mothers who delivered babies at Phra Mongkutklao Hospital, all mothers with study numbers ending with 0, 3, 5 and 6 were tested for HB_sAg by counterimmunoelectrophoresis and RIA and anti-HB_s by PHA. The results of these tests were compared to females of similar age from Huay Khwang who were (previously) tested with these methods (14). In general, the prevalence of HB_sAg was similar to that of Huay Khwang but antibody was less frequently found (Table 1).

Study Sample

Although the intention was to follow the infants of 30 mothers with HB_sAg, 30 with anti-HB_s and 60 negatives, this was not possible due to mistaken original classifications and study dropouts. Several mothers who were negative for anti-HB_s by RIAI and PHA were later found to have anti-HB_s at the time of delivery by AUSAB RIA. In addition, of 154 families selected for follow-up, 22 (14%) could not be adequately followed. The final study group consisted of 31 mothers with HB_sAg, 52 with anti-HB_s and 49 negatives. The final distribution was not considered detrimental to the interpretation of the results.

HBV Infections of Infants

During the first 12 months of follow-up, only infants of HB_sAg positive mothers became infected. Of the 31 infants with antigenemic mothers, 15 (48%) developed HB_sAg and/or anti-HB_s by the age of nine months (Figure 1). Two infants had high concentrations

Table 1. Frequency of HB_sAg and Anti-HB_s
in Bangkok Females

Source	Age Group	HB _s Ag			Anti-HB _s		
		No. Tested	RIA + No.	%	No. Tested	PHA + No.	%
PMKH	15-19	96	6	6.3	59	20	33.9
	20-29	282	17	6.0	206	66	32.0
	30-39	23	0	0.0	20	7	35.0
	40-49	1	0	0.0	1	0	0.0
	Total	402	23	5.7	286	93	32.5
Huay Khwang ¹	15-19	54	3	5.6	52	21	40.4
	20-29	71	4	5.6	67	28	41.8
	30-39	53	4	7.5	51	31	60.8
	40-49	56	3	5.4	52	33	63.5
	Total	234	14	6.0	222	113	50.9

¹ Data abstracted from Grossman, et al.
Amer. J. Epidemiol. 101:144-159, 1975

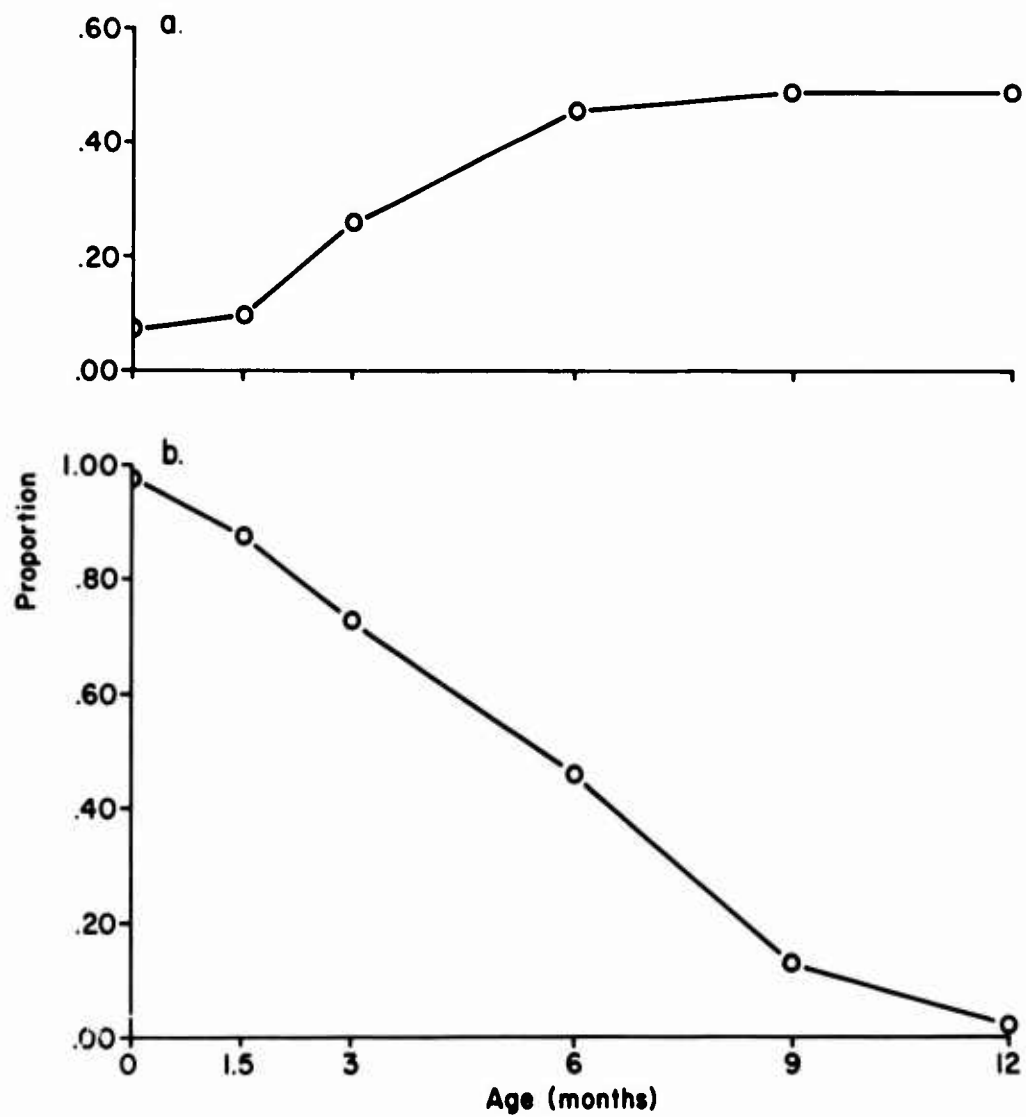


Figure 1. Infants with HB_sAg or anti-HB_s during the first year of life.
 a. 31 Infants of HB_sAg positive mothers.
 b. 52 Infants of anti-HB_s positive mothers.

of HB_sAg in their umbilical cord serum and remained persistently positive thereafter. Seven and five other infants were infected by ages three and six months, respectively. Nine of the infected infants (60%) developed persistent antigenemia. None of the infected infants developed overt signs of acute hepatitis.

Of the 52 infants of mothers with anti-HB_s, all but one had detectable antibody in the cord blood. The frequency of anti-HB_s declined steadily throughout the 12 month period (Figure 1). None of these infants had detectable HB_sAg or had lost and regained anti-HB_s during the first year. Similarly, none of the 49 infants of negative mothers showed evidence of infection with HBV.

At the beginning of the study, the mothers were divided into three groups on the basis of their serological test results. After the period of follow-up, four groups were differentiated: HB_sAg positive mothers with infected infants (15); HB_sAg positive mothers with non-infected infants (16); anti-HB_s positive mothers (52); and negative mothers (49). Analysis of factors influencing HBV transmission was based on these four study groups.

HBV Infections of Other Family Members

HBV infections among the family contacts of the infants included persons infected before, during, and after the birth of the infant (Table 2). Family contacts included anyone living in the home, such as relatives, maids or friends. Persons having no detectable HB_sAg or anti-HB_s at the time of the infant's birth were considered susceptible to infection. The greatest frequency of antigen carriers and fewest susceptible contacts were found in the families of the infants infected.

New infections of family contacts were infrequent and only occurred in four members of two families (Table 3). In one family (No. 97) there were 10 members. The mother had anti-HB_s at delivery and for 12 months; the infant had anti-HB_s until age 39 weeks. At the time of the infant's birth, an uncle was antigenemic and had elevated serum transaminase values. During the following 12 months, a 7 year-old sister and a 2 year-old brother developed anti-HB_s. A 22 year-old sister-in-law developed acute hepatitis with HB_sAg and elevated serum transaminase values.

In another family (No. 89), with five members, the mother and infant were negative for HB_sAg and anti-HB_s. A 3 year-old brother had persistent asymptomatic antigenemia. Another brother seroconverted at the age of 2 years. Although the only infants to be infected in this study had antigenemic mothers, older siblings were sometimes infected when other persons in the family had HB_sAg. Thus, horizontal transmission of HBV occurred occasionally in older siblings.

Table 2. Frequency of HB_sAg and Anti-HB_s in Family Contacts of Study Infants

Mother's Category	No. Families	Family Members					
		No.	Mean	HB _s Ag No. (%)	Anti-HB _s No. (%)	Negative No. (%)	
HB _s Ag-I *	15	42	2.8	25 (60)	15 (36)	2 (4.8)	
HB _s Ag	16	47	2.9	21 (45)	10 (21)	16(34)	
Anti-HB _s	52	166	3.2	21 (13)	99 (60)	46(28)	
Negative	49	157	3.2	4 (2.5)	58 (37)	100(64)	
Total	132	412	3.1	71 (17)	177 (43)	164(40)	

* HB_sAg carrier mothers who transmitted to their infants

Table 3. New HBV Infections in Family Contacts

Family Number	No. Members	Mother's Category	Person Infected (Age)	Time of Followup	HB _s Ag	Anti-HB _s	SGOT* SGPT
97	10	Anti-HB _s	Uncle (35)	At birth	Yes	NF**	305/95
			Sister (7)	28 weeks	No	Yes	39/15
			Brother (2)	56 weeks	No	Yes	29/10
			Sister-in-law (22)	56 weeks	Yes	Yes	402/294
89	5	Negative	Brother (3)	At birth	Yes	No	107/28
			Brother (1)	52 weeks	No	Yes	49/18

* Transaminase values at time seroconversion noted

** NF - not followed

Environmental Factors

The 132 families lived in homes scattered widely throughout the city in a distribution that was more representative of the maternity patients of the hospital than of the total population of Bangkok (Figure 2). Eighty two percent of the families lived in houses, 17% in apartment buildings (Table 4). Seventy one percent of the homes had one or two rooms, 27% had three or four rooms. Houses were usually wooden and had one or two floors and no basements. Apartments tended to be less than five stories high and were made of concrete block construction and wood.

No statistical differences were found between the groups with regard to the use of electricity, bottled gas, charcoal cooking fuel, source of drinking water, storage and preparation of drinking water, the number of indoor toilets and the type of sewage disposal. In addition there were no apparent differences in the method of food preparation, or cleaning of eating utensils. The most typical household description was as follows: The family lived in a house (82%) with less than three floors (71%) that had electricity (61%), bottled gas (31%), and cooked over charcoal (70%). City tap water was used for drinking (83%) and was boiled before consumption (96%). The families had an indoor toilet (92%). The mother prepared the food (81%) and washed vegetables with tap water (77%). The mother washed the dishes (84%) with tap water which was not previously treated (98%).

The mother provided the principal care for the baby in 83% of all families and 93% of the babies that were infected. Nearly all of the mothers (95) indicated an intent to breast feed their babies. Antigenemic mothers were more apt to breast feed for shorter periods and less frequently per day than those without antigenemia (Table 5). By the age of six weeks, most infants received supplemental baby food (51%), liquid formula (60%), and drank boiled water (94%).

None of the characteristics studied of the physical home environment were associated with HBV infections of infants.

Personal Contacts of Infants

Although the families were nearly equal in size, there were striking differences in the frequencies of HB_sAg carriers (Table 6). In families of infants who were infected, 60% of the household contacts were antigenemic. In families where the mother had antigen but the infant was not infected, only 45% of the household contacts had antigen. The difference was due to a larger number of antigenemic siblings in the first group.

BEST AVAILABLE COPY

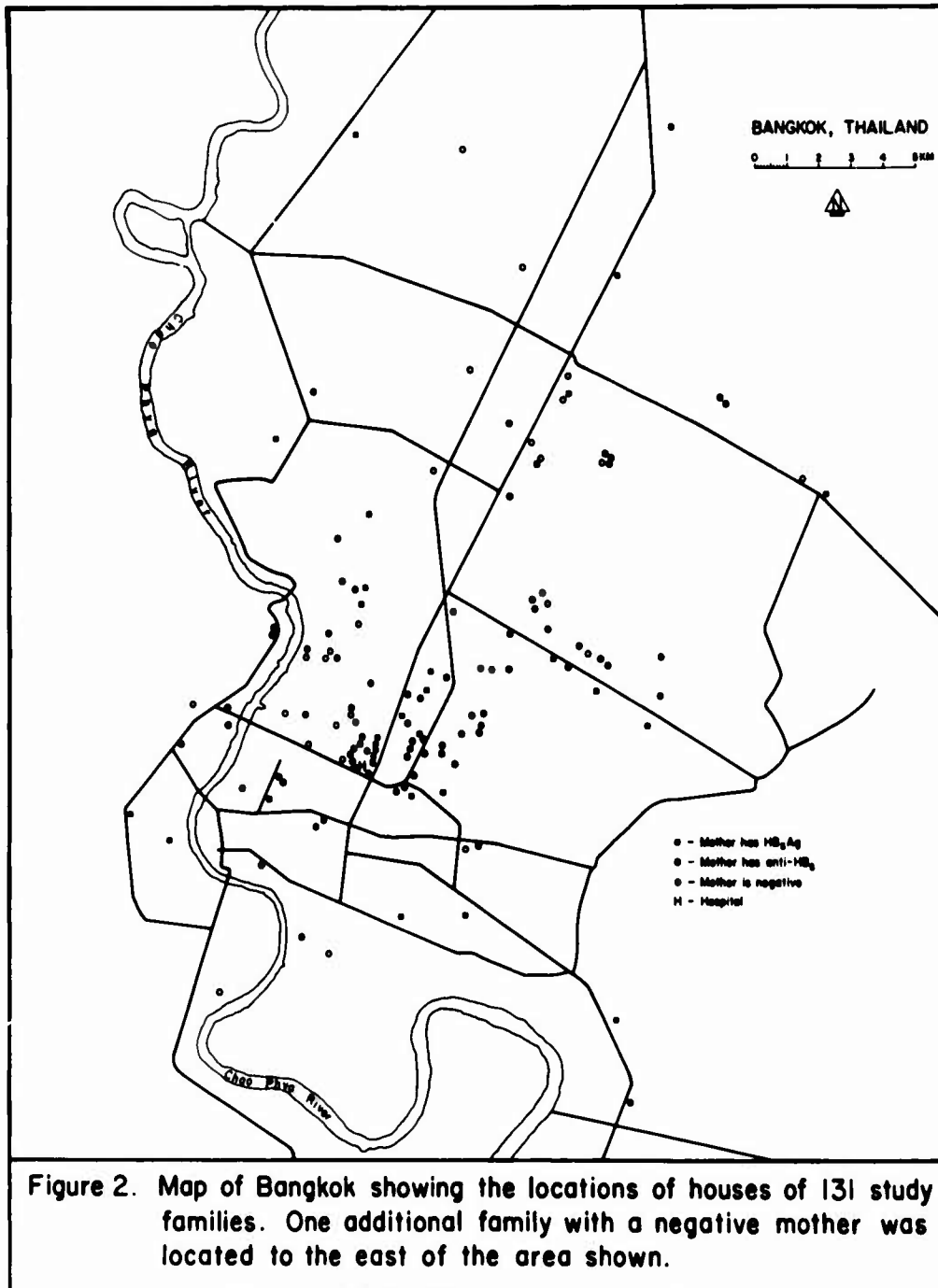


Table 4. Type of Dwelling

Mother's Category	Number of Dwellings	House	Apartment	Shop
HB _s Ag-(I)	15	.87*	.13	.00
HB _s Ag	16	.88	.13	.00
Anti-HB _s	52	.79	.19	.02
negative	49	.82	.16	.02
Combined	132	.82	.17	.02

* Proportion of dwellings

Table 5. Diet of Infants at Age 6 Weeks

Questionnaire	HB _s AG-I	HB _s Ag	Anti-HB _s	Negative
Number of Infants	15	16	52	49
Intent to Breast Feed				
Yes	87%	94%	96%	96%
Duration Breast Feeding (0 or 6 wk)	46%	50%	31%	31%
No. Breast Feedings per day				
1-5	51%	62%	43%	49%
6 or more	47%	38%	58%	52%
No. Formula Feedings per day				
None	27%	25%	37%	29%
1-5	20%	25%	25%	30%
6 or more	46%	50%	38%	40%
Other Baby Food				
Yes	47	32	60	49
No	53	69	40	51

Table 6. Household Contacts of 132 Infants

Mother's Category	No: of Families	Household Contacts		HB _s Ag+ Contacts	
		No.	Mean	No.	(%)
HB _s Ag- (1)	15	42	2.8	25	(60)
HB _s Ag	16	47	2.9	21	(45)
Anti-HB _s	52	165	3.2	21	(13)
Negative	49	157	3.2	4	(3)
Combined	132	411	3.1	71	(17)

Table 7. Frequency of Infant HBV Infections Related to Family Contacts

Family Member with HB _s Ag	Infants at Risk*	Infants Infected	
		No.	(%)
Father	8	2	(25)
Mother	31	15	(48)
Sibling	6	4	(67)
Mother and Sibling	5	4	(80)

* Infants with maternal antibody are excluded

In families of mothers with anti-HB_s, antigen carriers were four times as frequent as in the families of negative mothers. This difference suggests that children born with maternal antibody may be at greater risk of infection after they lose the antibody than children of negative mothers.

The study was based on the assumption that, regarding infection of infants with HBV, the mother was the most important contact for the infant. This assumption was supported by the relative frequency of infant infections when individual relatives were considered (Table 7). The results suggest that an antigenemic mother is more important than an antigenemic father and that antigenemic siblings may indicate the infants at greatest risk.

Mothers with HB_sAg

Sixteen mothers with HB_sAg were tested for anti-HB_c; all were positive (Table 8). In addition, one of 12 negative mothers had anti-HB_c. The infant of the HB_sAg negative mother with anti-HB_c did not become infected.

Twenty-eight mothers with HB_sAg were tested for e-antigen (Table 9). e-Antigen was more frequently found in mothers with infected infants, although two mothers with e-antigen did not have infected infants. The mother's serum CF titer of HB_sAg at the time of delivery was closely associated with infection of the infant (Table 10).

Predictability of HBV Infection in Infants

The following factors are associated with HBV infection during the first nine months of life:

1. Mothers with HB_sAg
2. Mothers with Serum titers of HB_sAg 1:64
3. Mothers with detectable e-antigen
4. HB_sAg positive siblings
5. Concentrations of HB_sAg in the umbilical cord blood detectable by counterimmunoelectrophoresis.

Impact of Infant Infections on the Adult Carrier Population

Any estimate of the contribution of infant infections to the adult carrier population must be based on three assumptions. First,

Table 8. Frequency of Hepatitis B Core Antibody* in Mothers

Mother's Category	No. Tested	Anti-HB _c No.	%
HB _s Ag- (I)	9	9	(100)
HB _s Ag	5	5	(100)
Anti-HB _s	1	0	(0)
Negative	12	1**	(8)

* Tests performed by Dr. G.R. Irwin, WRAIR, Washington, D.C.

** Infant did not become infected

Table 9. Frequency of e Antigen in Mothers with HB_sAg

e Antigen	Mothers	Infected Infants	
	No.	No.	(%)
Positive	8	6	(75)
Negative	20	8	(40)
Total	28	14	(50)

Table 10. Complement Fixation Titers
of HB_sAg Positive Mothers

Serum HB _s Ag CF Titer*	Mothers No.	Infected Infants No. (%)
≤ 1:32	15	2 (13)
≥ 1:64	13	12 (92)
Total	28	14 (50)

* At time of delivery of infant
Chi square = 14.35
p < .001

Table 11. Estimation of the Proportion of Adult HB_sAg Carriers
that are Due to Infection During Infancy

Frequency of HB _s Ag + mothers at PMKH	5.7%
Frequency of HBV infection in infants	48%
Frequency of infected infants becoming chronic carriers	60%
Prevalence of chronic HB _s Ag carriers among children of mothers delivering at PMKH:	
5.7% x .48 x .60	= 1.6%
Proportion of adult HB _s Ag carriers caused by infant infections	
$\frac{1.6\%}{5.7\%}$	= 0.28 = 28%

that over the period of at least one generation (approximately the next 25 years) the factors affecting the transmission of HBV within the Bangkok population will remain constant, so that the prevalence of adult carriers of HB_sAg will remain the same. Secondly, all infants who become chronic carriers will remain antigenemic until they reach child-bearing age. Thirdly, the mortality rates of children and young adults with HB_sAg are the same as for those without antigen.

Using these assumptions and the observations from this study, it is apparent that HBV infections during the first year of life cannot account for more than 28% of the adult carriers (Table 11). Conversely, up to 72% of the adult carriers in Bangkok are due to infections after the age of one year. Infections after the age of one year may be prevented by an effective vaccination program in the future.

SUMMARY: An analysis has been completed of the effect of HBV infections on infants and the factors influencing infection during the first year of life. All of the infected infants were asymptomatic and 60% developed persistent antigenemia. The factors most closely associated with infant infection were: an antigenemic mother; antigenemic siblings; e-antigen in the mother's serum; and a high CF titer of HB_sAg in the mother's serum. Evidence was found of horizontal HBV transmission among members of two families in which neither the mother nor the infant were infected. Only a minority of adult female antigen carriers can be accounted for by infant infections. The family follow-up period has been extended to two years to more clearly determine the frequency of HBV infections in youngsters.

2. Hepatitis B Virus Infection Among Americans Residing in Southeast Asia

OBJECTIVE: To determine the incidence of Hepatitis B virus (HBV) infections in Americans exposed to a population with endemic hepatitis and a high prevalence of Hepatitis B surface antigen (HB_sAg) carriers.

BACKGROUND: Until recently only clinical evidence was available to document infection with agents causing viral hepatitis. Over the past several years, however, the development of new serological tests has allowed for the detection of past infection with HBV. Data has been accumulated on the prevalence of HBV infections in various populations. Epidemiological investigations in Bangkok, Thailand, showed that almost ten percent of an urban Thai population were carriers of HB_sAg and that almost 50% had evidence of HBV infections at some time in the past. Age specific prevalence

of infection with HBV in this population increased until the age of 15 at which point it reached a plateau at approximately 70% (14). Studies of rural Thai populations have shown similar age acquisition curves for HBV infection (15).

In recent years, large numbers of Americans, mostly military personnel, have resided in Southeast Asia. These Americans came from an area in which HB_sAg has been found in only 0.1 to 1.0% of the population and evidence of HBV infection has been found in only five to twenty percent (16, 17).

This study was designed to determine the risk of becoming infected with hepatitis B, to define environmental and behavioral factors which increase the risk of infection, and to determine the ratio of clinically apparent to inapparent infections in a population of American enlisted men residing in Thailand.

DESCRIPTION: A description of the design of this study appeared in the SEATO Medical Research Laboratory Annual Report 1974-1975.

Subjects: Subjects were drawn from service men aged 18-27 years in enlisted grades E1-E5. These men were assigned to either the United States Army Support Group, Thailand or the United States Air Force 635th Combat Support Group. Shortly after arriving in Thailand a questionnaire was administered to volunteers to determine personal, demographic and medical information. During the ensuing year these men were interviewed three times at approximately four month intervals regarding social behavior and medical problems.

Serology: Serum was obtained initially and at the time of each interview. Serum was tested for HB_sAg by a counterimmunoelectrophoresis (CIEP) technique (SEATO Medical Research Laboratory Annual Report 1973-1974) and a commercially available radioimmune assay AUSRIA (Abbott Laboratories) using appropriate absorption procedures (14). CIEP positive sera were subtyped for HB_sAg determinants d, y, w and r, using an immunodiffusion technique (SEATO Medical Research Laboratory Annual Report 1972-1973). Antibody to HB_sAg (anti-HB_s) was detected by a commercially available radioimmune assay (AUSAB, Abbott Laboratories). This test was confirmed by a passive hemagglutination test using red blood cells coated with purified HB_sAg of subtype ad (Electronucleonics Laboratories) or by inhibition of the radioimmune assay by absorption of antibody activity with HB_sAg. All sera from each individual found to have HB_sAg or anti-HB_s at anytime were retested simultaneously. The presence of either HB_sAg or anti-HB_s was taken as evidence of prior infection. Sequential sera from a

sub-set of the men followed for one year in Thailand were submitted to the Walter Reed Army Institute of Research (WRAIR) to be screened for antibody to Hepatitis B core antigen (Anti-HB_c). Screening was done by a Radioimmune assay inhibition test developed at WRAIR.

PROGRESS: Study sample: Subjects were enrolled in this study between April and December 1973. Initial questionnaires were completed in December 1973 and the three follow-up interviews were completed in April, August, and December 1974 respectively. The first interview occurred 12 to 24 weeks, the second 24 to 38 weeks, and the last 39 to 65 weeks after entering Thailand. Individuals who missed interviews or whose interviews fell outside of the established time periods were considered incompletely collected for purposes of this study.

Demographic Data: There were 412 men who completed the initial questionnaire and submitted blood shortly after their arrival in Thailand. Examination of the questionnaires showed that 78% were white, 21% were black, and 1% were oriental. Although the age range was set from 18 through 27 years, over 80% of the men were between the ages of 20 and 24 years. Three quarters of the men had graduated from high school while five percent had completed college. Seventy-three percent of the men were enlisted rank E4 and above, but 12.5% were E2 reflecting the number of recently enlisted personnel.

Approximately 60% of the men were enlisted in the Army, and the remainder were in the Air Force. The Air Force personnel tended to be older; all of the men in grade E2 were in the Army. All of the Air Force and approximately two thirds of the Army were stationed in a large military complex on the east shore of the Gulf of Thailand near the town of Sattahip. The bulk of the remaining Army personnel were located in Bangkok, but a few were stationed at military posts further to the north. Contact with Thai civilians was available to all of these men.

Of the 412 men initially studied, 376 (91%) were seen at the first interview, 340 (83%) at the second, 326 (79%) at the third (Table 1). Some men were lost to follow-up due to curtailment of assignment in Thailand or to temporary duty elsewhere. There were 271 men who were followed at each interview (Table 2).

Prior Infections: Evidence of prior infections with HBV was detected in 31 (7.5%) of the 412 men studied on arrival in Thailand (Table 1). Examination of questionnaires revealed that 156 (38%) of these men

Table 1. Experience with Hepatitis B Infection Among American Enlisted Men Followed at Four Month Intervals for One Year

Observation	Initial	1st Interview	2nd Interview	3rd Interview
Weeks in Thailand	0	13-25	26-39	40-65
Total number studied	413	376	340	326
Prior HBV infection	31(7.5)*	23(7.4)	26(7.6)	21(6.4)
Acquired HBV infection	-	11(2.9)	14(4.1)	20(6.1)
Total HBV infection	31(7.5)	39(10.3)	40(11.8)	41(12.5)

*Percent of total number studied enclosed in parentheses

Table 2. Experience with Hepatitis B Infection Among 271 American Enlisted Men Followed at Four Month Intervals for One Year

Observation	Initial	1st Interview	2nd Interview	3rd Interview
Weeks in Thailand	0	13-25	26-39	40-65
Prior HBV infection	17(6.2)*	17(6.2)	17(6.2)	16(5.8)
Acquired HBV infection				
By interval	-	7(2.5)	5(2.0)	6(2.2)
Cumulative	-	7(2.5)	12(4.5)	18(6.7)
Total HBV infection	17(6.2)	24(8.7)	29(10.7)	34(12.5)

* Percent of total number studied enclosed in parentheses

Table 3. Prevalence of Experience with Hepatitis B Infection
Among 413 American Enlisted Men on Entering Thailand:
The Importance of a Prior Asian Tour

Prior Asian Tour	Prior Hepatitis B Experience		
	Yes	No	Total
Yes	20	136	156
No	11	246	257
Total	31	382	413

Chi square (1 df) = 9.0053 $p > 0.05$

Table 4. Frequencies of HB_sAg, Anti-HB_s and Anti-HB_c in Sera
Collected on Arrival in Thailand from 334 American Enlisted Men

	Anti-HB _c		Total
	Positive	Negative	
HB _s Ag Positive	1	1	2
HB _s Ag Negative Anti-HB _s Positive	15	10	25
Anti-HB _s Negative	3	304	306
Total Studied	19	315	334

reported a previous Asian assignment. HBV infections were significantly associated with experience in Asia (Table 3).

New Infections: There were six men who were hospitalized for icteric hepatitis during their one year in Thailand. If the 412 men initially studied were taken as the largest denominator, the minimum attack rate for clinical hepatitis was 14.6 cases/1000 men/year. Five of the six clinically apparent cases of hepatitis proved on serological testing to be HBV infections. In twenty additional men HBV infections were identified serologically by the development of either HB_sAg and/or anti-HB_s. Although these twenty infections were clinically mild, four men reported non-specific symptoms at approximately the time of infection. Thus, 83.3% of clinically diagnosed viral hepatitis in this group of young men during one year in Thailand proved to be HBV infections. The ratio of clinically inapparent to apparent HBV infections was 4:1, only 20% of HBV infections in this population manifesting themselves clinically.

Development of HB_sAg: HB_sAg was detected in ten individuals at one time or another during the observation period. In two people, HB_sAg was detected in the initial serum. One, a 19 year old white man, had cleared the HB_sAg and developed anti-HB_s by the first interview, four months after his arrival in Thailand. This man had been hospitalized for hepatitis just before coming to Thailand and admitted intravenous drug use prior to becoming ill. The second, a 22 year old black man, carried HB_sAg throughout his tour in Thailand. He denied any prior drug use or clinical hepatitis.

HB_sAg was first detected in eight individuals while they were residing in Thailand. HB_sAg was found in serum of one individual at the first follow-up bleed, three individuals at the second, and four at the third. All four of the men who developed antigen at the first and second follow-up bleed had cleared the antigen by the subsequent bleed and three of the four had developed anti-HB_s.

Subtypes of HB_sAg: Of the ten HB_sAg detected, subtypes were identified in six. Two were from individuals in whom antigen was detected upon arrival in Thailand. In the long term carrier, adw was detected while ayw was found in the man recovering from hepatitis. In four of the eight men who developed HB_sAg while in Thailand, adr was identified.

Antibody to Hepatitis B Core Antigen: Sequential sera from 334 men were submitted for anti-HB_c screening. Anti-HB_c was found in the initial sera of 19 of these men. It was associated with anti-HB_s in 15 individuals. In three sera, anti-HB_c was detected alone; in ten sera only anti-HB_s was found (Table 4).

The two individuals in whom HB_sAg was identified on arrival in Thailand were included in the 334 men tested for anti-HB_c. Anti-HB_c was found only in the initial blood of the 22 year old HB_sAg/adw carrier. In the 19 year old who was convalescing from HBV infection, anti-HB_c had appeared by the end of the first four months at a time when the HB_sAg had been replaced by anti-HB_s.

Among the 334 men screened for anti-HB_c, 24 developed HBV infection while in Thailand as evidenced by the appearance in their serum of HB_sAg and/or anti-HB_s. In two men, transferred to Thailand from Vietnam, anti-HB_c appeared in the initial serum four months prior to the time when anti-HB_s was detected. These men clearly had developed their HBV infections prior to entering Thailand despite the fact that the anti-HB_s was not present at the time of their arrival.

In eight of the remaining 22 men who developed HBV infection, HB_sAg was detected. Anti-HB_c was demonstrated in only three of the eight at the time the antigen was detected. In four of the eight, HB_sAg was first detected in serum collected at the last follow-up interview and no further follow-up could be carried out.

In the four men who could be followed HB_sAg had cleared by the time of the subsequent bleed. Although anti-HB_s was detected in only three of these men anti-HB_c was present in all of them by this time. Seventeen men developed anti-HB_s. Anti-HB_c was found, usually in the same sera, in 12 of them; in five, however, no evidence of anti-HB_c could be found. Once anti-HB_c was detected in an individual's serum, it was found to be present in all subsequent sera tested. Although the identification of anti-HB_c indicated an alteration of the time of infection in a few cases, screening for this antibody did not diagnose any additional infections other than those detected by HB_sAg and/or anti-HB_s.

HBV infections and Behavioral and Environmental Factors: Two hundred and seventy one men were studied four times at the stated intervals during their one year assignment in Thailand. Seventeen (6.3%) had experienced hepatitis B infection prior to their assignment but 18 (6.7%) acquired HBV infections while in Thailand (Table 2). Figures for the acquisition of HB infection in 271 individuals who were completely followed were not statistically different from those obtained from men bled at each interview, suggesting that the acquisition of hepatitis B infection in Thailand was not related to any behavior which led to curtailment of assignment.

Among the 271 men, the incidence of hepatitis B infections was approximately equal for each four month period (Table 2). However, if the population was limited to the 167 men who had no previous Asian experience, a difference in the incidence for each time period could be defined (Table 5). This difference probably reflected the large number of men who had been directly transferred to Thailand from elsewhere in the Far East and who may have been exposed to HBV prior to their arrival in Thailand.

Questionnaire and interview data on these 271 men were examined for patterns of behavior which were associated with the development of HBV infections in Thailand. There was no statistically apparent relationships between the development of HBV infection and age, rank, educational level or marital status. Blacks enlisted in the Army tended to have an increased risk of infection. Two patterns of behavior, which may be related, were statistically associated with HBV infection; residence in the Thai community, off post, and the use of drugs, especially cannabis.

SUMMARY: A group of American enlisted men were followed at four month intervals for a one year period in Thailand. The prevalence of prior HBV infection in these men was 7.5%. HBV infection was significantly associated with prior Asian experience. The incidence of HBV infection among men in this group with no prior Asian experience was 6%, with 90% of infections falling after the sixth month. Residence in the Thai community and to a lesser extent drug use, especially cannabis, significantly increased the risk of HBV infections in these men. Clinical evidence of disease was only found in 20% of those individuals who experienced HBV infections in Thailand.

Table 5. Experience with Hepatitis B Infection Among 167 American Enlisted Men with no Asian Experience Studied at Four Month Intervals for One Year

Observation	Initial	1st Interview	2nd Interview	3rd Interview
Weeks in Thailand	0	13-25	26-39	40-65
Acquired HBV infection Cumulative	-	1(0.6)	3(1.8)	6(3.6)

3. Continuing Studies of Hepatitis B Antigen Carriers
in Residents at Khao Yai National Park

OBJECTIVE: To determine the frequency of exposure to Hepatitis B virus (HBV) in residents at Khao Yai National Park (KYNP).

BACKGROUND: This is a continuation of work which was previously reported (4). The presence of Hepatitis B surface antigen (HB_sAg) and antibody (anti-HB_s) serves as evidence of prior exposure to hepatitis B virus (HBV). This study reports the frequency of both HB_sAg and anti-HB_s in a rural Thai population.

DESCRIPTION: HB_sAg carriers were identified using the counter-immunoelectrophoresis (CIEP) test and radioimmune assay (RIA). Anti-HB_s was identified using a radioimmune assay inhibition test (RIAI) described previously (4).

PROGRESS: Four hundred and eighty four sera from KYNP residents were studied for the presence of HB_sAg and anti-HB_s. The age specific prevalence of HB_sAg (Table 1) was previously reported (4). Additional data on the prevalence of anti-HB_s in this population was illustrated in Table 2. There were no statistically significant differences in the prevalence of anti-HB_s between males and females ($p > 0.317$). When the prevalence of HB_sAg and anti-HB_s carriers were combined (Table 3), however, evidence for HBV infection in males was more frequent than that found in females in almost every age group. This was due to higher prevalence of HB_sAg carriers in males. The differences in the frequency of prior exposure to HBV infection between males and females was statistically significant for total population ($p > .001$). The prevalence of prior infection by HBV was low in children and higher in the older age groups. The age specific prevalence of HBV infections reached a plateau of about 44% over the age of 20 years.

Subtypes were determined on six families in which more than one member was found to carry HB_sAg. There were five families, each with two HB_sAg positive members, in which only HB_sAg/adr was identified. In one family HB_sAg/adw was detected in two antigen positive boys. Anti-HB_s was detected in the parents of these two boys. There were no families in which more than one HB_sAg subtype could be detected.

SUMMARY: A well defined rural Thai population of 484 people, representing approximately 80% of the inhabitants of the KYNP have shown age specific prevalences of prior HBV infections which are similar to those seen in an urban Bangkok population (14) and in an urban Cambodian population (15). This suggests similar transmission of HBV in all three groups.

Table 1. Age Specific Prevalence of HBsAg in Khao Yai National Park Residents

Age (years)	Male			Female			Total		
	No. tested	+ve Ag	%	No. tested	+ve Ag	%	No. tested	+ve Ag	%
0-4	27	1	3.7	35	0	0	62	1	1.6
5-9	24	4	16.6	25	0	0	49	4	8.1
10-14	14	5	35.7	12	0	0	26	5	19.2
15-19	33	4	12.1	18	0	0	51	4	7.8
20-29	129	20	15.5	57	4	7.0	186	24	12.9
30-39	57	7	12.2	18	1	5.6	75	8	10.6
40+	28	3	10.7	7	0	0	35	3	8.6
Total	312	44	14.1	172	5	2.9	484	49	10.1

Table 2. Age Specific Prevalence of Anti-HBs in Khao Yai National Park Residents

Age (years)	Male		Female		Total	
	No. tested	Positive	No. tested	Positive	No. tested	Positive
		No. %		No. %		No. %
0-4	27	7 25.9	35	4 11.4	62	11 17.8
5-9	24	3 12.5	25	4 16.0	49	7 14.3
10-14	14	2 14.3	12	3 25.0	26	5 19.2
15-19	33	8 24.2	18	4 22.2	51	12 23.5
20-29	129	39 30.2	57	19 33.3	186	58 31.2
30-39	57	17 29.8	18	8 44.4	75	25 33.3
40+	28	11 39.3	7	2 28.6	35	13 37.1
Total	312	87 27.9	172	44 25.6	484	131 27.1

Table 3. Frequency of Prior Exposure to HBV in Khao Yai National Park Residents
(Combined HBsAg and Anti-HBs)

Age (years)	Male			Female			Total		
	No. tested	Positive		No. tested	Positive		No. tested	Positive	
		No.	%		No.	%		No.	%
0-4	27	8	29.6	35	4	11.4	62	12	19.3
5-9	24	7	29.1	25	4	16.0	49	11	22.4
10-14	14	7	50.0	12	3	25.0	26	10	38.4
15-19	33	12	36.4	18	4	22.2	51	16	31.4
20-29	129	59	45.7	57	23	40.3	186	82	44.0
30-39	57	24	42.1	18	9	50.0	75	33	44.0
40+	28	14	50.0	7	2	28.5	35	16	45.7
Total	312	131	41.9	172	49	28.5	484	180	37.1

4. Prevalence of Hepatitis B Virus Infections in Residents of Phnom-Penh

OBJECTIVE: To determine the experience with Hepatitis B virus of residents of Phnom-Penh.

DESCRIPTION: This work completes the study of a Phnom-Penh population reported in the SEATO Medical Research Laboratory Annual Report 1974-1975. This study was done in collaboration with the National Blood Transfusion Center and the Institute of Biology, Phnom-Penh, Khmer Republic. In June of 1973, questionnaire information and sera were obtained on residents of Phnom-Penh presenting for polio immunization. Prior to transport to SEATO Medical Research Laboratory sera were stored in Phnom-Penh in a -20°C freezer for approximately one year. Assays were performed for antibodies to several viruses known to be common in the tropics. Sera was screened at a dilution of 1:10 for antibodies against polio types one, two and three using a metabolic inhibition technique with LLC-MK₂ cells (18). Antibody to mosquito-borne togaviruses found in Southeast Asia was detected by a hemagglutination inhibition test using eight hemagglutinating units of antigen; antigens used were chikungunya (Ross) for alphavirus and dengue type two (Hawaii) for flavavirus. Sera with no detectable antibody at a dilution of 1:10 were considered negative. Hepatitis B surface antigen (HB_sAg) and antibody (anti-HB_s) were detected by commercially available radioimmune assays. For HB_sAg the AUSRIA II test (Abbott Laboratories) was used with confirmation by counterimmunoelectrophoresis (CIEP) or appropriate absorption of antigen by anti-HB_s. A radioimmune assay inhibition test (RIAI) using a standardized quantity of HB_sAg was employed for the detection of anti-HB_s. The sensitivity of the RIAI has been shown to be approximately equivalent to the passive hemagglutination test (4).

PROGRESS: Information and serology was completed for all viruses tested on 340 individuals of which 116 were male and 229 were female. Sexes were equally represented in the age group less than ten years old. The preponderance of females in the older age groups was probably due to collection of blood from mothers who brought their children in for immunization.

In the Phnom-Penh population described, the age specific prevalence for antibody to Alpha, Flavavirus, and poliovirus of all three types is presented in Table 1. The prevalence of prior Hepatitis B virus infections, as defined by the sum of those people with HB_sAg and with anti-HB_s, is also shown.

Table 1. Age Specific Prevalences of Viral Infections
in 340 Residents of Phnom-Penh

Age	No. Tested	Togaviruses		Poliovirus			Hepatitis B* Virus
		Alpha	Flava	One	Two	Three	
0-1	44	9 (20.4)	19 (43.2)	10 (22.7)	9 (20.5)	12 (27.3)	8 (18.2)
2-3	37	10 (27.0)	32 (86.5)	21 (56.6)	15 (40.5)	20 (54.1)	5 (13.5)
4-5	60	23 (38.3)	52 (86.7)	38 (60.3)	45 (75.0)	33 (55.0)	13 (21.7)
6-9	40	22 (55.0)	37 (92.5)	24 (60.0)	33 (82.5)	26 (65.0)	12 (30.0)
10-14	48	40 (83.3)	47 (97.9)	41 (85.4)	43 (90.0)	40 (83.3)	17 (35.4)
15-19	30	23 (76.7)	28 (93.3)	22 (73.3)	28 (93.3)	19 (63.3)	16 (53.3)
20-24	42	41 (97.6)	42 (100)	29 (69.0)	37 (88.1)	27 (64.2)	25 (59.5)
25-30	39	37 (94.6)	39 (100)	30 (76.9)	34 (87.2)	20 (51.3)	21 (53.8)
TOTAL	340	205 (60.3)	296 (87.1)	215 (63.0)	244 (71.7)	197 (57.9)	117 (34.4)

* Hepatitis B virus is the sum of those people with HB_sAg and anti-HBs

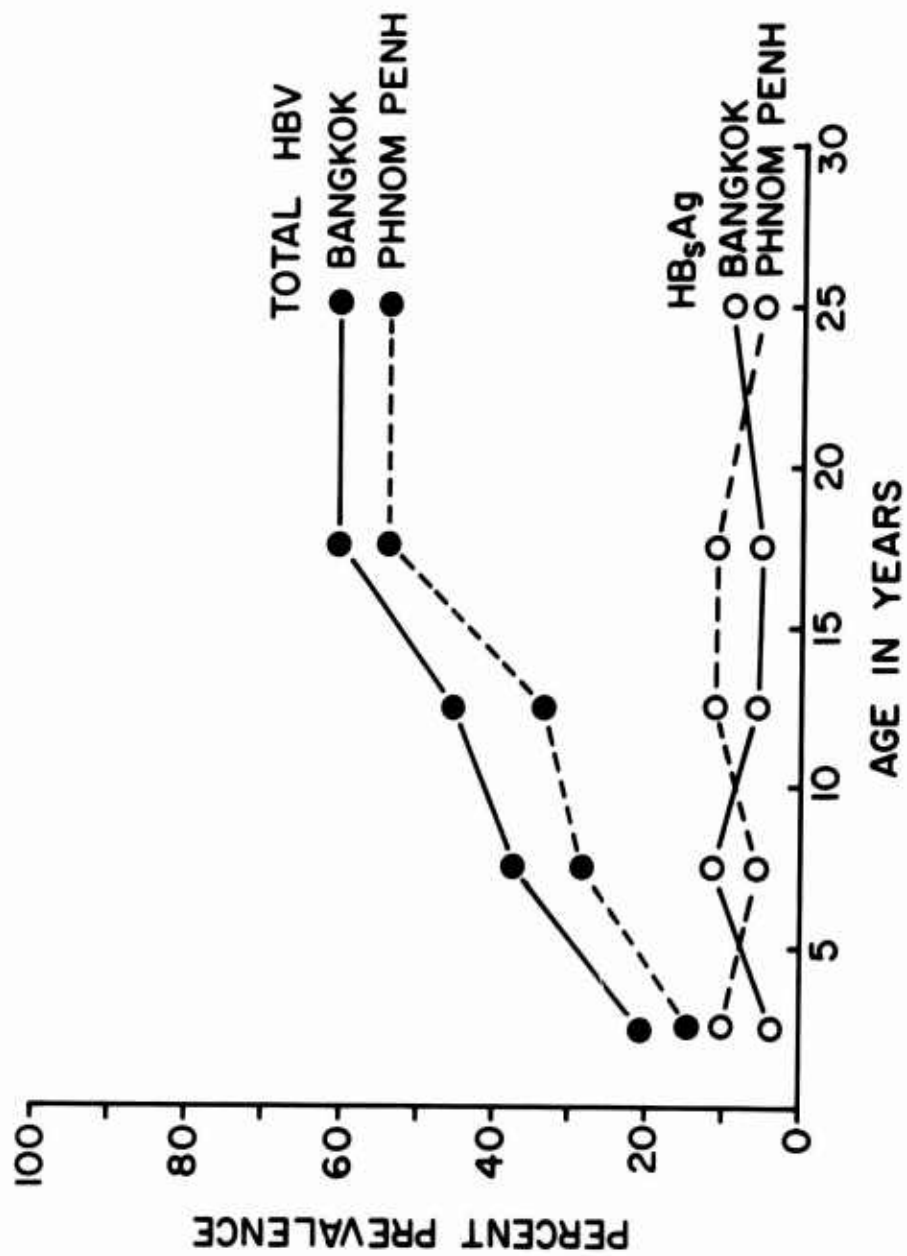


Figure 1. Age Specific Prevalences of Hepatitis B Infection in 697 Residents of Bangkok and 340 Residents of Phnom Penh.

DISCUSSION: As noted in the preliminary report (4), the acquisition of antibody to the three forms of polio, alphaviruses and flavaviruses, is extremely rapid in this population. This is probably due to the generally poor sanitation and high density of mosquitoes in the Phnom-Penh area at the time of the sampling. Development of the HB_sAg carrier state appeared to occur early in life and to be maintained at a relatively constant level through all age groups tested. Anti-HB_s appears to be acquired later; the data indicated that the prevalence of anti-HB_s does not reach a plateau until the age of 15 years. The prevalence of HB_sAg and anti-HB_s among these residents of Phnom-Penh is similar to that reported in a Bangkok population using a FHA test (Figure 1) (4). Similar age specific prevalence of experience with hepatitis B virus in these two cities suggests similar epidemiological conditions in other urban centers in Southeast Asia.

SUMMARY: Among a group of Phnom-Penh residents presenting for polio immunization, the age specific point prevalences of hepatitis B virus infections was similar to those seen in Bangkok, suggesting a similar epidemiology.

5. Transmission of Hepatitis B Virus to Gibbons by Exposure to Saliva Containing Hepatitis B Surface Antigen

OBJECTIVE: To determine if human saliva containing Hepatitis B surface Antigen (HB_sAg) contains infectious Hepatitis B Virus (HBV).

BACKGROUND: The presence of hepatitis B surface antigen (HB_sAg) in the whole mouth saliva of antigenemic people has been reported previously (19). The high prevalence of antigen and antibody to HB_sAg in members of the same family, children in institutions, sexual partners, and hepatitis in dental personnel has raised the possibility that saliva may be a vehicle for the transmission of Hepatitis B Virus (HBV) (20). A human bite has been implicated in the transmission of Hepatitis B virus in one case (21). Since no reports have demonstrated the infectivity of saliva, an attempt was made to transmit HBV by exposing captive gibbons to a pool of human saliva containing HB_sAg.

DESCRIPTION: Saliva Collection. Seven male and four female Thai adults, who were known to have HB_sAg positive sera, were asked to provide fresh saliva specimens. Since HB_sAg is inconstantly present in saliva (19), samples were collected from the donors on more than one day. Collections were made between 1000-1200 hours, at least two hours after eating any food or brushing the teeth. The mouth was rinsed with clear water before starting the collections. Each collection period lasted one to two hours.

Table 1. Frequency of HB_sAg in Two Types of Saliva Preparations

Donor No.	Whole Mouth Saliva				Parotid Secretion			
	No. Spec.	Occult Blood +	HB _s Ag + (RIA)	Mean Vol. (ml)	No. Spec.	Occult Blood +	HB _s Ag + (RIA)	Mean Vol. (ml)
1	2	2	2	12.5	1	1	1	45
2	7	7	6	11.3	2	2	0	10.5
3	2	2	0	14.5	2	1	0	7.5
4	6	6	4	4.3	1	1	0	7.5
5	6	6	4	6.4	2	2	0	3.8
Total	23	23	16		8	7	1	

Table 2. Whole Mouth Saliva Samples from Carriers of HB_sAg/adr Used in the Saliva Pool

Donor	Serum		Saliva Samples			
	HB _s Ag* Titer	e Antigen	Occult Blood Reaction	No. Tested	HB _s Ag +	Volume added to saliva pool (ml)
1. Male 36 years	1:128	+	4+	2	2	62.6
2. Male 36 years	1:32	-	4+ 3+ 2+ 1+	2 2 2 1	2 2 1 1	75.0
3. Female 35 years	1:28	-	4+ 3+ 2+	1 2 3	1 2 2	20.0
4. Male 33 years	1:11	N.T.**	4+ Trace	1 1	1 1	12.2
5. Female 28 years	1:11	+	4+	1	1	1.5
						171.3

* Geometric mean complement fixation titer

** Not tested

Two types of saliva collections were made. The first involved spitting into sterile containers. These samples were considered to be whole mouth saliva, since they included all oral secretions. No sialagogues were used to obtain these samples. The second type of collection utilized a circular hard plastic cup, 2 cm in diameter with a peripheral suction ring and a central drainage port attached to a flexible polyethylene drainage tube. The cup was positioned so as to completely cover the opening of one parotid duct and attached to the buccal mucosa by gentle suction. This type of collection was considered to consist of unilateral parotid secretions. During the collection of parotid secretions, the donor was sometimes permitted to suck on a hard candy in order to promote secretory activity. For both types of collections, the saliva samples were kept chilled in an ice bath. After removing a 1.0 ml aliquot for testing, the remainder of each sample was promptly frozen at -70°C until pooled. Samples were selected for inclusion in a saliva pool on the basis of the HB_sAg test results. All saliva samples were tested for occult blood using paper strips impregnated with a buffered mixture of organic peroxide and orthotolidine (Labstix; Ames Company, Elkhart, Indiana). Multiple serum samples were drawn from each donor in order to determine the geometric mean level of antigenemia during the period of saliva collection.

Preparation of the Saliva Pool

Five people provided samples of whole mouth saliva and parotid secretion; HB_sAg was present in 16/23 (61%) of the former and only 1/8 (12.5%) of the latter (Table 1). The single antigen positive parotid secretion sample had an unusually large volume suggesting the collection cup may have dislodged allowing HB_sAg to enter the sample from the oral cavity. Because of the low antigen yield, parotid secretions were not included in the final saliva pool.

Six of the 11 saliva donors had HB_sAg in one or more samples. Five of the six carried $\text{HB}_s\text{Ag}/\text{adr}$; the other had the adw subtype. In order to maintain uniformity of the antigen subtype, only 18 samples from the adr carriers were used (Table 2). Each sample was thawed quickly, mixed thoroughly together, centrifuged at 10,000 rpm ($12,100 \times g$) for 30 minutes at 4°C , and the supernate used as the saliva pool. The pool appeared visually clear, was positive for occult blood, and gave a reaction for HB_sAg by radio-immune assay only.

The pool was divided into aliquots for convenient use and cultured for bacterial growth. Portions reserved for subcutaneous injection

were treated with penicillin (1,000 units/ml) and streptomycin (1,000 mcg/ml). Bacterial cultures of the untreated saliva grew a few colonies of alpha streptococcus; the saliva containing antibiotics was bacteriologically sterile. All portions were kept frozen at -70°C until used.

Gibbon Management

Non-breeding white-handed gibbons (Hylobates lar) were housed in one wing of a Veterinary Medicine building designed to permit free circulation of air between the animal rooms and the outside area. Temperature and humidity were near ambient. Rooms were double screened to prevent the entry of insect vectors. The gibbons selected for exposure to the saliva were kept in one room apart from the others. Each gibbon was held in an individual, galvanized, metal cage spaced so as to prevent direct contact between animals. Animals were fed a commercial primate diet supplemented with fruits and vegetables. Each had an individual food and water supply. Soiled bedding from the cages was incinerated daily. Only veterinary personnel who had no HB_sAg were permitted to care for the study gibbons. All personnel wore clean protective outer clothing, face masks, and gloves when working with the animals.

Study Gibbons

Ten gibbons with no detectable HB_sAg or anti-HB_s were selected for exposure to the saliva pool. They included five males and five females that ranged in age from 1 to 11 years. Six were born in the SEATO Medical Research Laboratory gibbon colony; the remainder had lived there for 8 to 10 years. One other gibbon (B-66S) had anti-HB_s and was followed as a control for the methods of antibody detection. No studies of the transmission of viral hepatitis had ever been done in the gibbon colony before.

Exposure to the Saliva Pool

Ten gibbons were exposed to the saliva pool. Two gibbons (Pc-13, Pc-14) received subcutaneous injections of 1.7 ml of saliva every other day for three doses (Table 3). Eight other gibbons were divided into pairs and exposed to 1.0 ml of saliva on each of five successive days by either an aerosol spray of the nose; aerosol spray of the mouth; brushing the teeth with the saliva; or ingesting saliva injected into a banana. Each animal received a total of 5.0 or 5.1 ml of saliva pool over the same five day period. It may be assumed that a large portion of the saliva administered by the oral and nasal routes was swallowed. The antibody positive control gibbon (B-66S) was not exposed to the saliva.

Table 3. Method of Exposure of Gibbons to Human Saliva Pool

Saliva Pool		Gibbon		
Route of Exposure*	Total Dose (ml)	No.	Age (yr)	Sex
Subcutaneous	5.1	Pc-13	2	F
		Pc-14	2	M
Nasal Aerosol	5.0	Pc-20	2	M
		S-81	11	F
Oral Aerosol	5.0	Pc-21	1	F
		S-83	8	M
Toothbrush	5.0	Pc-16	1	F
		P-16	9	M
Banana	5.0	P-24	1	F
		B-40	9	M

* The subcutaneous doses were given on Days 0, 2 and 4. All other methods of exposure were used on Days 0 to 4 inclusive.

Method of Follow-up

The first day of exposure to the saliva pool was designated Day 0. On every subsequent day each animal was observed for altered behavior and the rectal temperature was recorded. Once a week, each gibbon was weighed and examined by a veterinarian. Following sedation with a rapidly acting intramuscular anesthetic (phencyclidine hydrochloride or ketamine hydrochloride), a blood sample was drawn for a complete blood count, serum transaminase levels (SGOT, SGPT), and hepatitis B serological tests.

Detection of HB_sAg, Anti-HB_s and e-Antigen

All serum and saliva samples were tested for HB_sAg by solid phase radioimmune assay (AUSRIA II, Abbott Laboratories, Inc., North Chicago, Illinois) without preliminary treatment. Positive reactions were confirmed by a 50% or more reduction of test serum reactivity after incubation with a human serum containing anti-HB_s. Serum antigen titers were determined by complement fixation. HB_sAg subtypes were detected by immunodiffusion using subtype-specific rabbit antisera. e-Antigen was detected in serum by immunodiffusion using serum from a Thai blood donor as antibody.

Anti-HB_s was detected by a solid phase radioimmune assay (AUSAB, Abbott Laboratories, Inc.) interpreted according to the manufacturer's recommendations. Positive reactions were confirmed whenever possible by a 50% or more reduction in reactivity after incubating the test sera with human serum containing HB_sAg/adr. Antibody titers were determined by passive hemagglutination (Electronucleonics Laboratory Inc., Bethesda, Maryland) using erythrocytes coated with HB_sAg/ad.

Immune Electron Microscopy

Dane particles were sought in the saliva pool by immune electron microscopy. First, 6.0 ml of the saliva pool were centrifuged at 19,000 rpm (43,500 x g) in a Sorvall RC2-B centrifuge with an SS-34 rotor for 3 hours at 4°C. As a control for the saliva preparation, human plasma containing HB_sAg/adr was diluted 1:400 in saliva collected from a donor without antigenemia and centrifuged in an identical manner. Second, 5.0 ml of each supernate were centrifuged in a Beckman L350 ultracentrifuge using an SW 39L rotor at 35,000 rpm (approximately 100,000 x g) for 16 hours at 4°C. The top 4.85 ml of supernate was removed with a pipette. The yellow brown sediment was easily resuspended in the remaining 0.15 ml of supernate to give a 33X concentration of antigen.

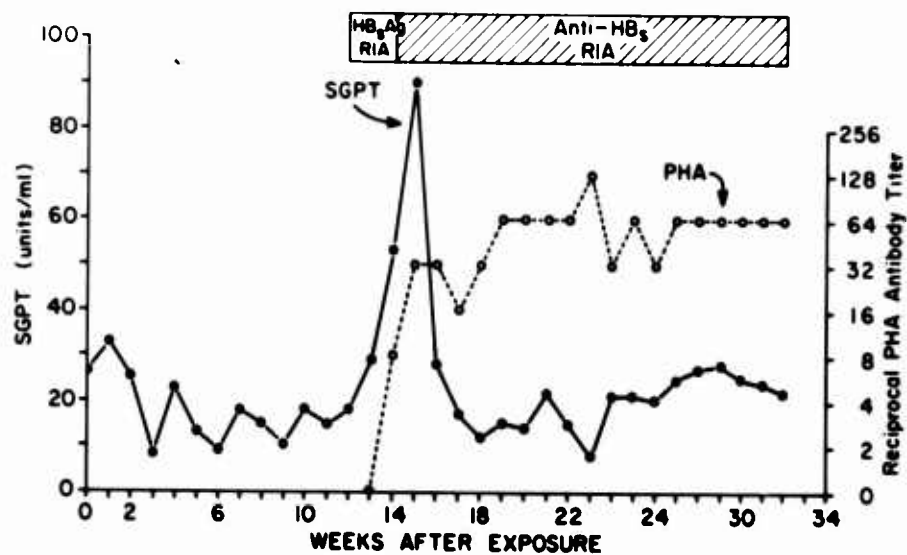


Figure 1 Response of gibbon Pc-13 to the subcutaneous injection of a pool of human saliva containing HB_sAg. Antigenemia (HB_sAg) was followed sequentially by hypertransaminosemia and the appearance of antibody detected by passive hemagglutination (PHA) and radioimmune assay (RIA).

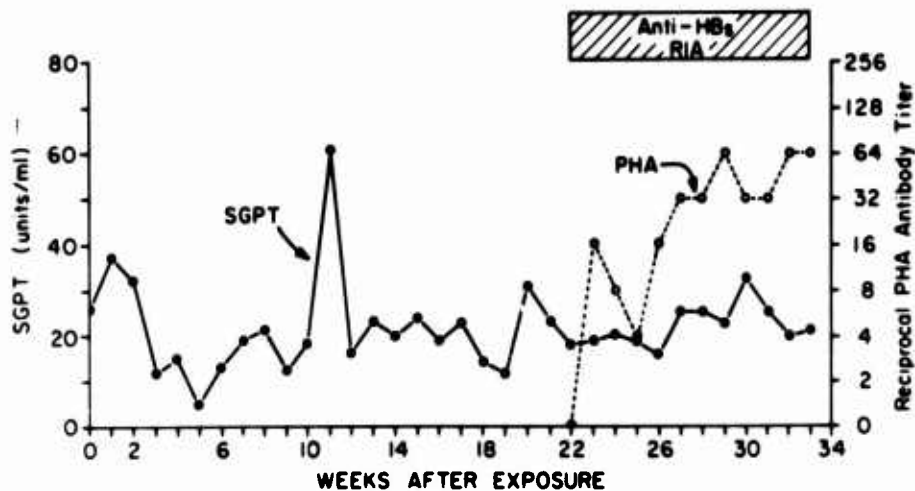


Figure 2 Response of gibbon Pc-14 to subcutaneous inoculation of a pool of human saliva containing HB_sAg at 0 weeks. Hypertransaminosemia at 11 weeks immediately followed a surgical procedure on one hand. Anti-HB_s was first detected by radioimmune assay (RIA) at 22 weeks. HB_sAg was not detected.

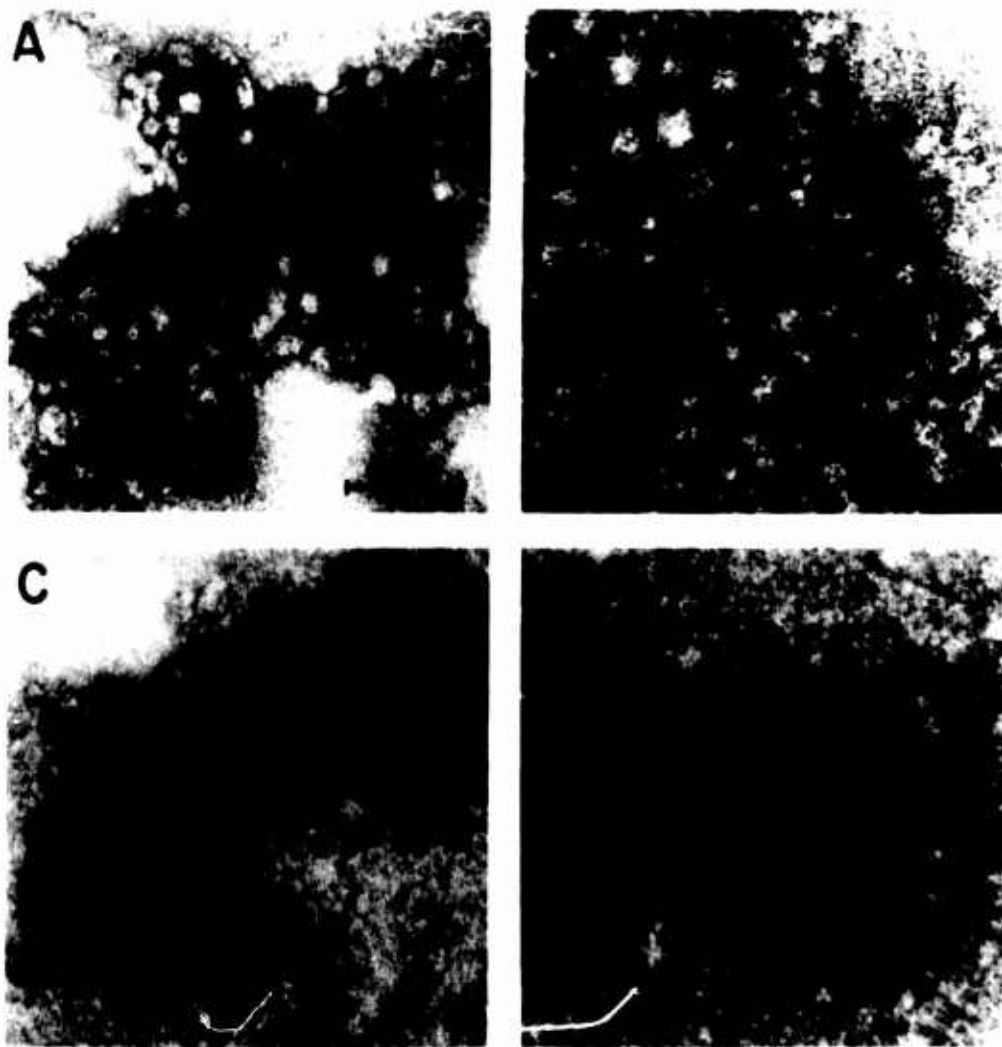


Figure 3. Electron micrographs of clumps of HB_sAg particles derived from human plasma and saliva. A; partially purified HB_sAg/adr (EHO17) with normal rabbit serum. C; EHO17 with rabbit anti-HB_s. B, D; similar small and large particles from the human saliva pool precipitated with rabbit anti-HB_s. Filamentous forms were not found in the saliva. The bar represents 100 nm (X 192,000).

As a third step, each saliva concentrate was divided in two and 0.07 ml mixed with either 0.1 ml of a 1:50 dilution of rabbit anti-HB_s/adr or an equal dilution of serum from the same rabbit obtained before it was immunized. The rabbit antiserum was absorbed to excess with normal human serum. In addition, separate preparations were made of a partially purified HB_sAg/adr in cesium chloride which had previously been shown to contain small particles, filaments and Dane particles. Each sample was incubated at 25°C for one hour, then at 4°C overnight.

The fourth step involved centrifugation of each sample at 17,000 rpm in the Sorvall RC2-B for 90 minutes (22). The entire supernate was removed with a pipette and each sediment resuspended in three drops of distilled water. One drop of each sample was placed on a collodion-carbon coated copper 200 mesh grid and stained with 2% uranyl acetate before it was examined under a Hitachi HU-11C electron microscope.

RESULTS: HBV Infections in Exposed Gibbons. On 3 September 1975 12 weeks after the first inoculation of the saliva pool, gibbon Pc-13 was positive for HB_sAg (Figure 1). This gibbon had detectable antigenemia for the next two weeks accompanied by a distinct rise in the SGPT values. Anti-HB_s was detected in all subsequent sera. During the period of antigenemia, Pc-13 had a normal temperature, normal eating behavior, and no change in weight. Jaundice, hepatomegaly, lymphadenopathy, skin rash, and other abnormal physical findings were not found. As soon as antigenemia was detected, this gibbon and the other that was inoculated (Pc-14) were transferred to an empty room to reduce any possibility of inadvertent exposure of the remaining eight study animals.

Gibbon Pc-14 remained well until 10 weeks and 4 days when he escaped from his cage and was bitten severely on the hand by gibbon P-16. Following a surgical repair, during which one finger was amputated, the SGPT value rose to 61 units and the SGOT to 45 units (Figure 2). Both values were at normal levels one week later. At 22 weeks, the serum first contained anti-HB_s by radio-immune assay. The following week the PHA titer of anti-HB_sAg was 1:32. HB_sAg was not detected in any serum specimen. The incubation period is assumed to be 22 weeks, although it could have been as short as 11 weeks based on the SGPT. It is not possible to be certain whether or not the rise in transaminase was entirely due to the traumatic injury. Since gibbon P-16 showed no evidence of HBV infection, there is no reason to suspect infection of Pc-14 resulted from the bite. None of the gibbons exposed to the saliva by the oral or nasal routes developed HB_sAg or anti-HB_s at any time.

HBV Infections in Unexposed Gibbons

Blood samples collected from gibbons living in the colony between March 1973 and December 1975 permitted tests of 55 animals. While some animals left the colony for a variety of reasons during this period, others were born into it. Two gibbons were found to be chronic carriers of HB_sAg in March 1973; only one of these was still present during the experimental period. Throughout the 34 month observation period, none of the unexposed gibbons developed HB_sAg or anti-HB_s. Gibbon B-66S, the antibody control, was consistently positive for anti-HB_s.

Immune Electron Microscopy

The partially purified HB_sAg/adr (EH017) was found to contain some clusters of particles even without the addition of rabbit anti-HB_s. In the presence of antiserum, particle clusters were more abundant. The mean dimensions of the principal morphological forms were: small spheres, 21.6 ± 3.6 nm diameter; filaments up to 177 nm long; and large spheres, 36.1 nm (Figure 3). Small particles similar to those in EH017 were seen in the normal saliva containing HB_sAg/adr serum only after rabbit antiserum was added.

The saliva pool contained no clusters of particles in the absence of antiserum. The addition of specific antibody, however, precipitated many clusters which included small particles with a mean diameter of 23.3 ± 2.7 nm and large spheres averaging 41.8 nm in diameter (Figure 3). Filamentous forms were not found in the saliva pool.

DISCUSSION: It is certain gibbon Pc-13 and Pc-14 were infected with Hepatitis B virus following an exposure to the pool of human saliva. Because of the precautions taken to avoid other exposures to HBV, it is highly unlikely they were infected in any other way. During the study period, one other gibbon and four animal technicians were known to carry HB_sAg. All five antigen carriers were kept separated from the study gibbons. Throughout 34 months of observations, none of the other gibbons developed serologic evidence of infection, whether or not they were included in this experiment. The detection of large particles in the saliva pool, similar in size to Dane particles (23), adds additional weight to the probability that the saliva was the source of the infection.

Why didn't the eight gibbons exposed by the oral and nasal routes become infected? It is probable the concentration of infectious material was too low. It can be assumed that each animal swallowed some of the saliva. Drugman (24) demonstrated that MS-2 serum was highly infectious by mouth. The MS-2 serum contained a concentration of HB_sAg detectable by immunodiffusion. The saliva

pool used in this study had antigen detectable only by radioimmune assay. The relative insensitivity of immunodiffusion compared to radioimmune assay suggests the MS-2 serum contained a much higher titer of HB_sAg than the saliva and probably more infectious particles as well.

The incubation periods of 12 and 22 weeks observed for the gibbons were similar to the ranges reported for chimpanzees, 2 to 15 weeks; rhesus monkeys, 12 to 15 weeks; and human children, 4 to 15 weeks. The presence of detectable HB_sAg in saliva correlated in general with high serum titers of antigen and the concentration of occult blood. Exceptions to this generalization were observed, however. Perhaps the presence of 3-antigen in the donor's blood is also important for selecting infectious saliva donors.

SUMMARY: A pool of whole mouth saliva collected from five human carriers of HB_sAg/adr, was found to contain antigen particles with mean diameters of 23.3 and 41.8 nm by immune electron microscopy. Two gibbons received subcutaneous injections of the pool and developed serological and, in at least one animal, biochemical evidence of Hepatitis B virus infection at 12 and 22 weeks, respectively. Although none of eight other gibbons that were exposed by the nasal or oral routes were infected, the experiment demonstrated that human saliva can serve as a vehicle for the transmission of hepatitis B virus.

6. The Prevalence of Hepatitis B with e-Antigen and Antibody in Thai Blood Donors

OBJECTIVE:

1. To determine the prevalence of e-antigen and antibody in Thai blood donors.
2. To develop and test methods for detection of the e-antigen and antibody (anti-e).

BACKGROUND: Hepatitis B Virus (HBV) has now been associated with three apparently independent antigens; hepatitis B surface antigens, (HB_sAg), hepatitis B core antigen (HB_cAg) and e-antigen. HB_sAg is present on the surface of the putative virus; the 40 nm "Dane" particle. It also appears to be produced in over-abundance in the form of particles and rods, 28 nm in diameter. HB_cAg is associated only with the Dane particle and is found only in the nuclear capsid of the particle. Recently a third antigen, the e-antigen has been described. This antigen is associated with the intact Dane particle and appears to be a marker of infectivity (25). Antibody has been identified against each of

these antigens. This reports a study of e-antigen and antibody (anti-e) in HB_sAg positive Thai blood donors and also describes the preliminary testing of a counterimmunoelectrophoresis test (CIEP) for the e-antigen and antibody.

DESCRIPTION: HB_sAg positive sera, collected from Thai blood donors were examined for the presence of e-antigen and antibody using a routine immunodiffusion test (ID). The ID test used a micro Ouchterlony technique with two-seven well patterns punched in 0.8% agarose gel diluted in a buffer pH 7.6 coating microscope slide. Reference human serum containing anti-e (EH-421) was placed in the central well of the left pattern and in the top well of the right pattern. Reference human serum containing e-antigen (Donor No. H. 50483) was placed in the central well of the right pattern and the top well of the left pattern. Serum to be tested were placed in coordinate wells of both patterns. The loaded slides were placed in a moist chamber, incubated at room temperature for 48 hours and observed for the presence of precipitin lines between test and reference sera. A counterimmunoelectrophoresis test (CIEP) for detection of e-antigen and anti-e was developed. The conditions of the test were similar to that used for detection of HB_sAg (26). A reference sera, donor-H-50483 was used for e-antigen, and EH-421 was used for anti-e. Appropriate controls were included in each test.

PROGRESS: Table 1 illustrates the prevalence of e-antigen and anti-e among HB_sAg positive blood donors. e-Antigen was found in 14% of 105 HB_sAg positive Thai blood donors. Of the 105 donors 87.6% were HB_sAg/adr and 12.4% were HB_sAg/adw. The prevalence of e-antigen was the same in each subtype. Anti-e was found only in HB_sAg/adr positive sera, however, the number of HB_sAg/adw positive individuals was small.

Comparisons were made of the ID and the CIEP test for the detection of e-antigen and anti-e. A 12% (3/25) increase in the detection of e-antigen and a 42% (3/7) increase in detection of anti-e were found using the CIEP test (Table 2 and 3). The CIEP also detected anti-e in sera negative for HB_sAg.

DISCUSSION: The e-antigen has been detected in the sera of Thai blood donors using a reference serum (EH-421) containing anti-e. Anti-e also has been detected in Thai blood donors using sera containing e-antigen; detected in Thailand. A selection of those sera in which e-antigen and anti-e were detected have been sent to Dr. George L. Le Bouvier, Yale University for confirmation (Table 4). The e-antigen and anti-e were detected using both an ID and a CIEP technique. In preliminary testing the CIEP technique appeared to be more sensitive than the ID.

Table 1. Prevalence of e-Antigen and Antibody Among HB_sAg Carrier Blood Donors (Immunodiffusion Test)

HB _s Ag Subtype	e-Ag			Anti-e		
	No. tested	Positive		No. tested	Positive	
		No.	%		No.	%
adr	92	13	14	83	4	5
adw	13	2	15	10	0	0
Total	105	15	14	93	4	4

Table 2. The Comparison of e-Ag Tested by ID and CEP Methods

HB _s Ag (IEOP)	No. tested	Test for e-Antigen			
		ID		CEP	
		No. positive	Percent	No. positive	Percent
Positive	51	22	43	25	49
Negative	25	0	0	0	0
Total	76	22	28	25	32

Table 3. The Comparison of Anti-e Tested by ID and CEP Methods

HB _s Ag	No. tested	Test for Anti-e			
		ID		CEP	
		No. positive	Percent	No. positive	Percent
Positive	28	4	14	4	14
Negative	25	0	0	3	12
Total	53	4	14	7	13

Table 4. Red Cross Blood Donors Sera Containing
e-Antigen and Anti-e

e-Antigen	H. 50475	*H. 50685
	H. 50480	*H. 50720
	H. 50482	*H. 50771
	H. 50483	*H. 50772
	H. 50485	*H. 50773
	H. 50624	*H. 50778
	*H. 50636	*H. 50826
	H. 50662	
Anti-e	H. 47960	*H. 50655
	H. 47994	*H. 50664

* Sent to Dr. George L. Le Bouvier for confirmation.

A similar prevalence of e-antigen was found among blood donors carrying either HB_sAg/adr or HB_sAg/adw. This suggested that e-antigen is not subtype specific. Since e-antigen has been suggested as a marker for hepatitis B infection, further studies of this antigen will be carried out.

SUMMARY: The e-antigen and anti-e has been detected in Thai blood donors. A CIEP test has been developed which appears to increase the sensitivity of detection. Further studies are planned to determine the usefulness of e-antigen as an indicator of infectivity of hepatitis B virus.

7. Radioimmune Assays for Antibody to Hepatitis B Surface Antigen

OBJECTIVE: To compare three assays for antibody to Hepatitis B surface antigen (Anti-HB_s): A direct radioimmuno assay, a passive hemagglutination test and a radioimmuno assay inhibition test. To develop a systematic method for screening sera for hepatitis B surface antigen (HB_sAg) and anti-HB_s.

BACKGROUND: The radioimmune assay inhibition test (RIAI, based on the AUSRIA II, Abbott Laboratories) and the passive hemagglutination test (PHA, Electronucleonics, Inc.) for anti-HB_s were shown to have similar sensitivity for the detection of antibody to hepatitis B surface antigen. (SEATO Medical Research Laboratory Annual Report 1974-1975). These tests, as well as a counterimmunoelectrophoresis (CIEP) technique have been used routinely at the SEATO Medical Research Laboratory for the past three years. In 1976 a new direct radioimmune assay for anti-HB_s (AUSAB, Abbott Laboratories), became commercially available. This necessitated an evaluation of the new test and the development of a new system for screening serum.

DESCRIPTION: Comparative tests of the CIEP, RIAI, PHA, And AUSAB were carried out. The PHA and AUSAB were performed using the instructions of the manufacturer and the CIEP and RIAI followed the method described previously (SEATO Medical Research Laboratory Annual Report 1971-1972 and 1974-1975). Appropriate controls were included for each test. In cases where one test was not confirmed by another, the presence of anti-HB_s was substantiated by neutralization with HB_sAg.

PROGRESS: A panel of 100 sera from a population of Thai females collected in 1973 and known to have a high prevalence of anti-HB_s were tested by the four methods. Of the 100 sera tested, antibody was detected in 66 by AUSAB, 37 by RIAI and 34 by PHA. Twenty-six

Table 1. Comparison of Four Tests for Anti-HB_s
Results of 100 Sera

Test	Pattern of Positive Results						Sera positive for each test
AUSAB	X	X	X	X		X	66
RIA-I	X	X	X			X	36
PHA	X	X			X		34
IEOP	X						11
Total sera positive by tests indicated	11	19	6	4	1*	26*	

* Neutralized with HB_sAg

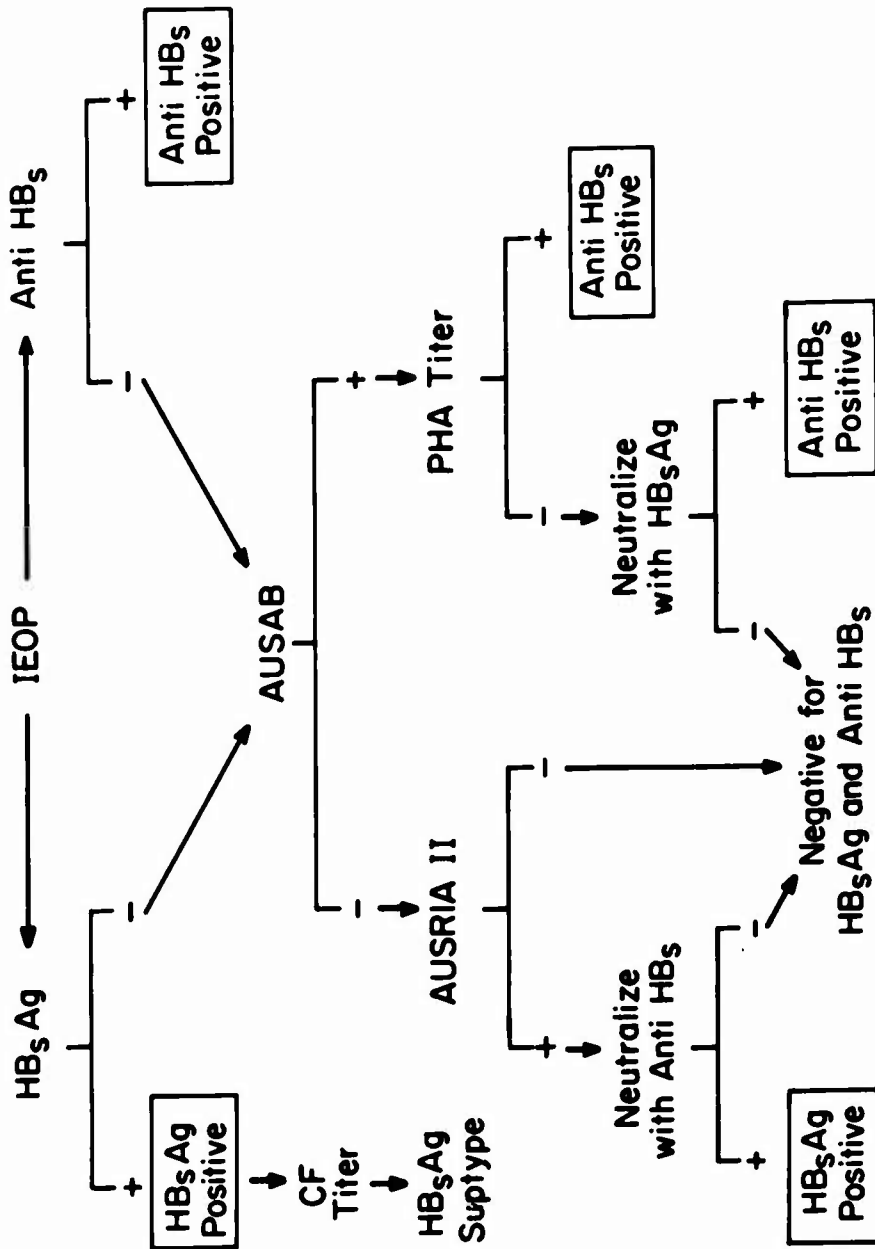


FIGURE 1. TEST SEQUENCE FOR HEPATITIS SERUM

sera were detected by AUSAB only; almost twice as many as were detected by the RIAI (Table 1).

The marked increase in sensitivity without loss of specificity or reproducibility, shown by the AUSAB over previously performed antibody detection methods led to the abandonment of the RIAI and the PHA for routine antibody screening. Because of this a new protocol for HBV testing had to be designed.

The laboratory now submits all sera to a testing sequence designed to identify HB_sAg and anti-HB_s by the most efficient but inexpensive method possible (Figure 1). Sera is initially screened for HB_sAg and anti-HB_s by CIEP. The CIEP was shown in previous studies to identify over 80% of HB_sAg containing sera and up to 10% of anti-HB_s containing sera (SEATO Medical Research Laboratory Annual Report 1972-1973 and 1974-1975). Sera that are negative for HB_sAg and anti-HB_s by IEOP are tested by AUSAB. AUSAB positives are confirmed and titrated by PHA or, if negative by PHA, they are substantiated by neutralization with HB_sAg. A serum is considered neutralized if the counts per minute are less than or equal to 50% of the counts per minute of non-neutralized serum. Those rare sera in which neutralization does occur are called false positives, and are considered negative. The sera which are negative by AUSAB are tested by RIA (AUSRIA II) for HB_sAg. Sera found to be positive for HB_sAg by this technique are neutralized by absorption with anti-HB_s and are considered negative for HB_sAg if no neutralization takes place.

8. The Frequencies of Hepatitis B Antigen Subtypes in Various Parts of Thailand

OBJECTIVE: To determine the relative frequency of hepatitis B surface antigen (HB_sAg) subtypes in the different regions of Thailand.

BACKGROUND: On the surface of the hepatitis B antigen particle there are at least five different antigenic determinants; the common determinant a, and two pairs of usually mutually exclusive determinants d/y and w/r. In Asia the y determinant is uncommon and the majority of antigens are subtypes adr (HB_sAg/adr).

A gradient of increasing frequency from north to south of the r subtype has been reported in Japan (27). This observation suggested that similar geographic gradients might be found in other Asian nations and an investigation was undertaken in Thailand of the prevalence of HB_sAg subtypes in different regions.

DESCRIPTION: Available information on the HB_sAg subtypes in Thailand indicates some differences in subtype frequency between samples collected from Bangkok, the northern part of the country (Chiang Mai)

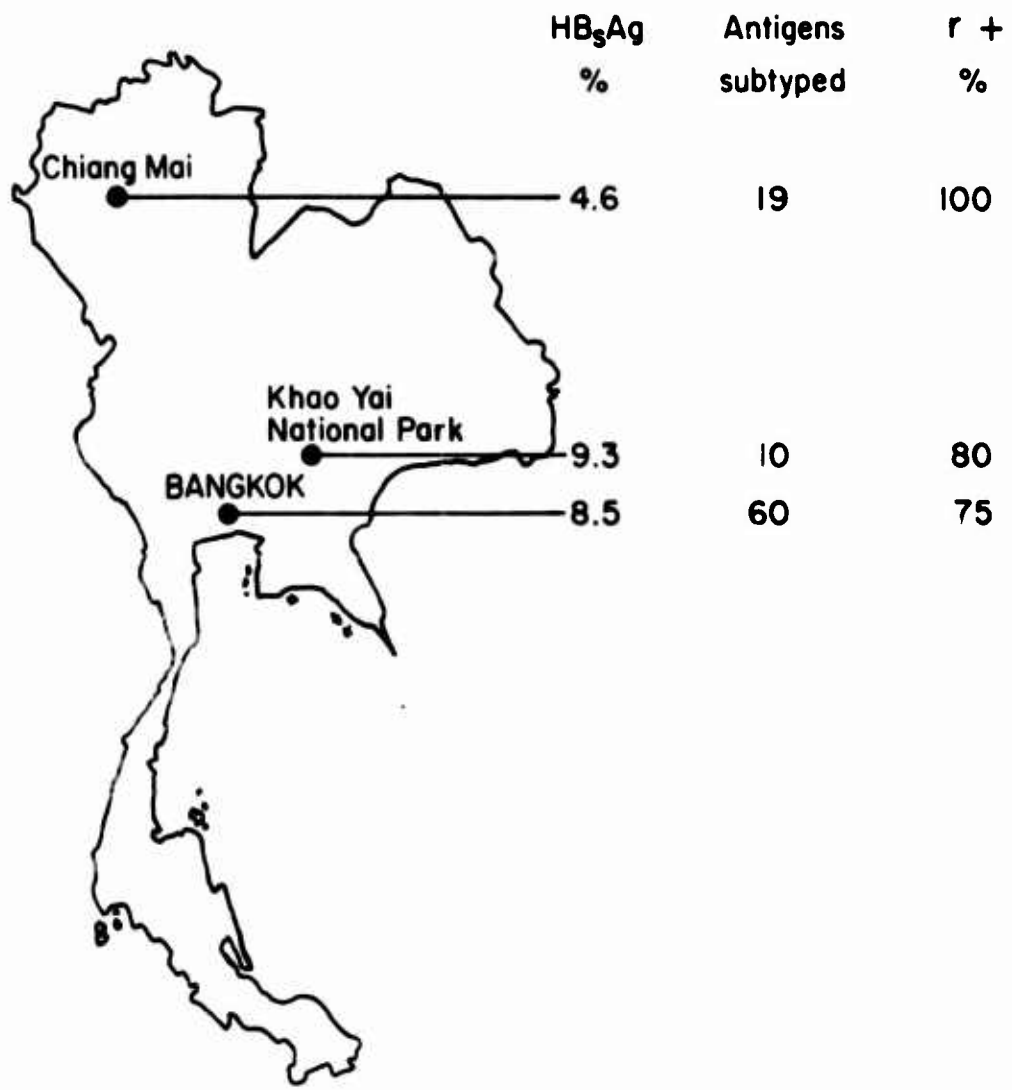


Figure 1. Frequency of Detection of the r Determinant in Thailand

and Khao Yai National Park (Figure 1). Additional information on the subtypes of HB_sAg may or may not support the preliminary findings.

This study was done in collaboration with the Thai Red Cross Center, Bangkok. Blood donors presenting to the blood collection centers in the central and eastern regions of Thailand were sampled by the National Blood Bank Service of the Thai Red Cross Center. Sera was tested for the presence of HB_sAg by counterimmunoelectrophoresis (CIEP) at the Thai Red Cross Laboratory for subtype determination.

All HB_sAg positive blood donors provided the following information:

- | | |
|--|---|
| a. Name | b. Age |
| c. Sex | d. Present residence |
| e. Previous blood donation and transfusion | f. History of clinical hepatitis and jaundice |

Antigen subtypes were determined by immunodiffusion (ID) (28), using specific rabbit antisera.

PROGRESS: Six hundred and fifty-four sera containing HB_sAg were tested. All of them were found to have the d determinant. Eighty-six percent of 311 HB_sAg positive sera collected from blood donors residing in Bangkok were HB_sAg/adr (Table 1). Seventy-six percent of 252 HB_sAg positive sera from the central region of Thailand, collected in Nontaburi, Phathumdhani, Ayudhya, Lopburi, Samutprakarn, Kanchanaburi, Samutsakorn, Samutsongkram, Rajburi, Nakorn Pathom, Petchaburi and Prachuab Kirikan were HB_sAg/adr. Eight-four percent of 91 sera collected in the eastern region from Choburi, Trat, Chanthaburi, Chacherngsoa and Prachinburi were HB_sAg/adr. The combination of all of the HB_sAg positive sera collected from Bangkok and other provinces in the central region show 81% HB_sAg/adr. So far we have detected no statistical differences in the prevalence of HB_sAg/adr in the central part of Thailand.

DISCUSSION: The data on the frequency of HB_sAg subtypes collected from the central districts of Thailand have not revealed any regional differences in subtype prevalences; however, HB_sAg positive blood donors from the north and the south of Thailand remain to be tested.

All of the 654 blood donors tested in this study have had the d determinant. However, a HB_sAg obtained from the serum of a 15-year old Karen girl, living in Sangkhlaburi, Kanchanaburi, a

remote section of Thailand, has been identified in our laboratory as HB_sAg/ayw. This antigen showed lines of identity in immunodiffusion to a reference HB_sAg/ayw antigen (WRAIR-DI-387, JF 019) and to D2-2 antigen (confirmed in Paris, France, April 1975 as HB_sAg/ayw). This is the first serum of an indigenous Southeast Asian in which the y determinant has been detected. Further information is being sought on the contacts of this Karen woman, in an attempt to determine the source of this anomalous subtype.

SUMMARY: Sera from 654 HB_sAg positive blood donors collected in the central and eastern regions of Thailand were tested for HB_sAg subtypes. Eighty-one percent of the sera contained HB_sAg/adr. There were no statistical differences in the prevalences of subtypes in different regions. Subtype prevalence in the northern and southern regions of Thailand are being examined.

Table 1. The Relative Frequency of HB_sAg Subtypes in Blood Donors from Various Parts of Thailand

Region	HB _s Ag Positive Sera	Subtype Frequency (%)		
		adr	adw	ad(?)
Bangkok	311	86	12	2
Central	252	76	17	7
East	91	84	13	3

9. Serum Immunoglobulin Levels in Infants with and without Hepatitis B Virus Infection

OBJECTIVE:

1. To determine the serum levels of IgM and IgG during the first year of life.
2. To determine if Hepatitis B virus (HBV) infection alters the serum immunoglobulin levels in infants.

BACKGROUND: Little information is available on IgM and IgG levels in Thai infants. No information exists on the effect of HBV infections on immunoglobulin levels of Thai infants. This

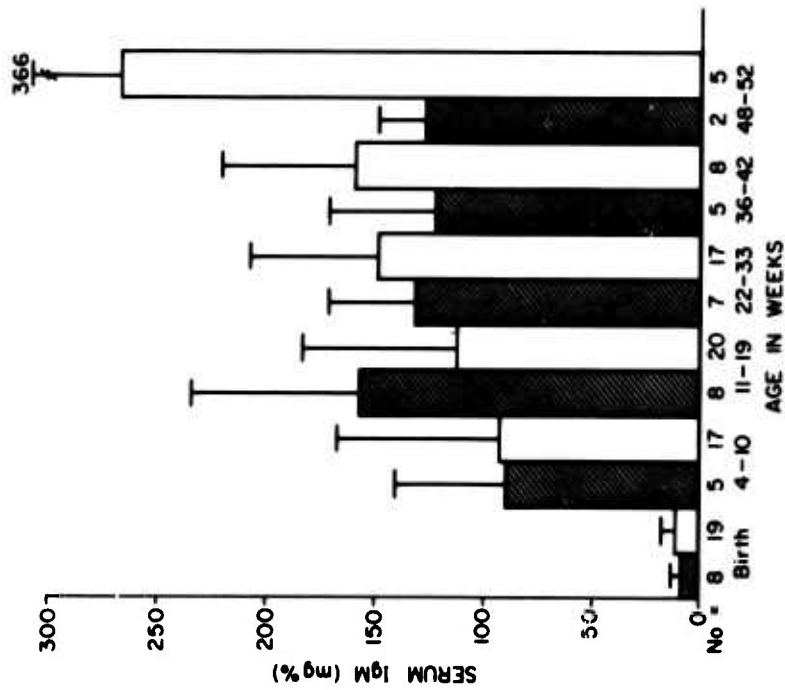


Figure 1. Serum IgM levels during the first year of life. One standard deviation indicated. Crosshatched bars represent infants re-infected with hepatitis B virus before the age of 36 weeks. Open bars are non-infected infants.

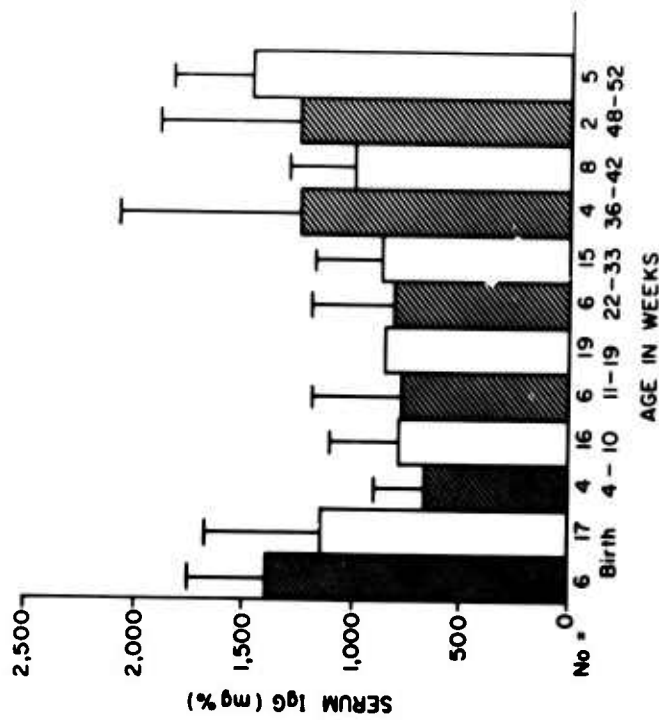


Figure 2. Serum IgG levels during the first year of life. One standard deviation indicated. Crosshatched bars represent children infected with hepatitis B virus before the age of 36 weeks. Open bars represent non-infected infants.

Table 1. Serum IgM Levels (mg%) During the First Year of Life

Infant Group		Age in Weeks					
		Birth	4-10	11-19	22-33	36-42	48-52
HBV Infected	Mean	9.5	90	157	132	123	128
	S.D.	3.2	51	76	39	48	11
	S.E.	1.1	23	27	15	21	8
	N.	8	5	8	7	5	2
Non-infected	Mean	11.9	93	113	149	159	267
	S.D.	5.5	74	70	58	62	99
	S.E.	1.3	18	16	14	22	44
	N.	19	17	20	17	8	5
Combined	Mean	11.2	92	126	144	145	227
	S.D.	5.0	69	73	53	58	106
	S.E.	1.0	15	14	11	16	40
	N.	27	22	28	24	13	7

Table 2. Serum IgG Levels (mg %) During the First Year of Life

Infant Group		Age in Weeks					
		Birth	4-10	11-19	22-33	36-42	48-52
HBV Infected	Mean	1344	660	771	809	1249	1255
	S.D.	384	138	419	387	824	629
	S.E.	157	69	171	158	412	445
	N.	6	4	6	6	4	2
Non-infected	Mean	1143	785	857	867	998	1465
	S.D.	536	323	375	313	303	365
	S.E.	130	81	86	81	107	163
	N.	17	16	19	15	8	5
Combined	Mean	1195	760	836	850	1081	1405
	S.D.	501	296	379	327	509	407
	S.E.	104	66	76	71	147	154
	N.	23	20	25	21	12	7

investigation was incorporated into a larger study of the effects of HBV infections on Bangkok children during the first year of life.

DESCRIPTION: Serial serum samples were collected during the first year of life from infants of mothers who carried hepatitis B surface antigen (HB_sAg), antibody (anti-HB_s) or were negative at the time of delivery.

Serum samples were stored at -20°C until tested for HB_sAg and anti-HB_s by techniques previously described (4). Serum IgM and IgG levels were determined by testing all serum samples using commercial radial immunodiffusion kits (Hyland Laboratories). Questionable determinations were repeated on each individual simultaneously. For purposes of analysis, eight infants who developed HBV infections were compared to 21 infants who did not. All of the mothers of the eight infected infants were HB_sAg positive; the mothers of the non-infected infants included two with anti-HB_s (FM69, 535), five with HB_sAg (FM68, 487, 555, 573, 578) and 14 negatives.

PROGRESS: IgM and IgG concentrations for each age are shown in Tables 1 and 2, respectively. Although few infants had complete serum collections to test, there was a general trend toward rising IgM levels in both groups of infants during the first 33 weeks (Figure 1). IgG levels fell immediately after birth, then showed a progressive rise (Figure 2). The wide range of values in members of both study groups prevented the recognition of any difference between them due to infection with HBV.

SUMMARY: A small pilot study of serum IgM and IgG levels in Bangkok infants was completed. Combined data for all infants showed IgM levels rose progressively after birth as infant IgM production began. Serum IgG levels fell during the period when maternal antibody was declining but recovered as intrinsic IgG production increased. There was no discernible difference between eight infants with HBV infections and 15 non-infected infants.

C. RABIES

1. Animal Rabies in Thailand: Rabies Diagnostic Laboratory Services

OBJECTIVE: To provide rabies diagnostic services to US military personnel in Southeast Asia and the Western Pacific.

DESCRIPTION: Every brain submitted was examined by the fluorescent antibody test and confirmed by mouse inoculation.*

PROGRESS: Of 750 brain specimens examined, 230 (31%) were positive (Table 1). The prevalence of rabies in the dog (37%) and in the cat (4%) was slightly less than in recent years.

Withdrawal of US armed forces from Thailand resulted in a decrease in rabies specimens of military origin. For instance, during the first quarter of 1976, rabies specimens submitted by US armed forces accounted for 20% of the total. By the end of the second quarter of 1976, US armed forces specimens accounted for only 11% of the total.

* As described in Laboratory Techniques in Rabies, Third Edition, WHO Monograph Series No. 23, (1973).

Table I. Summary of Rabies Diagnoses 1 April 1975-31 March 1976

Species	Number of Specimens	Number Positive	Percent Positive
Canine	586	217	37
Feline	101	4	4
Human	5	4	80
Rodent	9	0	0
Primate	9	1	11
Rabbit	10	0	0
Bat	6	0	0
Squirrel	5	0	0
Monkey	8	0	0
Others *	11	4	36
Total	750	230	31

* 1 avian, 3 mice, 4 bovine, 1 horse, 1 otter, 1 pig.

III. PARASITIC DISEASES OF MAN AND ANIMALS

A. FILARIASIS

1. Ecology of Bancroftian Filariasis

OBJECTIVE: To investigate the ecology of bancroftian filariasis in rural areas of Sangkhlaburi district, Kanchanaburi Province with the following specific aims:

1. To identify the vector(s) of Wuchereria bancrofti by recovery of filariae from wild-caught mosquitoes and by feeding laboratory-reared strains of suspected vector species on known microfilaria-carriers.

2. To gather data on the distribution, larval habitats and bionomics of vector species and to collect correlated series of larvae, pupae and adults of these mosquitoes for taxonomic studies.

DESCRIPTION: In 1970, Harinasuta and associates (29) described an endemic focus of bancroftian filariasis in rural villages located near the headwaters of the Kwai River in the Sangkhlaburi district of Kanchanaburi Province. Microfilaremia in infected villagers was nocturnally subperiodic, with peaks near 2000 hours but microfilariae were also present in significant numbers in the peripheral blood during daylight hours. Infective stage larvae of W. bancrofti were found only in wild-caught mosquitoes belonging to the Aedes (Finlaya) niveus complex. These mosquitoes are among the most common diurnal man-biting mosquitoes in the forested areas of Southeast Asia; but many of the species of this complex cannot be differentiated with certainty at the present time.

During the 1974-1975 SEATO Medical Research Laboratory report period, a total of 5141 mosquitoes were caught in biting collections made from villagers during daylight and early evening hours in five villages in the Sangkhlaburi district, Lawa, Wang Kalang, Nithae, Nong Padong and Kupadu. Of these, 45 mosquitoes belonging to eight species (Aedes niveus group species "A," Aedes desmotes, Aedes gardnerii, Aedes mediopunctatus, Aedes imprimens, Armigeres annulitarsis, Armigeres flavus and Mansonia dives) were found infected with filarial larvae (4). While mature larvae found in "Species A" were typical of W. bancrofti, the larvae from the other species were not identified at the time of the last report.

Table 1. Results of Dissection of Mosquitoes for Filariae-Sangkhlaburi, 1975-76.

Species	Number Dissected	Number Positive
<u>Aedes niveus</u> group (species "A")	73	3
<u>Aedes desmotes</u>	3	1
<u>Aedes gardnerii</u>	4	1
<u>Armigeres subalbatus</u>	3	1
<u>Mansonia dives</u>	117	2
<u>Anopheles barbirostris</u> group	35	0
<u>Anopheles aconitus</u>	29	0
<u>Anopheles balabacensis</u>	66	0
<u>Anopheles maculatus</u>	69	0
<u>Anopheles minimus</u>	307	0
<u>Anopheles nivipes</u>	189	0
<u>Culex vishnui</u> complex	102	0
Other species*	105	0

*Less than 20 specimens per species dissected.

Table 2. Mosquitoes fed on microfilaria carriers-
Sangkhlaburi, 1975.

Patient No.	Mfl.per cmm.	Mosquito	No. Fed	No. Diss	No. Pos.	Percent Pos.
154	5	<u>Anopheles balabacensis</u>	27	13*	0	0
"	"	<u>Anopheles maculatus</u>	27	9*	1	11
305	21	<u>Anopheles balabacensis</u>	100	68*	16	24
"	"	<u>Anopheles maculatus</u>	29	7*	2	28

*Mosquitoes which were alive after 21 day incubation period.

Colonized strains of Aedes aegypti, A. albopictus, A. togoi, Armigeres annulitarsis and Culex quinquefasciatus were fed upon villagers circulating microfilariae of W. bancrofti, but development to the infective stages of the parasite was observed only in A. togoi and C. quinquefasciatus.

PROGRESS: One of the principal objectives of this study was to identify the member(s) of the Aedes niveus complex involved in the transmission of W. bancrofti in the Sangkhlaburi area. Therefore, the collection and dissection of these and other diurnal mosquitoes was emphasized during the previous reporting period. During the present period, from April 1975 to February 1976, we concentrated on the examination of nocturnal mosquitoes, especially anophelines. Night-time human-bait collections were made and CDC light traps operated in the five study villages, yielding a total of 1102 mosquitoes for dissection. Essentially the same species of mosquitoes that were positive in the 1974-75 studies were again found infected (Table 1). Infective stage larvae of W. bancrofti were present only in species "A" of the Aedes niveus complex. The filariae found in Aedes desmotes, A. gardnerii, Armigeres subalbatus and Mansonia dives during this period have not yet been identified. However, most of the filariae recovered from these same species of mosquitoes during the 1974-75 season have been identified as Dirofilaria sp. (30). There is some uncertainty about a few of these determinations because first and second stage filarial larvae were found in the thoracic muscles of the mosquitoes rather than in the malpighian tubules which are the typical developmental sites of most Dirofilaria species. None of the anophelines dissected during the present period were infected, although Harinasuta et al. reported finding early stage filariae in Anopheles maculatus, An. minimus and An. vagus during their investigations (29).

Colonized strains of Anopheles balabacensis and Anopheles maculatus were transported to Sangkhlaburi and fed on two known microfilaria carriers. Infective stage larvae of W. bancrofti were found in the head and mouthparts of both species 21 days after feeding on a patient circulating approximately 21 microfilariae per cmm of blood (Table 2). When these mosquitoes were fed on a patient circulating significantly fewer microfilariae, development to infective stages was observed in a single An. maculatus.

Mosquito larvae, collected from bamboo oviposition cups set out in the study villages throughout the 1975-76 rainy season, were

reared to maturity, and their larval and pupal skins, together with the correlated adults, were preserved for taxonomic study. Members of the Aedes niveus complex most frequently identified from these collections included species "A," Aedes niveoides Barraud and Aedes nipponicus La Casse and Yamaguti. The females of species "A" closely resemble those of Aedes albopictus (Theobald), but the terminalia of the males are distinctly different. Descriptions of all stages of this apparently undescribed species are being prepared for publication.

2. Establishment of An Animal Model for Use in Filariasis Studies

OBJECTIVE: To trap wild rodents of Genus Rattus infected with nematodes of Superfamily Filarioidea, to maintain these in the laboratory and to transmit the filarial infections from them, through mosquitoes, to laboratory albino rats.

BACKGROUND: The need for a consistently reproducible laboratory infection with nematodes of Superfamily Filarioidea in a readily available, genetically controlled, laboratory animal has been expressed (31). Litomosoides carinii naturally found in the wild cotton rat can be experimentally transmitted to the laboratory albino rat and the Mongolian gerbil but not by a mosquito. A mosquito-transmitted filaria-laboratory rat system would more closely meet the requirements of the desired experimental model (32). Attempts to transmit Brugia tupaia through mosquitoes, to various laboratory animals were made at the SEATO Medical Research Laboratory (SMRL) in 1969 and 1970 (33,34) but were unsuccessful. One mosquito-transmitted filarial parasite, Brientalia booliati, was recently reported in Malaysia and laboratory rats have been successfully infected with this nematode at the University of Singapore (35,36).

A preliminary study in which wild rodents in Thailand were trapped and screened for microfilaria revealed the presence of unreported filarial nematodes in several species. This study was initiated to evaluate the model potential of some of these.

DESCRIPTION: Small mammals were live-trapped, using bananas as bait, in 8 different locations in Southeastern and South Central Thailand from late August, 1975 through May, 1976 (Figure 1). The habitats trapped ranged from evergreen forests on mountain sides to the water front area of Bangkok. Traps were set in the evening and picked up in the morning. Blood was drawn from



Figure 1. Sites where animals were trapped in South-eastern and South-central Thailand

the rats and examined for microfilariae in late morning and early afternoon, generally between 1130 and 1530 hours.

The animals were anesthetized with ether and 1/4 to 1 1/2 cc of blood was withdrawn by cardiac puncture with a heparin-wet syringe. The blood was mixed with 20 to 30 cc of normal saline and passed through a 3 or 5 micron Millipore filter. The filter was placed on a clean glass slide with one or two drops of saline, a coverslip was added, and the filter was examined at a magnification of 100 X. Movement of the microfilariae was readily discernible and an estimate of size could be made from these live mounts. By using this procedure, relatively large numbers of animals could be examined quickly and the animals determined to be positive or negative within 10 minutes of bleeding. All negative animals were released within 3 to 4 hours of bleeding and positive animals were transferred to laboratory rat cages.

Although no attempts were made to study periodicity of microfilaria, different bleeding times were used occasionally to see if the percentage of positive animals varied. Animals were bled as early as 0900 hours and as late as 2400 hours and no significant difference was noted.

Any positive animals that died as a result of handling or bleeding were necropsied and examined for adult filariae. If any were found, they were placed in 10% glycerin-alcohol fixative. Microfilaremic animals in which the adult filariae were not found were placed in 10% neutral buffered formalin and returned to the laboratory for histopathological examination. Live positive animals were returned to the laboratory for mosquito feedings and as a source of adult filariae for taxonomic study.

For mosquito feedings, donor rats were restrained in wire cloth cylinders and placed in a cage containing laboratory reared Aedes togoi, Anopheles balabacensis, and Armigeres subalbatus, respectively. Mosquitoes that fed on donor rats were dissected after 2 to 3 weeks to detect filarial infections. If larval stages of filaria were found, mosquitoes from that group were fed on albino laboratory rats by the procedure described above. After 3 months thick blood films were made from the laboratory rats and examined for microfilaria.

To study the morphology of the microfilariae, smears from wild caught rats were made at the laboratory, dried overnight at

room temperature, dehemoglobinized for two minutes in tap water, fixed for 30 seconds in methyl alcohol, and stained in Giemsa and/or Field's stain.

Some positive animals were later sacrificed in the laboratory to obtain adult filariae. These nematodes and thick blood films from some of the animals in which they were found were sent to the Filariasis Research Division of the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia for identification.

PROGRESS: One thousand six hundred and ninety four (1964) animals of 15 different species were trapped, 235 of which were microfilaremic. The trapping locations, the number and species of animals trapped and the number and percent of those that were microfilaremic are given in Table 1. Six different types of microfilariae were evident on the live mounts and this was confirmed when stained thick films were studied. Brientalia booliati, Brugia tupaia, and Dunnifilaria ramachandri have tentatively been identified from the microfilariae, the host species, and the location and size of the adult filariae. Adult filariae of five of the six species have been submitted to the IMR for identification but these studies are incomplete. Unfortunately, the six positive Hylomys suilis all died shortly after trapping, and the adult worms were not found at necropsy.

Mosquitoes were infected with B. booliati which was found in Rattus rattus, Rattus neilli, and Rattus koratensis. Aedes togoi, which fed on infected Rattus rattus, became infected and when dissected later third stage larvae were found in these mosquitoes. These Aedes togoi were allowed to feed on laboratory rats. There were no microfilariae on thick smears made from these rats 3 months later. Unsuccessful attempts were made to infect mosquitoes with an unidentified filaria from R. rattus. Mosquito transmission was not attempted with animals infected with Brugia tupaia or Dunnifilaria ramachandri

Screening histopathology sections proved to be an unrewarding method of locating adult filariae in the animals and was discontinued.

SUMMARY: One thousand six hundred and ninety four (1964) small wild mammals were trapped in Thailand and screened for microfilaremia. Two hundred and thirty five were found to be positive. Identification of the species of filariae found and mosquito transmission studies of the filariae to laboratory rats are incomplete.

SPECIES	LOCATION										Total By Species							
	Bangkok		Chantaburi		Nakhon Rajasima		Prachinburi		Sai Yok		Sa Kao		Sakaree		Sangkhla Buri		Total	Pos
<i>Bandicota indica</i>	0	0	14	0	0	0	0	0	0	0	5	0	0	0	0	19	0	0
<i>Hylomys suillus</i>	0	0	5	0	0	0	0	0	0	0	0	0	46	6	0	51	6	11.8
<i>Menetes berdmorei</i>	0	0	12	2	0	0	0	0	0	0	0	5	1	52	4	69	7	10
<i>Rattus berdmorei</i>	0	0	31	1	0	0	0	0	0	0	0	0	0	4	0	35	1	2.9
<i>Rattus exulans</i>	17	0	0	0	3	0	26	0	0	0	15	0	0	39	0	100	0	0
<i>Rattus koratensis</i>	0	0	80	20	0	0	0	0	0	0	0	15	9	0	0	95	29	30.5
<i>Rattus losea sakeratensis</i>	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0
<i>Rattus neilli</i>	0	0	0	0	0	0	0	0	5	2	0	0	0	0	0	5	2	4.0
<i>Rattus bukit</i>	0	0	5	0	0	0	0	0	0	0	0	40	4	2	0	47	4	8.5
<i>Rattus norvegicus</i>	106	0	0	0	5	0	77	0	0	0	0	0	0	0	0	188	0	0
<i>Rattus rattus</i>	0	0	63	2	0	0	0	0	1	0	0	117	46	99	1	280	49	17.5
<i>Rattus sabanus</i>	0	0	1	1	0	0	0	0	0	0	0	49	34	1	0	51	36	70.6
<i>Rattus surifer</i>	0	0	146	1	0	0	0	0	3	0	0	386	15	9	0	544	16	2.9
<i>Suncus murinus</i>	11	0	0	0	0	0	22	0	0	0	0	0	0	0	0	33	0	0
<i>Tupaia glis</i>	0	0	46	23	0	0	0	0	4	4	0	78	38	47	20	175	85	48.6
Total By Location	134	0	403	50	8	0	125	0	13	6	22	0	736	154	253	25		

0 Total Table 1. Results of Trapping and Screening for Microfilaria by Location and Species of Animal Trapped.
+ Positive.

IV. MISCELLANEOUS

1. An Epizootic of Canine Ehrlichiosis (Tropical Canine Pancytopenia) in Thailand

OBJECTIVE: To study the epizootiology of Canine Ehrlichiosis (TCP) in a population of military working dogs, and to evaluate the efficacy of currently recommended prophylactic and therapeutic measures in a natural outbreak.

BACKGROUND: Canine ehrlichiosis was first suspected in Thailand in March 1974 among a group of 7 German Shepherd dogs working at Lopburi, Thailand. The methods used to confirm this tentative diagnosis and the control measures instituted thereafter at the Military Working Dog Center, (MWDC) Pakchong, Thailand from June 1974 through March 1975 have been previously described (37, 4). This report consists of a continuation of those studies plus the extension of the serologic testing to include some of the Royal Thai Army (RTA) and Royal Thai Air Force (RTAF) installations in the provinces where most of the older, trained dogs actually work.

DESCRIPTION: The population of working dogs at the MWDC has increased from an average of 175 dogs, during the period June 1974 - March 1975, to 262 dogs in March 1976. This number includes only those dogs actually present at the MWDC at this time and over six months old. As of March 1976 we have identified and serologically tested for ehrlichiosis, at least once, 514 dogs. This increase (from 301 in March 1975) is partially due to the increase in the numbers of dogs reaching six months of age at the MWDC and partially due to testing at RTA and RTAF installations in some of the more remote provinces.

It has not proved to be practical to attempt to regard the dogs at the MWDC and the dogs at remote installations as two populations since almost all of the dogs come from the MWDC originally and return there periodically. To date, however, almost all of the control measures have been centered at the MWDC although many serologically positive dogs have been treated upcountry and tick control recommendations were made at all installations that were visited.

In September 1975, RTA and RTAF installations in Lopburi, Chiang Kam, Phitsanulok, Chiang Mai, Nan, Chiang Rai, Takhli, Lopburi, Nakhon Pathom, Prachuab, Nakhonsrithamaraj, Hat Yai, and Don Muang (Figure 1) were visited and 120 dogs were bled.



Figure 1. Thai Military Installations Where Dogs Were Tested for Canine Ehrlichiosis

Table 1. Results of Serologic Studies at Pakchong - June 1974 to July 1975

Date	Cumulative Number of Dogs Studied(a)	Serologically Positive Dogs			Serologically Negative Dogs			Cumulative Known Positive Dogs (a)	Cumulative Known Negative Dogs (a)	Percent Positive
		Converted To Positive	Added To Study	Total New Positive	Converted To Negative	Added To Study	Total New Negative			
4 Jun 75	176	-	86	86	-	90	90	86	90	49%
25 Jul 74	200	30	17	47	8	13	21	120	80	60%
Sep-Oct 74	242	13	12	25	6	33	39	136	106	56%
Dec 74-Jan 75	294	3	2	5	45	53	98	93	201	32%
Mar - Apr 75	308	1	2	3	24	17	41	69	240	22%
Jun-Jul 75	345	10	2	12	9	36	45	72	273	21%
(a) Dogs which have died and dogs which were no longer under control of the Center are excluded.										
Sep-Oct 75	446	28*	13	41	27	73	100	94	352	27%
March	514	10	0	10	15	63	78	87	427	17%

* 19 at Pakchong, 9 up-country.

Dogs were bled at the MWDC on June 4-5 1975, July 18, 1975, September 10-11, 1975, October 6, 1975 and on March 29-30, 1976. The results of this serologic testing are shown in Table 1 which also includes all testing done upcountry in September 1975. Unfortunately, due to problems with antigen production at WRAIR and the University of Illinois, the results for the July, September and October serology were received in March 1976.

All dogs found to be serologically positive were treated with tetracycline (30 mgm/lb/day for 14 days). Dogs entering the MWDC for any reason were quarantined for 14 days, dipped for ticks four times and given 3 mgm tetracycline/lb/day for 14 days. Recommendations for tick control were made at all installations that were visited. Prophylactic tetracycline (3 mgm/lb/day) was not given to any dogs (except for those in quarantine) this year. All other control measures that had been instituted at the MWDC (4) were continued.

PROGRESS: As of 31 March 1976, 514 dogs have been tested serologically at least once. There are currently 87 dogs that were positive on their last test (which was, in some cases, two years ago) for a positive percentage of 17% overall. This is the lowest positive percentage since the initiation of testing in June 1974 (Table 1). There were 241 dogs tested at the MWDC in March 1976 and of these there were 23 positives (9.5%). There were 10 serologic conversions to positive at the MWDC between September 1975 and March 1976. Four of these were old positives that had had one negative test at the 1:10 dilution.

From March 1975 through March 1976 there was one case of ehrlichiosis seen at the MWDC Veterinary Hospital. On March 1, Kerchief, tattoo number 5217, a 2 year old female German Shepherd, was returned from upcountry to the hospital with epistaxis, anorexia and a temperature of 105.6°. Tetracycline therapy was instituted 1 March 1976 on the basis of clinical symptomatology. She was serologically positive when bled on 29 March. No other cases were clinically diagnosed and serologically confirmed during this period.

During June 1975, 10 dogs in the breeding section converted to positive and a recommendation to treat all of the 16 serologically positive dogs was made. In September 1975 there were 2 more new positives in the breeding section and in March 1976 there were 2 more. All of these were treated.

DISCUSSION: The report in the 1975 SEATO Medical Research Laboratory (SMRL) Annual Report (4) stated that prophylactic tetracycline was being given to all dogs, except the young adults at the MWDC. This was a misunderstanding between personnel at SMRL and those at the MWDC. Actually tetracycline prophylaxis was administered in June, July and August 1974 and in January, February and March 1975 and has not been used since.

Due to budgetary limitations it was not possible to administer prophylactic tetracycline to any dogs this year. During the same period heavy demands for dogs were made from the field resulting in some dogs going out to work at one year rather than 16 months as in the past.

Despite problems with test results, non-availability of drugs, rapid turnover and frequent movement of dogs the number of obvious clinical cases of ehrlichiosis and the percentage of dogs found to be positive continued to decline indicating that application of the recommended control measures will control the disease even when they are used under somewhat less than perfect conditions.

2. Neonatal Diarrhea with Sepsis in the Nursery of the Phra Mongkutklao Hospital

OBJECTIVE: To define the cause of and to make recommendations on methods of terminating an epidemic of neonatal diarrhea with sepsis.

BACKGROUND: Neonatal sepsis is a worldwide problem. Because of the hospitalization of mothers for delivery and the subsequent placing of the newborn infant in common nurseries, nosocomial neonatal infections are and will continue to be an aggravating and potentially severe problem. The extent of this problem may be partially estimated by the output of scientific articles dealing with it. A computerized literature search resulted in retrieval of an average of 2,500 references/year over the past 10 years.

For at least the first half of 1975, the nursery at the Phra Mongkutklao Hospital (PHK) experienced neonatal diarrhea. During this period between two and ten cases of diarrhea occurred per month.

During the third quarter of 1975 a marked increase in the incidence of neonatal diarrhea, up to 20-30 cases per month, was noted by the nursing staff. Because of the increase in the

number of cases the pediatric service of the Phra Mongkutklao Hospital requested the assistance of the SEATO Medical Research Laboratory in investigating the source of the epidemic and in formulating recommendations for eradicating this problem.

DESCRIPTION: Five lines of investigation were followed:

1. A review of the nurseries, clinical and laboratory records for the preceding year to determine the extent, cause and, if possible, source of the epidemic.

2. A bacteriologic survey of a sample of infants in the nursery to determine if colonization of infants with pathological organisms was occurring.

3. A bacteriological survey of the nursery to identify the types of organisms found and to indicate possible areas of contamination.

4. A bacteriological survey of the personnel entering and/or working in the nursery and delivery areas to identify carriers of pathogenic organisms.

5. A review of antibiotic sensitivities on bacterial isolates from the nursery.

Review of Clinical and Laboratory Records:

During the first half of 1975 hospital records showed diarrhea related morbidity rate of 1.9% among approximately 1600 deliveries at the Phra Mongkutklao Hospital. In the third quarter the diarrhea related morbidity rate jumped from 2% in July to 12.2% in August, to 8.4% in September and to 9.4% in October.

Review of the isolations from rectal swabs sent for routine culture to the Microbiology Department of the SEATO Medical Research Laboratory from October 1974 through September 1975 revealed the continuous presence of pathogenic Escherichia coli. Of 245 specimens received, pathogenic E. coli were detected in 107 or 44%. Other pathogenic organisms such as Shigella and Salmonella were occasionally cultured. There were 10 serotypes of pathogenic E. coli isolated (Table 1), However one subtype 0127:B8 was consistently present throughout the year and represented 75% of all pathogenic E. coli isolated.

Environmental Survey of the Nursery:

Several site visits were made to the nursery in mid-October 1975. The nursery was located on the second floor of a ten year old

Table 1. Monthly Isolation of Pathogenic Organisms
from Rectal Swabs Submitted by the Pramongkhat Klao Hospital
from October 1974 - September 1975

Pathogenic Organisms	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	
<u>Escherichia coli</u>													
0127:B8	4	20	28	7	1	2	7	1		2	3	12	80(32.7%)*
0119:B14	4	1	1						2		2	1	11 (4.5%)
0125:B15				1				1			6		8 (3.3%)
0112:B11						1		4					5 (2.0%)
025:B19:B23		1		1		1			1				4 (1.6%)
026:B6	2			1					1				4 (1.6%)
0128:B12						3							3 (1.2%)
086:B7				1						1			2 (0.8%)
055:B5												1	1 (0.4%)
0111:B4	1												1 (0.4%)
<u>Total E. coli</u>	11	22	29	11	1	7	7	6	4	3	11	14	119(43.7%)
Other pathogens	0	2	5	6	4	0	7	2	0	0	3	5	27(11.0%)
<u>Total pathogens</u>	11	24	34	17	5	7	7	8	4	3	14	19	146(54.7%)
<u>Total samples</u>	12	31	52	24	20	8	7	19	15	8	25	31	245

* Percent of total cultures

Obstetrics and Gynecology building. It lay within the Obstetric Department; adjacent to the delivery area (Figure 1).

The neonatal unit was composed of a series of interconnecting rooms. Infants were housed in one of four bays which were separated from each other by glass and screen partitions. A service area, along one end of the bays, was partially partitioned from them by a glass wall with screening at the top. The four bays were loosely designated: 1) normal newborn bay, 2) premature infant bay, 3) potentially septic and intensive care bay, and 4) septic bay. There were separate entrances from the service area into each of the four nursery bays but air could circulate freely between bays through the screened partitions.

The nursery bays were individually ventilated, with window air conditioners. Each bay was provided with a sink and separate waste and diaper disposal pails.

The formula preparation area was located adjacent to the nursery area. Formula was prepared from powdered preparations by the addition of boiled tap water. No terminal sterilization was used.

The bacteriological survey identified widespread contamination of the neonatal unit and formula preparation area. Pathogenic organisms were isolated from ten of 60 cultures obtained from sites throughout the nursery bays and the food preparation area. These sites included floors, preparation tables, door handles and lids to diaper pails. One culture grew Shigella boydii and the remaining nine grew pathogenic E. coli of three serotypes; two strains each of 0128:B12 and 0127:B8 and five strains of 0119:B14.

Bacterial Survey of Infants:

Stool cultures were obtained from 30 babies housed in the newborn nursery on 15 October 1975, 21 of these were housed in the normal or premature nursery bays, seven were housed in the potentially septic bay and two were in the septic bay. From these infants six potential pathogens were isolated, five pathogenic E. coli serotypes 0127:B8 (three) and 0119:B14 (two), were isolated from seemingly healthy infants and one E. coli, serotype 0128:B12, from an infant with diarrhea.

BEST AVAILABLE COPY

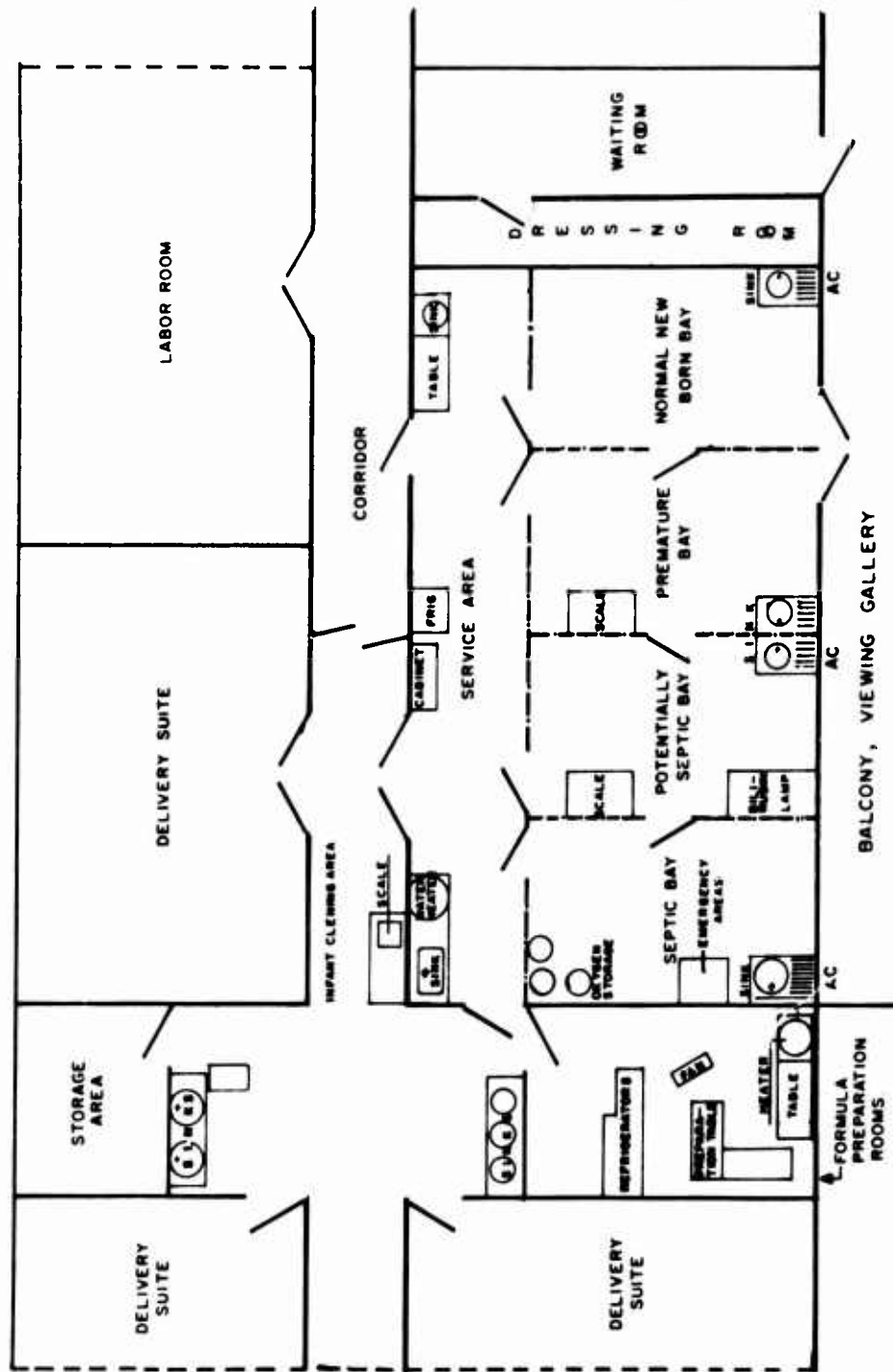


FIGURE 1. FLOOR PLAN FOR NURSERY PRAMONGKHUT KLAO HOSPITAL

Personnel Survey:

Ninety-seven people were reported to frequent the nursery. Sixty-two of these people provided an adequate perineal swab for culture. Fourteen pathogenic organisms were isolated (Table 2), one was a Salmonella B. and the remaining 13 were pathogenic E. coli of six serotypes. Eight, (over half of the pathogenic organisms isolated), were pathogenic E. coli serotype 0127:B8. Thus 22.5% of the personnel who frequented the nursery and were adequately sampled, were found to be carriers of pathogenic organisms.

Sensitivity of Isolates:

We were able to recover the antibiotic sensitivity data on 39 strains of pathogenic E. coli collected from infants in the nursery at PKH from June to September 1975 and tested by the Kirby Bauer Disc Technique (Table 3). The isolated pathogenic E. coli were generally found to be susceptible to trimethoprim-sulfamethoxazole (one resistant strain out of 39 strains tested). The next best drugs appeared to be colimycin (seven resistant strains out of 39 strains tested), and gentamycin (12 resistant strains out of 39 tested).

Discussion and Recommendations:

Investigation of the epidemic of neonatal diarrhea and sepsis in the nursery at PKH indicated five possible contributors to the epidemic:

1. Carriers of pathogenic organisms among personnel assigned to the nursery.
2. Large number of individuals, (nearly 100) who frequent the nursery and delivery areas.
3. General contamination of the nursery and formula preparation area with pathogenic organisms.
4. Failure to use terminal sterilization.
5. Open nursery bays allowing for free movement of air from contaminated to uncontaminated areas.
6. Introduction of new strains of pathogenic organisms from septic deliveries.

Table 3. Sensitivity Patterns on Rectal Isolates from Infants at the Pramongkhit Klao Hospital June - September 1975

Organism	AMP*	CHL*	COL*	GEN*	KAN*	NEO*	SDZ*	TRM* SMZ	STR*	TET*	Total Organisms
<u>Escherichia coli</u>											
0127:B8	17/0**	17/0	5/12	4/13	15/2	16/1	17/0	0/17	17/0	16/1	17
0119:B14	3/3	3/3	1/5	1/5	4/2	4/2	4/2	0/6	5/1	3/3	6
0112:B11	5/0	5/0	0/5	0/5	0/5	0/5	5/0	0/5	5/0	5/0	5
0125:B15	7/0	7/0	0/7	7/0	1/6	1/6	7/0	0/7	7/0	7/0	7
026:B6	1/0	1/0	0/1	0/1	1/0	1/0	1/0	0/1	1/0	1/0	1
086:B7	1/0	1/0	1/0	1/0	1/0	1/0	1/0	1/0	1/0	1/0	1
025:B19:B23	1/0	1/0	0/1	0/1	1/0	1/0	1/0	0/1	1/0	1/0	1
055:B5	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1
All organisms	35/4	35/4	7/32	13/26	23/16	24/25	36/3	1/38	37/2	32/5	39

* AMP = Ampicillin, CHL = Chloramphenicol, COL = Colymycin, GEN = Gentamicin, KAN = Kanamycin, NEO = Neomycin, SDZ = Sulfadiazine, TRM/SMZ = Trimethoprim-sulfamethoxazole, STR = Streptomycin, TET = Tetracycline.

** Resistant/sensitive

Table 2. Isolations of Pathogenic Organisms from Nursery Personnel at the Pramongkhut Kiao Hospital

Personnel Frequenting the Nursery		97
Personnel Adequately Cultured		62
Carriers of Pathogenic Organisms		
<u>Salmonella B</u>	1	
Pathogenic <u>E. coli</u> 0127:B8	8	
026:B	1	6
0119:B14	1	
0124:B17	1	
0125:B15	1	
0126:B16	1	
Total carriers	14 (23%)*	

* Percent of adequately cultured

No one organism could be implicated as the cause of the epidemic. The major group of pathogenic organisms isolated from rectal swabs on affected infants were pathogenic E. coli of at least ten serotypes. These organisms were also isolated from environmental surfaces in the nursery and from perineal swabs from 22.5% of the nursery personnel who submitted adequate cultures. E. coli serotype 0127:B8 was the most prevalent organism cultured in both the rectal swabs of affected babies and in the perineal swab of the nursing personnel. Ninety-seven percent of 39 organisms tested were sensitive to trimethoprim-sulfamethoxazole, 82% were sensitive to Colimycin and 66% were sensitive to Gentamycin.

Recommendations were made to the hospital in eight general categories:

1. General decontamination of the nursery and delivery areas.
2. Treatment of all personnel associated with the nursery with an appropriate antimicrobial therapy.
3. Suspension of all new admissions to potentially contaminated areas and admission of all new babies to a decontaminated nursery.
4. Alterations in the preparation of formula to allow for terminal sterilization.
5. Decrease traffic in and through the nursery area.
6. Minor structural alterations to decrease air circulation, increase isolation and facilitate formula production.
7. Increase emphasis on sterile precautions in the nurseries.
8. Changes in the linen and waste disposal systems.

Follow-up:

The first two recommendations were implemented in mid-November 1975. The nurseries and the food preparation areas were thoroughly decontaminated using a glutaraldehyde preparation and all personnel frequenting the nursery were provided with adequate amounts of trimethoprim-sulfamethoxazole for a ten day period of therapy.

Following this the neonatal morbidity rate due to diarrhea fell from 9.4% in October to 1% in December, with a corresponding decline in the sepsis related mortality rate from 7% to 0%. During the same period the number of rectal swabs submitted from the nursery to the Microbiology Department at the SEATO Medical Research Laboratory fell from 75 in October to 15 in December and the number of pathogenic organisms isolated fell from 27 isolates, of seven different pathogenic strains of E. coli in October, to two isolates of one pathogenic strain of E. coli (0127:B8) in December.

SUMMARY: During 1975 the neonatal nursing at the Phra Mongkutklao Hospital experienced an epidemic of neonatal diarrhea. Investigation revealed that the epidemic was due to pathogenic E. coli of several serotypes.

The organisms had contaminated the nursery environment, were colonizing the infants and were carried by over 20% of the nursery staff. Decontamination of the nursery and treatment of the nursing staff with Trimethoprim-sulfamethoxazole led to a marked decrease in diarrhea cases.

3. Pathogens of Medically Important Mosquitoes of Thailand

OBJECTIVE: To determine the kinds of mosquito pathogens occurring in medically important species of mosquitoes in Thailand and to elucidate the biology of selected pathogens sufficiently to assess their potential as biological mosquito control agents.

BACKGROUND: An examination of the slide-mounted mosquito larva collection in the Department of Medical Entomology, SEATO Medical Research Laboratory, and a preliminary survey for pathogens in Culex quinquefasciatus in Bangkok confirmed the occurrence of pathogens in the Thai mosquito fauna (4). The present study involved a survey for mosquito pathogens in Aedes aegypti and C. quinquefasciatus at 20 locations in Thailand. These mosquito species were selected for study because of their primary medical importance, because they can be easily collected in relatively large numbers and because their breeding habits make them vulnerable to biological control by pathogens.

DESCRIPTION: From four to 40 man-days were spent collecting larvae of Ae. aegypti and C. quinquefasciatus at the locations in Figure 1, excluding Bangkok. Because of its size and proximity to the laboratory, more extensive collecting was done in Bangkok, here considered to include Thon Buri, Nonthaburi, and Pathum Thani. Collections from Sangkhlaburi were from numerous small villages along the Khwae Noi River between Sai Yok and Cha Deng Cheng, and up the Sang Kalia River as far as Sang Kalia. Collections from Ko Samui were from several villages on adjacent islands as well as from the villages of the main island.

C. quinquefasciatus larvae were collected primarily from polluted water discharged from dwellings and market places. Ae. aegypti larvae were collected from domestic water containers in and around homes. Other species of larvae were collected occasionally and were processed and examined, also, although they were not necessarily pertinent to the study. Larvae were examined successively in white enamel pans and in black pans under a bright light to detect gross signs of pathology. Watched for were any abnormality in color, size, shape or behavior. Larvae displaying such signs were separated and identified. Part were smeared on microscope slides, five per slide, in discrete smears, and the remainder were preserved in 7% neutral buffered formalin. Smears were air dried, fixed with methanol, and stained with Giemsa stain in Tris buffer at pH 7.2. Formalinized material was embedded in paraplast, sectioned and stained with hematoxylin and eosin. Specimens were examined at a magnification of 500X. A sample of larvae showing no gross signs of infection was also taken from each collection. These were identified, smeared, processed, and examined for evidence of covert infections. With the exception of eugregarines and ciliates, only those organisms observed in host tissue or hemolymph were reported. Some preliminary studies have been made to determine the infectivity, mode of transmission and host specificity of some of the pathogens found.

RESULTS: Twenty-five host-pathogen associations were found in 20,000 specimens prepared from eight species of mosquitoes. These are summarized in Table 1 and briefly discussed below.

A. Protozoans

1. Ciliates - Ciliates were frequently seen in the midguts and occasionally in the hemolymph of C. quinquefasciatus.

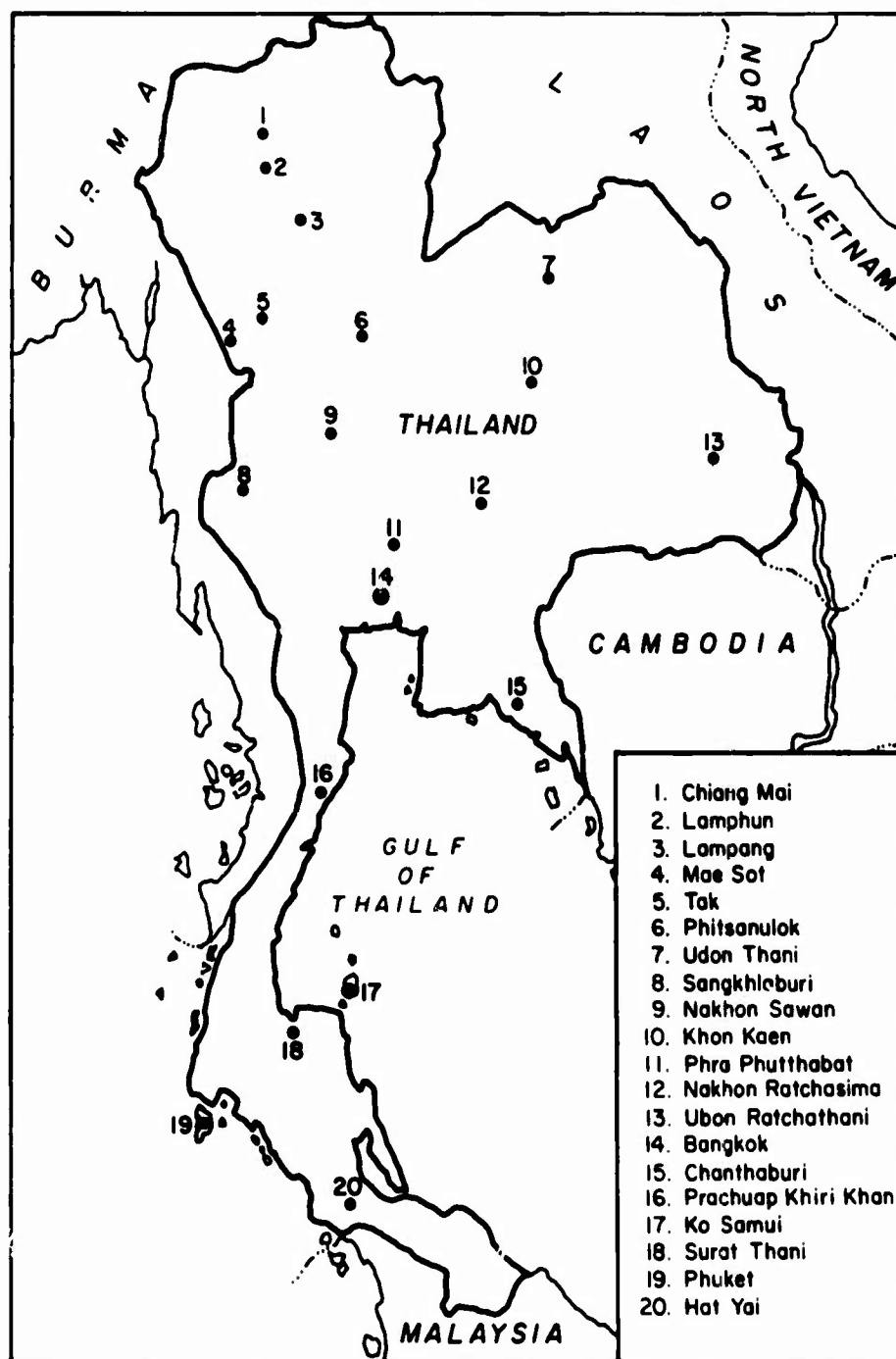


Figure 1. Locations where Aedes aegypti and Culex pipiens quinquefasciatus were surveyed for mosquito pathogens.

Table 1. Summarization of pathogens found in eight species of mosquitoes in Thailand.

Pathogens	Host							
	<u>Aedes</u>			<u>Culex</u>			<u>Armigeres</u>	<u>Anopheles</u>
	<u>aegypti</u>	<u>albopictus</u>	<u>chryso-lineatus</u>	<u>pipiens quinque-fasciatus</u>	<u>minor</u>	(<u>Lutzia</u>) sp.	<u>subalbatus</u>	<u>vagus</u>
Protozoans								
Ciliates				X				
Microsporida	X							
	X							
	X							
	X							
				X				
								X
						X		
Helicosporida	X			X				
Eugregarines	X	X		X			X	
Fungi	X			X				
					X			
Bacteria	X			X			X	
	X	X					X	
				X				
Unidentified			X					

Although some species of ciliates are pathogenic for mosquitoes, they generally are regarded as having little bio-control potential.

2. Microsporidans - Seven apparently distinct microsporidan species were found, four in Ae. aegypti and one each in C. quinquefasciatus, Anopheles vagus and Culex (Lutzia) sp. Of those in Ae. aegypti, one was a Stempellia sp., two were Thelohania spp., and one was a Nosema sp.

The Stempellia had pyriform spores $8.0 \times 4.0 \mu\text{m}$ in wet mounts, and was present at 17 of the 20 locations studied. It has been transmitted in the laboratory both per os and transovarially, and about 8.0×10^5 spores were obtained per patently infected larva. The dose-infection rate curve was linear between infection rates of 15 and 85 percent, and infection rates of 100 percent have been obtained. Mortality rates approaching 100 percent occurred, but these rates varied widely in response to unknown factors in addition to infection rates.

Thelohania spp. of Ae. aegypti were found respectively at Ubon Ratchathani and at Hat Yai. One was a pathogen of the midgut epithelium, with oval spores $2.0 \times 1.5 \mu\text{m}$. The tissue preference of the other is unknown, because it was found only in smears. The spores were oval and $3.0 \times 2.0 \mu\text{m}$.

The microsporidan in C. quinquefasciatus was a Stempellia sp. It was found at 9 of the 20 locations studied but was highly prevalent only at Hat Yai. The spores were pyriform and $5.0 \times 2.75 \mu\text{m}$ in wet mounts. Chapman (38) reported seeing a Stempellia sp. in C. quinquefasciatus in Bangkok and considered it identical to a species of Stempellia in the United States, subsequently described and named S. milleri by Hazard and Fukuda (39). Hazard and Fukuda did not report having examined material from Thailand, so the identity of this species is still in doubt. We have transmitted it per os, and further studies are underway.

Anopheles vagus at Lamphun were infected with an unidentified genus of Microsporida. The spores were $5.0 \times 3.0 \mu\text{m}$ and had structural features which will require electronmicroscopy for resolution. It was distinctly different from others encountered in this study and was found primarily in the fatbody. Attempts to transmit it per os to Anopheles maculatus were unsuccessful.

Culex (Lutizia) sp. at Sangkhlaburi were infected with a microsporidan with pyriform spores 7.0 x 4.0 μ m. The body cavity of infected larvae was filled with sporoblasts, though few spores were seen. The sporoblasts were usually disassociated, but some were associated in groups of eight. Insufficient material was examined to conclude that it was Genus Thelohania, so identification will require further study.

3. Helicosporidans - Apparently the same species of Helicosporidium was found widely distributed and moderately prevalent in Ae. aegypti and C. quinquefasciatus. The spores were spherical and 5.5 μ m in diameter. It has been transmitted per os to Ae. aegypti, C. quinquefasciatus and Anopheles balabacensis. There is only one previous report of Helicosporida in mosquitoes (40). The order is currently considered monotypic (41).

4. Eugregarines - These agents were commonly found in Ae. aegypti, aedes albopictus, C. quinquefasciatus, and Armigeres subalbatus. They are generally regarded as having little bio-control potential.

B. Fungi

Apparently the same species of Entomophthora was found in Ae. aegypti and C. quinquefasciatus at Sangkhlaburi. It was transmitted through several passages in the laboratory and was highly virulent though not very infectious. A fungus of Genus Coelomomyces was found in Culex minor at Sangkhlaburi.

C. Bacteria

In several locations, Ae. aegypti, C. quinquefasciatus, and Ar. subalbatus were found infected with minute (about 1 μ m long), gram negative, highly motile, vibrioform bacteria that produced an extremely virulent septicemia. Chapman (38) reported seeing possibly the same species in Bangkok and commented that the agent was found many places in the world but had never been cultured. Our attempts to culture this organism so far have been unsuccessful.

An endospore-forming bacillus was found in Ae. aegypti, Ae. subalbatus from Sangkhlaburi, but only in preserved material.

An apparent bacterial agent, which turned the infected host red, was found in C. quinquefasciatus in Bangkok.

D. Unknown

A pathogen of undetermined identity was found in the midgut epithelium of Ae. chrysolineatus from Sangkhlaburi. Epithelial cells were greatly enlarged and contained small, non-staining particles less than 0.5 μ m in diameter. The particles resemble a rickettsia of Genus Enterella.

The following information reflects the prevalence of mosquito pathogens in Bangkok. Ae. aegypti was collected 73 times at 55 collecting sites. At least one kind of pathogen was found in 37 of the 73 collections and at 45 of the 55 collection sites, C. quinquefasciatus was collected 151 times at 90 collecting sites. At least one kind of pathogen was found in 137 of the 151 collections and at 81 of the 90 collecting sites.

No viruses were detected during the survey.

4. Zoological Aspects of SMRL Studies

OBJECTIVE: To provide information on the native fauna to those departments whose work on human diseases in Thailand involves natural reservoir hosts.

BACKGROUND: In support of studies on Japanese encephalitis, now concluded, an intensive capture-mark-and-release program of birds and mammals necessitated delving into taxonomy and accumulation of a scientific vertebrate collection. Groups which particularly needed clarification on species-limits and which species live in Thailand were rats, mice, and gibbons.

PROGRESS: Taxonomic revisions were concluded, based on morphology, ecology, distribution, karyology, and additionally, for the gibbons, analysis of tape-recordings of their songs.

Rat hosts of fleas and chiggers subject to systemic poison which do not harm the rat) were identified for the Department of Entomology. Rattus rattus in the forest was the principal host of chiggers; whereas in the town, R. exulans and R. rattus had fleas. During trapping at Pakdongchai, Mr. Vandee discovered the field mouse, Mus cervicolor, living in houses at the market.

A study of natural hosts of filarial worms by Department of Veterinary Medicine was supported through identifying the various rodents and Tupaias which naturally harbored the worms.

These included Rattus sabanus, R. berdmorei, R. surifer, R. bukit, R. neilli (which we found at Saiyok - a large extension of known range for this newly discovered species), R. koratensis and R. rattus. They are easily identified in life, except for the last two. Unfortunately, the conditions of the experiment denied us access to examination of the carcasses to ascertain the number of mammae (12 in koratensis, 10 in rattus). Of the many dozens of each species that were sacrificed in order to obtain the adult worms we received only 6 skulls, which are necessary for the identification. All from Sakaerat, they all had the characteristic skull of Rattus koratensis although 4 of them had been identified as R. rattus by the veterinary field team.

Taxonomic studies of native mice included sending live colonies to Laboratory of Cell Biology, National Cancer Institute, where Dr. Michael Potter found biochemical traits distinguishing the various species. There, a type C virus of leukemia was discovered in our Mus caroli (37), which is the same as that in gibbons, and different from that of the laboratory mouse. NCI's expanding search for the virus led to an agreement for full-time support of SMRL personnel in collecting and shipping to NCI native live mice, especially those species which share forest habitats with gibbons such as Mus shortridgei, M. pahari, M. cookii and Mus cervicolor popaeus. Shipments of about 100 mice per month to NCI have been received in good condition.

We initiated the programmed release of conditioned gibbons from the laboratory colony to a safe forest area at Saiyok, Kanchanaburi Province, through the cooperation of the Protein Expansion Project (Ministry of Defense). Of the twelve animals so far liberated, all took immediately to the trees and found natural food and water. We conclude that domestic-reared gibbons instinctively revert to a natural life and need not be extensively rehabilitated. The only problem is that tame, human oriented animals have to be taken far enough into the hills so that they will not follow people back to civilization. The release program did not include breeding pairs.

5. Bacterial Etiology of Leucorrhea

OBJECTIVE: To determine the aerobic and anaerobic bacteria associated with leucorrhea in cervicitis patients.

BACKGROUND: Leucorrhea is one of the most frequently encountered conditions seen in females attending the obstetric and gynecological

clinic of the Royal Thai Army Hospital, Bangkok, Thailand. The condition may be the result of infection with bacteria, fungi, parasites or viruses. The purpose of this study is to determine the aerobic and anaerobic organisms present in discharges from cervicitis patients.

DESCRIPTION: Specimens were obtained from cervicitis patients attending the obstetrics and gynecology clinic of the Royal Thai Army Hospital. Routine cervical swabs were immediately streaked on Thayer-Martin chocolate agar, 5% sheep blood agar, and MacConkey agar poured in standard petri plates. After specimens were obtained they were sent to the bacteriology section of the SEATO Medical Research Laboratory within two hours. The chocolate agar plates were incubated in candle extinction jars for the detection of Neisseria gonorrhoea. Blood agar and MacConkey agar plates were incubated aerobically. All cultures were incubated at 37°C, and were examined at 24 and 48 hours. An additional cervical swab was obtained for the culture of anaerobic organisms. This swab was placed in tubed cooked meat broth media, incubated at 37°C in anaerobic jars using Gas-Paks (Baltimore Biologicals, Cockeysville, Md.) to establish an oxygen free atmosphere. Cultures were observed seven days for growth with routine sub-cultures to biochemical media being made when indicated.

RESULTS: During the period July 1975 - April 1976 a total of 78 specimens were cultured for aerobic organisms, and 69 for anaerobes. Neisseria gonorrhoeae was isolated from two patients (2.5%). This result was similar to that found in a 1972-73 study of gonorrhoea in asymptomatic females performed in the same hospital where 3.5% were found to be harboring the gonococcus.

Aerobic bacterial isolates from cervical cultures are presented in Table 1. Staphylococcus epidermidis and alpha hemolytic streptococci were found frequently, occurring in 54% and 36% of the patients respectively. Candida albicans was isolated in 15 of 78 patients (19%). This yeast was also isolated in throat cultures from three patients whose cervical cultures revealed C. albicans. Anaerobic bacterial isolates are shown in Table 2. Sixty nine specimens were submitted for anaerobic cultures. Clostridium perfringens was isolated in five patients (7.3%). However, this organism is often found as a harmless inhabitant of the vagina. Other anaerobic organisms isolated include the Peptostreptococcus, Peptococcus, Bacteroides, Veillonella and Fusobacterium groups. Anaerobic bacteria were not isolated in 19 of the 69 cultures examined.

Table 1. Aerobic Bacterial Isolated from 78 Patients with Cervicitis

Organisms Isolated	No. Isolated	% Isolated
<u>Neisseria gonorrhoeae</u>	2	2.5
<u>Staphylococcus epidermidis</u>	42	53.8
<u>Staphylococcus aureus</u>	1	1.2
Micrococcus species	11	14.1
alpha streptococcus	28	35.8
beta streptococcus (not group A)	1	1.2
<u>Streptococcus fecalis</u>	3	3.8
Diphtheroids	16	20.5
<u>Candida albicans</u>	15	19.2
Yeast species	2	2.5
Mimae species	3	3.8
<u>Escherichia coli</u>	7	8.9
<u>Enterobacter aerogenes</u>	3	3.8
<u>Enterobacter cloacae</u>	1	1.2
<u>Enterobacter hafnia</u>	1	1.2
Intermediate coliform	3	3.8
<u>Proteus rettgeri</u>	1	1.2

Table 2. Anaerobic Bacterial Isolates from 69 Patients with Cervicitis

Anaerobic Organisms	No Isolated	% Isolated
<u>Clostridium</u>		
<u>C. perfringens</u>	5	7.3
<u>C. acetobutylicum</u>	1	1.4
<u>Peptostreptococcus</u>		
<u>Ps. intermedius</u>	17	24.6
<u>Ps. productus</u>	2	2.9
<u>Ps. anaerobius</u>	4	5.8
<u>Ps. parvulus</u>	1	1.4
<u>Peptococcus</u>		
<u>Pc. asaccharolyticus</u>	7	10.2
<u>Pc. magnus</u>	14	20.3
<u>Pc. prevotii</u>	13	18.8
<u>Pc. constellatus</u>	1	1.4
<u>Bacteroides</u>		
<u>B. melaninogenicus</u>	8	11.6
<u>B. fragilis</u>	7	10.2
<u>Veillonella</u>		
<u>V. alcalescens</u>	3	4.3
<u>V. parvula</u>	4	5.8
<u>Fusobacterium</u>		
<u>F. nucleatum</u>	1	1.4
No anaerobic bacteria isolated	19	27.5

Project 3A762759A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 074 Tropical and subtropical diseases in military
medicine

Literature Cited:

References:

1. Halstead, S.B.: Observations Related to Pathogenesis of Dengue Hemorrhagic Fever. IV. Hypothesis and Discussion. Yale J. Biol. Med. 42:350, 1970.
2. Russell, P.K.: Pathogenesis of the Dengue Shock Syndrome: Evidence for an Immunologic Mechanism. Pages 426-435 in P.A. Miescher, (ed.) Immunopathology, Schwabe, Basal, 1970.
3. Nimmannitya, S., Halstead, S.B., Cohen, S.N. and Margiotta, M.R.: Dengue and Chikungunya Virus Infection in Man in Thailand, 1962-1964. I. Observations on Hospitalized Patients with Hemorrhagic Fever. Am. J. Trop. Med. Hyg. 18:954, 1969.
4. SEATO Medical Research Laboratory Annual Report 1975.
5. Rosen, L. and Gubler, D.: The Use of Mosquitoes to Detect and Propagate Dengue Viruses. J of Trop. Med. 23:1153, 1974.
6. McNeil, B.J., Keeler, E. and Adelstein, S.J.: Primer on Certain Elements of Medical Decision Making. New Eng. J. Med. 293:211, 1975.
7. Top, F.H., Jr., Gunakasem, P., Chantarasri, C., et al: Serologic Diagnosis of Dengue Hemorrhagic Fever Using Filter Paper Discs and One Dengue Antigen. Southeast Asian J. Trop. Med. and Pub. Hlth. 6:18-24, 1974.
8. SEATO Medical Research Laboratory Annual Report 1974.
9. Hunter, W.M. and Greenwood, F.C.: Preparation of Iodine-131 Labeled Human Growth Hormone of High Specific Activity. Nature (London), 194:495, 1962.
10. Lampe, R.M., Kirdpol, S., Mansuwan, P., Benenson, M.W.: Glucose-6-Phosphate Dehydrogenase Deficiency in Thai Children with Typhoid Fever. J. of Ped. 87:576, 1975.

11. Morrór, R.H., Smetana, H.F., Sai, F.T. and Edgcomb, J.H.: Unusual Features of Viral Hepatitis in Aerea, Ghana. Ann. Intern. Med. 68:1250, 1968.
12. Gall, J.C., Brewer, G.J. and Dern, R.J.: Studies of Glucose-6-Phosphate Dehydrogenase Activity of Individual Erythrocytes: The Methemoglobin-Elution Test for Identification of Females Heterozygous for G-6-PD Deficiency. Am. J. of Human Genetics, 17:359, 1965.
13. Magnius, L.O. and Espmark, J.A.: New Specificities in Australia Antigen Positive Sera Distinct from the Le Bouvier Determinants. J. Immunol. 109:1017-1021, 1972.
14. Grossman, R.A., Benenson, M.W., Scott, R. McNair, Snitbhan, R., Top, F.H., Jr. and Pantuwatana, S.: An Epidemiological Study of Hepatitis B Virus in Bangkok Thailand. Amer. J. Epidemiol. 101:144-159, 1975.
15. SEATO Medical Research Laboratory Annual Report 1976.
16. Lander, J.J., Holland, P.V., Alter, H.J. et al: Antibody to Hepatitis-Associated Antigen. Frequency and Pattern of Response as Detected by Radioimmunoprecipitation. JAMA. 220:1079, 1972.
17. Cherubin, C.E., Purcell, R.H., Lander, J.J. et al: Acquisition of Antibody to Hepatitis B Antigen in Three Socioeconomically Different Medical Populations. Lancet 2:149, 1972.
18. Lennette, E.H. and Schmidt, N.J. (ed): Diagnostic Procedures for Viral and Rickettsial Infections. Am. Pub. Hlth. Assoc. 1969.
19. Irwin, G.R., Allen, A.M., Bancroft, W.H., Karwacki, J.J., Brown, H.L., Pinkerton, R.H., Willhight, M., Top, F.H., Jr.: Hepatitis B Antigen in Saliva, Urine and Stool. Infection and Immunity 11:142-145, 1975.
20. Vaisrub, S.: Hepatitis B - Traffic in the Dentists' Office. JAMA 232:1270, 1975.
21. MacQuarrie, M.B., Forghani, B., Wolochow, D.A.: Hepatitis B Transmitted by a Human Bite. JAMA 230:723-724, 1974.
22. Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T.S., Kalica, A.R., Chanock, R.M.: Visualization by Immune Electron Microscopy of a 27-nm Particle Associated with Acute Infectious Nonbacterial Gastroenteritis. J. Virol. 10:1075-1081, 1972.

23. Dane, D.S., Cameron, C.H., Briggs, M.: Virus-like Particles in Serum of Patients with Australia-Antigen-Associated Hepatitis. *Lancet* 1:695-698, 1970.
24. Drugman, S., Giles, J.P., Hammond, J.: Infectious Hepatitis. Evidence for Two Distinctive Clinical Epidemiological and Immunological Types of Infection. *JAMA* 200:365-373, 1967.
25. Okada, J., Kamiyama, I., Inomata, M., Imai, M., Miyakawa, Y., and Mayumi, M.: e-Antigen and anti-e in the Serum of Asymptomatic Carrier Mothers as Indicators of Positive and Negative Transmission of Hepatitis B Virus to Their Infants. *New Eng. J. Med.* 294:746, 1976.
26. SEATO Medical Research Laboratory Annual Report 1971-1972.
27. Yamashita, Y., et al: South-North Gradient in Distribution of r Determinant of Hepatitis B Surface Antigen in Japan. *J. Inf. Dis.* 131:567, 1975.
28. Snitbhan R., et al: Subtype of Hepatitis B Surface Antigen in Southeast Asia. *J. Inf. Dis.* 131:708, 1975.
29. Harinasuta, C. et al.: Bancroftian Filariasis in Thailand, A New Endemic Area. *SE Asian J. Trop. Med. Pub. Hlth.* 1:233-245, 1970.
30. Muller, R.: Personal Communication.
31. WHO Expert Committee on Filariasis 2nd Report, WHO Tech Report, 1967, No. 359.
32. Schacher, J.F: Laboratory Models in Filariasis: A Review of Filarial Life-Cycle Patterns. *SE Asian J. Trop. Med. Pub. Hlth.* 4:336-349, 1973.
33. SEATO Medical Research Laboratory Annual Report, 1969
34. SEATO Medical Research Laboratory Annual Report, 1970.
35. Singh, M., Ho, B.: Brienlia booliat. A Filaria of the Malayan Forest Rat, Rattus sabanus. *J. of Hel.* 77:127-133, 1973.
36. Singh, M., Ho, B. and Booliat, L.: Preliminary Results on Experimental Transmission of a New Filarial Parasite, Brienlia booliat from Malayan Wild Rats to Laboratory Albino Rat. *SE Asian J. Trop. Med. Pub. Hlth.* 3:622, 1972.

37. Davidson, D.E., Dill, G.S., Tingpalapong, M., Premabutra, S., Nguen, P.L., Stephenson, E.H. and Ristic, M.: Canine Ehrlichiosis (Tropical Canine Pancytopenia) in Thailand. SE Asian J. Trop. Med. & Pub. Hlth. 6:540-543, 1975.
38. Chapman, H.C.: Biological Control of Mosquito Larvae. Ann. Rev. Entomol. 19:33, 1974.
39. Hazard, E. and Fukuda, T.: *Stempellia milleri* sp. n. (Microsporida: Nosematidae) in the Mosquito *Culex pipiens quinquefasciatus* Say. J. Protozool. 21:497, 1974.
40. Chapman, H.C., Woodard, D.B. and Petersen, J.J.: Pathogens and Parasites of Louisiana Culicidae and Chaoboridae. Proc. N. J. Mosq. Exterm. Assoc. 54:54, 1967.
41. Kudo, R.R.: Protozoology. 5th Ed. C.C. Thomas, Publishers. Springfield. 1966.

Publications:

1. Bailey, C.L. and Gould, D.J.: Flight and Dispersal of Japanese encephalitis Vectors in Northern Thailand. Mosquito News. 35:172-178, 1975.
2. Bailey, C.L., Vongpradist, S. and Mongkolpanya, K.: Observations of an Unusual Oviposition Habit in *Armigeres (Leicesteria) annulitarsis* (Leicester). Mosquito News. 35:564-565, 1975.
3. Bancroft, W.H., Scott, R.M., Snitbhan, R., Weaver, R.E., and Gould, D.J. Isolation of Langkat Virus from *Haemaphysalis papuana* in Thailand. Am. J. Trop. Med & Hyg. 25:500-504, 1975.
4. Benenson, M.W., Scott, R.M., Snitbhan, R., Top, F.H., Jr. and Pantuwatana, S., Grossman, R.A. An Epidemiological Study of Hepatitis B Virus in Bangkok, Thailand. Am. J. of Epidemiol. 101:144-159, 1975.
5. Davidson, D.E., Dill, G.S., Tingpalapong, M., Premabutra, S., Nguen, P.L., Stephenson, E.H. and Ristic, M.: Canine Ehrlichiosis (Tropical Canine Pancytopenia) in Thailand. SE Asian J. Trop. Med. Pub. Hlth. 6:540-543, 1975.
6. Markvong, A., J.T. Marshall, Pathak, S. and Hsu, T.C. Chromosomes and DNA of *Mus*: The Karyotypes of *M. fulvidentris* and *M. dunni*. Cytogenetics and Cell Genetics. 14:116-125, 1975.

7. Marshall, J.T. Karyotypes of Asian Species of Mus. Mammalian Chromosomes Newsletter. 16:19-25, 1975.
8. Marshall, J.T. and Marshall, E. Letters to the Editor (Critical Evaluation of published karyotypes of Gibbons). Mammalian Chromosomes Newsletter. 16:30-33, 1975.
9. Marshall, J.T. and Marshall, E. Gibbons and Their Territorial Songs. Science. 193:235-237, 1976.
10. Ratanaworabhan, N. and Panthusiri, P.: An Illustrated Key to The Female Culicoides of Chiangmai Valley, Thailand. Bangkok: SEATO Medical Research Laboratory, 1-49, 1975.
11. Scott, R.M., Chiewsilp, D. and Bancroft, W.H. The Effect of the Offspring of Maternal Hepatitis B Antigenemia Pediatric Research. 61:299, 1975.
12. Scott, R.M., Nimmannitya, S., Bancroft, W.H. and Mansuwan, P. (Abs. 1976). Serum Complement Levels in Primary Dengue with Shock Syndrome. Malasian Soc. of Pura and Trop. Med. P. 4
13. Snitbhan, R., Scott, R.M., Bancroft, W.H., Top, F.H., Jr. and Chiewsilp, D. Subtypes of Hepatitis B Antigen in Southeast Asia. J. of Infect. Dis. 131:708-711, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL DD DR&E (AR) 636	
3 DATE PREV SUMMARY 75 07 01	4 KIND OF SUMMARY D. Change	5 SUMMARY SCTY ^c U	6 WORK SECURITY ^d U	7 REGRADING ^e NA	8A DISTR INSTR ^f 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 ^g	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62760A	3A762759A831	00	075			
B. CONTRIBUTING							
	CARDS 114F						
11 TITLE (Precede with Security Classification Code) ^h (U) Rickettsial Diseases of Military Personnel							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ⁱ 010100 Microbiology							
13 START DATE 55 08	14 ESTIMATED COMPLETION DATE CONT	15 FUNDING AGENCY DA	16 PERFORMANCE METHOD C. In-House				
17 CONTRACT GRANT			18 RESOURCES ESTIMATE PRECEDING FISCAL YEAR 76	19 PROFESSIONAL MAN YRS 4	20 FUNDS (In thousands) 164		
A. DATES/EFFECTIVE NA	EXPIRATION		CURRENT 77	4	170		
B. NUMBER ^j							
C. TYPE	4. AMOUNT:						
D. KIND OF AWARD	F. CUM. AMT.						
19 RESPONSIBLE DOD ORGANIZATION NAME ^k Walter Reed Army Institute of Research ADDRESS ^l Washington, DC 20012 RESPONSIBLE INDIVIDUAL NAME Joy, Robert J.T., COL TELEPHONE 202-576-3551				20 PERFORMING ORGANIZATION NAME ^k Walter Reed Army Institute of Research Div of CD&I ADDRESS ^l Washington, DC 20012 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME ^m Osterman, Joseph V., DR. TELEPHONE 202-576-2146 SOCIAL SECURITY ACCOUNT NUMBER [REDACTED] ASSOCIATE INVESTIGATORS NAME ⁿ Shirai, Akira, DR. NAME ⁿ Eisenberg, George H.G., Jr., MAJ, MSC			
21 GENERAL USE Foreign intelligence not considered							
22 KEYWORDS (Precede EACH with Security Classification Code) ^o (U) Rickettsial infections; (U) Laboratory diagnosis; (U) Vaccines; (U) Epidemiology							
23 TECHNICAL OBJECTIVE, ^p 24 APPROACH, ^q 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Detection of rickettsial diseases. 2. Improvement of methods for clinical serologic diagnosis. 3. Immunopathological studies in host response to infection with wild type and attenuated rickettsial agents, which represent a serious hazard to troops operating in the field. 24. (U) 1. Development of a monocyte culture technique for early diagnosis of rickettsial disease. 2. Comparison of histopathology following infection with strains of different virulence for mice. 3. Comparison of physical and chemical methods for production of experimental scrub typhus immunogens. 25. (U) 75 07-76 06. 1. A monocyte culture technique for the early diagnosis of Rocky Mountain Spotted Fever has been developed. The technique allows diagnosis from 4-6 days after onset of febrile illness and employs equipment commonly available in any tissue culture laboratory. 2. Host defenses in experimental scrub typhus have been characterized at the histopathological level. Using the inbred BALB/c mouse model, a comparative study was performed on the pathology of sublethal immunizing Gilliam infection, lethal Karp infection and Karp infection of Gilliam-immunized mice. 3. A comparative study of the immunizing capacity of formalinized and gamma irradiated rickettsiae has been performed. Gamma irradiated organisms are clearly superior to formalinized preparations, eliciting higher levels of protection with fewer organisms, and providing substantial protection against heterologous challenge. 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.							

PII Redacted

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 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 075 Rickettsial Diseases of Military Personnel

Investigators.

Principal: Joseph V. Osterman, Ph.D.; Akira Shirai, Ph.D.;
MAJ George H. G. Eisenberg, Jr., MSC
Associate: Janis Campbell; SP4 John Hallam

Description.

To develop new techniques for diagnosis of rickettsial disease and to characterize both the humoral and cell-mediated immunological responses to rickettsial infection.

Progress.

I. Development of new diagnostic techniques.

A. Early diagnosis of Rocky Mountain Spotted Fever using the primary monocyte culture technique.

Rickettsial disease continues to be of great actual and potential significance in the world. During the last six years the incidence of Rocky Mountain Spotted Fever (RMSF) in the United States has increased from an average of 400 cases per year in the 1960's to 774 cases in 1974. Five to seven percent of the infected patients died. As with other rickettsial diseases, diagnosis is based initially on clinical findings because serological confirmation is available only late in the illness. The development of a rapid laboratory diagnostic technique for RMSF would be of great immediate usefulness.

Buhles et al. (1973) identified Rickettsia rickettsii in cultures of circulating monocytes from four experimentally infected guinea pigs at 10 to 14 days after inoculation. Gambrill and Wisseman (1973) have described in detail both the morphology of cultured macrophages and the growth of typhus rickettsiae in experimentally infected human macrophage cultures. The characteristic adherence of monocyte cells to a glass surface was used by both groups of investigators to select and cultivate this subpopulation of circulating cells.

Work by Badger (1933) and Saslaw et al. (1966) has shown the monkey to be a useful host for study of RMSF. They demonstrated that the animal was not only susceptible to the disease, but developed a seemingly identical clinical and histological picture to that seen in man.

This study was performed to further investigate the usefulness of the monocyte culture technique as a diagnostic procedure for human rickettsial disease.

Four 10-12 pound male rhesus monkeys (Macaca mulatta) were assigned to two groups and inoculated subcutaneously with one milliliter (ml) of a yolk sac suspension of R. rickettsii (Sheila Smith strain) adjusted such that two received 1×10^4 and two 1×10^5 plaque forming units (PFU). Monkeys were monitored by daily physical examination and measurement of rectal temperatures. Complete blood count, platelet count, serology by complement fixation, monocyte culture, and plaque assay were performed prior to inoculation and on the second, fourth, and tenth days after the onset of fever. Because the temperature of monkeys varies with excitement, the designation of "second febrile day" was made only after two consecutive days of a rectal temperature greater than 104F.

The monocyte culture technique was performed basically as described by Nyindo et al. (1971). Sixteen ml of blood were drawn aseptically from each monkey into a heparinized (20 units/ml) disposable sterile plastic syringe containing 8 ml of 3% dextran (Pharmachem) in normal saline. The syringe was inverted with the needle end up and allowed to stand for 30 to 60 minutes until the supernatant was clear. The supernatant fluid, consisting of a mixture of dextran and white blood cells, was transferred to a second sterile plastic syringe and gently agitated to distribute the cells throughout the mixture. One ml of this mixture was placed into each of eight sterile Leighton tubes containing alcohol-washed 8 X 35 mm borosilicate coverslips. Culture tubes were incubated horizontally at 35C in an atmosphere of 5% CO₂ for 24 hours, then washed three times with Hanks' balanced salt solution to remove unattached cells. Cultures were maintained in Eagle's minimum essential medium with Earle's balanced salt solution supplemented with 1% glutamine and 20% homologous heat-inactivated monkey serum. Cultures were refed every three days. No antibiotics were employed in any aspect of the monocyte culture technique.

Two coverslips were removed from Leighton tubes on the second, fifth, eighth, and tenth day of culture. One coverslip was stained by the Gimenez technique and one was stained with specific fluorescein-conjugated anti-R. rickettsii rabbit serum. Specificity of the anti-serum was established before use in the study. Uninfected monocyte cultures were used for control purposes. Identification of rickettsiae was based on typical morphological and tinctorial or immunofluorescent characteristics of organisms observed intracellularly. When possible, 250 cells were counted before calling a culture negative.

The disease course in the monkeys was related to the infecting dose. Both monkeys receiving the higher dose of rickettsiae (#1 and #3) became febrile by the fourth post-inoculation day. Monkey #1 remained febrile for three days, showed slight decreases in appetite and activity but did not develop a rash. Monkey #3 remained febrile, became increasingly lethargic and anorectic, and manifested a generalized petechial rash on the fourth febrile day. The monkey died one day later. Histological studies demonstrated diffuse necrotizing vasculitis, typical of *R. rickettsii* infection. In the lower dose group, Monkey #2 was febrile by the fourth post inoculation day, but became afebrile within four days. No clinical signs of the disease were noted other than the fever. Monkey #4 had an elevation in temperature for only one day and did not become clinically ill. Since the hyperthermia could have been induced by excitement, this monkey was not considered to have become febrile.

The estimated levels of rickettsemia by the plaque-formation technique are shown in Table 1. Transiently febrile Monkeys #1 and #2 had approximately 300 rickettsiae/ml on the second febrile day and none subsequently. Monkey #3, which succumbed, had a persistent rickettsemia. Monkey #4, which never became febrile, was not rickettsemic when tested on day 10.

In the surviving monkeys specific complement fixing antibody titers developed by the tenth febrile day. Moderate anemia, leukopenia, and thrombocytopenia developed in the three febrile animals, while Monkey #4 never showed significant hematologic changes.

The results of staining of coverslip cultures of monocytes from Monkeys #1, #2, and #3 by the Gimenez and direct immunofluorescent techniques are shown in Table 2. Rickettsiae were demonstrated by fluorescent staining as early as the fourth and not later than the sixth day after the onset of fever. The fluorescent antibody technique was more sensitive for observing rickettsiae very early in the disease, but by seven days into the illness organisms were clearly discernable by the Gimenez staining technique. Surprisingly, rickettsiae also were demonstrated from a culture taken on the 13th post-inoculation day from Monkey #4, which never became febrile.

The two levels of rickettsial inocula selected for this study produced the full spectrum of clinical illness, from asymptomatic to fulminating and fatal. In the fatal case, clinical and histological observations supported a diagnosis of RMSF similar to that seen in seriously ill humans. The asymptomatic infection seen in Monkey #4 was substantiated by significant increases in specific complement fixing antibodies and demonstration of organisms in monocyte culture preparations. The period of rickettsemia detected by plaque assay correlated well with the febrile response. This technique may have minimized levels of rickettsemia, since detection of small numbers of plaques in undiluted blood was obscured by the presence of red blood cells.

The small size of the monkeys used, and attendant problems with anemia, limited the volume of blood obtained and the frequency of sampling for monocyte culture. Nevertheless, the animals receiving 1×10^5 PFU of rickettsiae had positive cultures which allowed a diagnosis of RMSF to be made as early as four days into their illness. The group receiving 1×10^4 PFU was more difficult to diagnose but were positive by febrile day six. It is possible that, with more frequent sampling and larger numbers of replicates, the time required for diagnosis could be reduced further.

Our work concentrated on diagnosis from onset of the febrile illness to days 9 and 12 when serological data becomes clinically useful. Although all animals had positive cultures by 12 days, data from this time onward are not reported because they lacked diagnostic significance.

No available laboratory method allows diagnosis of RMSF during the critical period from onset of symptoms to development of serological response, which occurs after about 12 days of the illness. The supporting equipment for monocyte culture is available in laboratories with tissue culture capabilities. The technique is relatively simple and the volume of blood necessary is minimal. We believe the success demonstrated here in diagnosis of RMSF warrants investigation of this technique as a diagnostic tool in human rickettsial disease.

Table 1. Estimated levels of rickettsemia (PFU/cc)

Monkey number	Infecting dose (PFU)	Day after onset of febrile illness when culture taken (a)		
		2	4	10
1	1×10^5	3×10^2	0	0
3	1×10^5	9×10^2	2×10^2	ND ^(b)
2	1×10^4	3×10^2		0
4 ^(c)	1×10^4	ND	ND	0

(a) (0) indicates no rickettsemia detected, ND indicates not done.

(b) Monkey #3 died on day 5 after onset of febrile illness.

(c) Monkey #4 was cultured only on day 10.

Table 2. Identification of Rickettsia rickettsii in monocyte cultures from blood of infected monkeys

Monkey number	Infecting dose (PFU)	Monocyte staining technique	Day after onset of febrile illness when culture was first positive (a)
1	1×10^5	Gimenez Fluorescent	7 6
3	1×10^5	Gimenez Fluorescent	7 4
2	1×10^4	Gimenez Fluorescent	6 6

(a) Cultures were read on days 0, 4, 6, and 7. Cultures read on days 4 and 7 were planted on day 2, whereas cultures read on day 6 were planted on day 4 after onset of febrile illness.

II. Characterization of humoral and cell-mediated immunity to rickettsial infection.

A. Host defenses in experimental scrub typhus - Histopathological correlates.

In other studies, we have verified previous observations that the virulence of the Karp strain of Rickettsia tsutsugamushi was far greater than that of the Gilliam strain in mice and that pre-inoculation with an infectious dose of a less virulent strain of scrub typhus protected mice from subsequent challenge with a lethal strain. Finally and most importantly, it was observed that cell-mediated immunity induced by Gilliam immunization was principally responsible for the initial stages of heterologous protection.

The ultimate goal of studies such as these is an understanding of the mechanisms of immune protection. An essential prerequisite for achieving this goal is a thorough knowledge of the pathobiology of rickettsial infection. Although others have studied the pathology of scrub typhus, such reports have not focused on the pathological consequences of infection with a sublethal dose of Gilliam strain nor on the modifications in lethal Karp pathology effected by prior heterologous immunization with Gilliam. Therefore, we decided to compare and contrast the pathology of sublethal, immunizing Gilliam infection with lethal Karp infection and also Karp infection of Gilliam-immunized mice.

Sequential pathological alterations were observed after intraperitoneal inoculation of *R. tsutsugamushi*. There were three experimental groups: (1) animals infected with a lethal dose (1,000 MLD₅₀) of the Karp strain; (2) animals infected with a nonlethal dose (100 MID₅₀) of the Gilliam strain, which, in fact, resulted in immune protection against otherwise lethal Karp challenge; and (3) animals immunized with the Gilliam strain, as above, 3 days before challenge with 1,000 MLD₅₀ of the Karp strain. Groups of three mice were sacrificed on the first and third days postinfection and every third day subsequently. At each time point, a comparable control group was also sacrificed. Regardless of the lethality of the infecting strain, the elapsed time postinfection, or the state of immunity, the histopathological changes were confined to the peritoneal cavity, the peritoneal surfaces of abdominal organs, and the spleen and liver. Lesions outside the peritoneal cavity (excluding the spleen and liver) were not observed in these experiments at the level of resolution of the light microscope. Pathological differences between the three groups of infected mice were usually of a quantitative rather than a qualitative nature, and similar classes of lesions differing only in severity could be demonstrated in each group.

Titration of the stock suspension of Karp in BALB/c mice indicated identical values of 10⁹ MLD₅₀/ml and 10⁹ MID₅₀/ml. The stock suspension of Gilliam was similarly titrated and found to contain 10⁵ MLD₅₀/ml and 10^{8.2} MID₅₀/ml of rickettsiae. The similarity of MLD₅₀ and MID₅₀ values for Karp confirmed its lethality for this strain of mice. The difference between MLD₅₀ and MID₅₀ values for Gilliam reflects its reduced virulence for mice and was the basis for selection of the 100 MID₅₀ immunizing dose. Since the MLD₅₀ and MID₅₀ values were identical for Karp, the inoculum of 1,000 MLD₅₀ contained approximately 10-fold more infectious doses of rickettsiae than the inoculum of 100 MID₅₀ of Gilliam used for immunization.

The peritoneal fluid of a normal mouse was clear, straw-colored, and contained cells free in suspension. These cells were mostly mononuclear, consisting of both lymphocytes and macrophages, but occasional polymorphonuclear leukocytes (PMNs) and mast cells were also observed. During the first 6 days after infection with either strain of *R. tsutsugamushi*, there was a definite increase in the number of peritoneal cells observed, particularly mononuclear cells (Fig. 1). At this time, macrophages were principally of the monocytoïd type. They were of medium size and did not contain prominent granules, vacuoles, or projections (Fig. 2). Rickettsial organisms were not identifiable during this period, but there were numerous examples of lymphocyte-macrophage interaction with occasional lymphoid cell rosette formation around macrophages (Fig. 3). During the initial 6-day period, peritoneal scrapings from the three groups of mice were indistinguishable, but differences began to appear 9 days after infection. At that time, peritoneal fluid from mice bearing lethal Karp infections was thick,

tenacious, and pale reddish-gray, suggesting a fibrinous peritonitis. Microscopically, numerous macrophages containing large numbers of intracellular coccobacillary organisms identical in morphology to scrub typhus rickettsiae were observed (Fig. 4). Further, a sharp increase in PMNs also occurred, but no rickettsiae were observed in these cells. In sharp contrast to lethal Karp challenge, rickettsial organisms were rarely observed in either the group infected with a nonlethal dose of Gilliam strain or the group immunized with Gilliam and subsequently challenged with Karp. The animals from these groups both exhibited an increased number of peritoneal macrophages with large empty vacuoles (Fig. 5), in contrast to the organism-filled vacuoles demonstrated in Fig. 4. This morphological appearance suggested that these macrophages were able to destroy the intracellular rickettsiae. Again in contrast to lethal Karp infection, no comparable increase in PMNs was observed, and fibrinous peritonitis did not occur.

Until 3 days after infection, spleens of infected mice were indistinguishable from those of control animals (Fig. 6). After that time, infection with either Karp or Gilliam resulted in a marked increase in spleen size due principally to an enlargement of the white pulp (Fig. 7). Also evident at this time was an increased prominence of germinal center formation. This trend of splenomegaly due to increase in white pulp continued with time and reached its peak at 12 days in mice infected with Gilliam and in Karp-infected animals, some of which were moribund. The group of mice immunized with Gilliam and subsequently challenged with Karp showed the most striking development of white pulp (Fig. 8), which was first evident at 3 days after Karp challenge. Rickettsial organisms were not observed in histological sections of the spleen in any of the three groups.

Infection with either Karp or Gilliam led to a generalized prominence of Kupffer cells during the first 6 days. At low magnification, the prominent nuclei of the usually flattened Kupffer cells were readily identifiable. After day 6, definite nodular aggregates of cells could be identified, scattered randomly throughout the hepatic parenchyma (Fig. 9). At higher magnification, it was observed that these nodules lacked PMNs and were composed of mononuclear cells, mainly Kupffer cells (Fig. 10,11). After day 9, these mononuclear aggregates or "granulomas" decreased in size and number in animals infected with Gilliam and in animals immunized with Gilliam and subsequently challenged with Karp. However, the converse occurred in Karp-infected animals - the number and size of such granulomas increased until time of death. At no time were rickettsial organisms observed by light microscopy in these granulomas or elsewhere in the hepatic parenchyma. Only Karp-infected animals eventually evidenced a severe fibrinous peritonitis (Fig. 12).

Our results have shown a clear difference between the pathobiology of a nonlethal immunizing infection with Gilliam strain and lethal infection with Karp, as well as the moderating effect of prior Gilliam immunization on lethal Karp challenge. It must be understood, however, that we have purposely selected a small (100 MID₅₀) nonlethal dose of Gilliam for study because it provides effective heterologous immunization and allows the study of cross-immunity between strains of scrub typhus. If we had used a larger, lethal dose of Gilliam, it is possible that the pathological manifestations of infection would more closely resemble those seen with Karp.

The results presented confirmed the reports of others that intraperitoneal scrub typhus infection remains largely confined within the peritoneal cavity. However, two histological observations deserve mention before further discussion of the particular results: (1) the lymphocyte-macrophage interactions depicted in Fig. 3 have been previously described *in vitro* (peripoleosis) and *in vivo* and are thought to represent afferent events in an immune response, probably related to the presentation of antigen by macrophages to immunocompetent lymphocytes; (2) although organisms were not demonstrable within hepatic granulomas, it seemed likely that their appearance and growth were due to the presence of rickettsiae or their products. Such local proliferation of Kupffer cells has been observed in the livers of mice stimulated by Corynebacterium parvum vaccine. However, in the absence of radioautographic and immunofluorescent confirmation, this remains speculative.

In animals infected with R. tsutsugamushi, two phenomena occurred in parallel: (1) those related to proliferation of rickettsia in macrophages and presumably in hepatic granulomas; and (2) those related to concomitant immunization and splenic lymphoid hyperplasia. In the case of Gilliam-infected animals, the phenomena relating to rickettsial proliferation were minimal and transient. Thus, hepatic granulomas appeared briefly and regressed, and there was minimal and finite proliferation of rickettsia in peritoneal macrophages (Fig. 5). These findings were consistent with the host's ability to mount a sufficiently vigorous immune response to deal with the infectious burden. The cellular site of rickettsiacidal activity appeared to be the macrophage. This cell undergoes extensive morphological alterations during infection from the "inactive" monocytoïd state (Fig. 2) to the "angry" macrophage (Fig. 5), capable of destroying rickettsial organisms in its lysosomal complex.

Similarly, an immune response seems to be initiated in a lethal Karp infection, as is evidenced by splenic lymphoid hyperplasia. In contrast to the Gilliam infection, however, rickettsial proliferative

phenomena in peritoneal macrophages (Fig. 4) and the number and size of hepatic granulomata (Fig. 9 to 12) continued to increase until death. These findings are consistent with a host immune response inadequate to deal with the proliferative effects of a highly lethal infection.

It is suggested that survival in scrub typhus infections in mice is the result of a delicate balance between the proliferation of the organism and the intensity of host's immune response. In support of this concept is the experiment in which Gilliam-immunized mice were infected with Karp. Although an equally lethal dose of Karp was given to immune as well as to nonimmune animals, the immune animals displayed a rickettsial proliferative component similar to that observed with a nonlethal Gilliam infection. These immune animals, however, displayed the most striking splenic lymphoid hyperplasia observed in this series of experiments. This suggested that an extremely vigorous host immune response overcame the proliferative capacity of Karp. In this context, preimmunization with Gilliam seems to "prime" the immune system to produce and mobilize sufficient effector cells to deal with the lethal Karp challenge.

III. Development of an inactivated scrub typhus immunogen.

A. Experimental scrub typhus immunogens - Gamma-irradiated and formalinized rickettsiae.

Primary scrub typhus infection renders man solidly immune to reinfection by the homologous strain of Rickettsia tsutsugamushi for at least one year, but protection against the several known heterologous strains wanes rapidly with susceptibility to disease reappearing within months. A similar period of protection against homologous challenge is seen in rodent animal models, but the duration of heterologous immunity is less clearly defined since most studies have tested heterologous resistance within 1-2 months following initial infection. Attempts to develop a safe, effective vaccine for scrub typhus have not been successful, although at least three conceptual approaches have been employed. Vaccination with strains of R. tsutsugamushi considered to be "attenuated" by laboratory or clinical criteria resulted in scrub typhus infections that were indistinguishable from those occurring after natural exposure. Inoculation of pathogenic rickettsiae combined with chemoprophylaxis had limited practical applicability because the technique required a delicate balance between the individual and the rickettsial infection to allow sufficient replication of the organism for adequate immunogenicity, while suppressing overt disease by chemotherapy. Immunization with formalin or merthiolate treated rickettsiae was ineffective in providing protection for man against natural infection, but did protect laboratory animals against moderate homologous challenge.



FIG. 1 Peritoneal scraping prepared 6 days after Gilliam infection. Cellular composition is predominantly mononuclear, consisting of darker-staining small lymphocytes and larger macrophages with pale cytoplasm. Organisms were not identified. (Giemsa, X440)



FIG. 2 Peritoneal scraping prepared 6 days after Karp infection. The cells are predominantly mononuclear macrophages lacking prominent vacuolization. Organisms were not identified. Arrow indicates degenerating PMN. (Giemsa, X1,000)



FIG. 4 Peritoneal scraping prepared 9 days after Karp infection. Arrows indicate large activated macrophages containing cocco-bacillary forms in vacuoles. (Giemsa, XI,000)

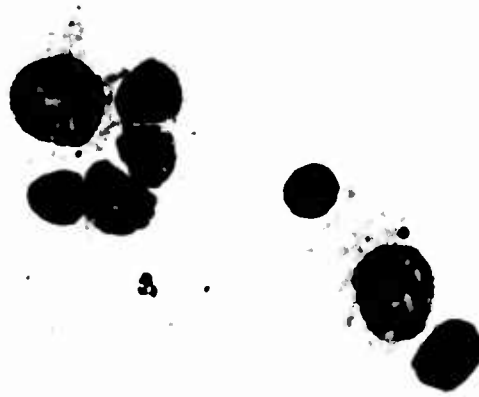


FIG. 3 Peritoneal scraping prepared 3 days after Karp infection. Early in the infectious period with either Karp or Gilliam, intimate contact between lymphocytes and macrophages was observed but was not seen at later times. (Giemsa, XI,000)

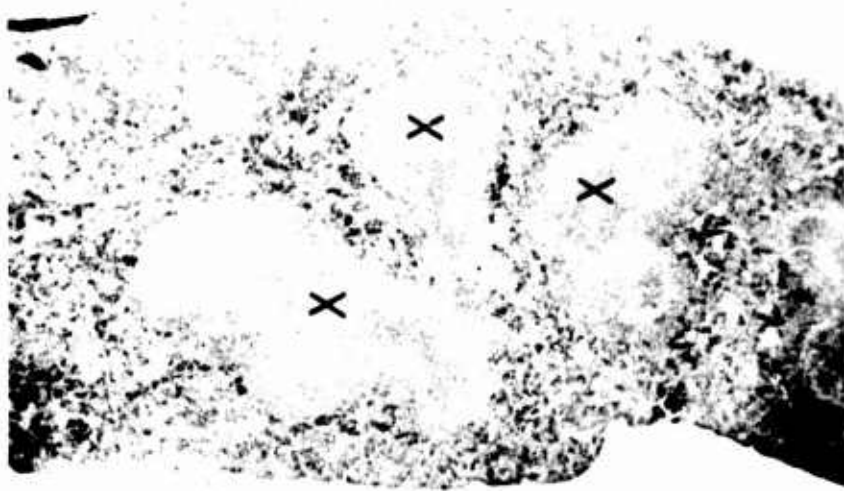


FIG. 6 Low-power view of a spleen 1 day after Gilliam infection. White pulp area is marked by X. This histological appearance is indistinguishable from uninfected animals. (Hematoxylin and eosin, X35)

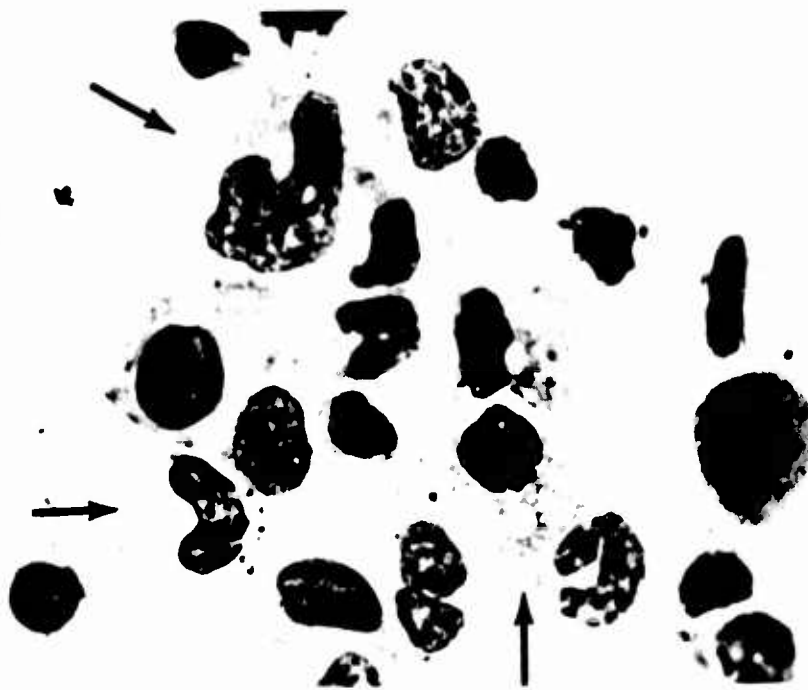


FIG. 5 Peritoneal scraping prepared 13 days after Karp challenge of a Gilliam-immunized animal. Although organisms are not seen, the exudate contains large, highly vacuolated macrophages (arrows). (Giemsa, X1,000)



FIG. 7 Low-power view of a spleen 3 days after Gilliam infection. Note the development of the white pulp (X) at the expense of the red pulp (compare with Fig. 6). (Hematoxylin and eosin, X40)

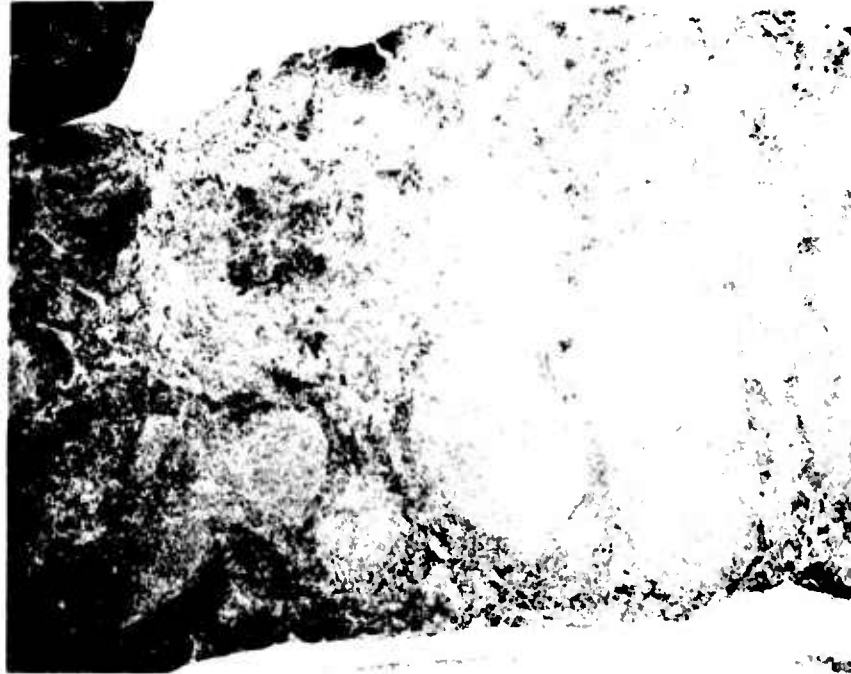


FIG. 8 Low-power view of a spleen 6 days after Karp challenge of a Gilliam-immunized animal. The spleen is composed almost completely of white pulp. (Hematoxylin and eosin, X40)

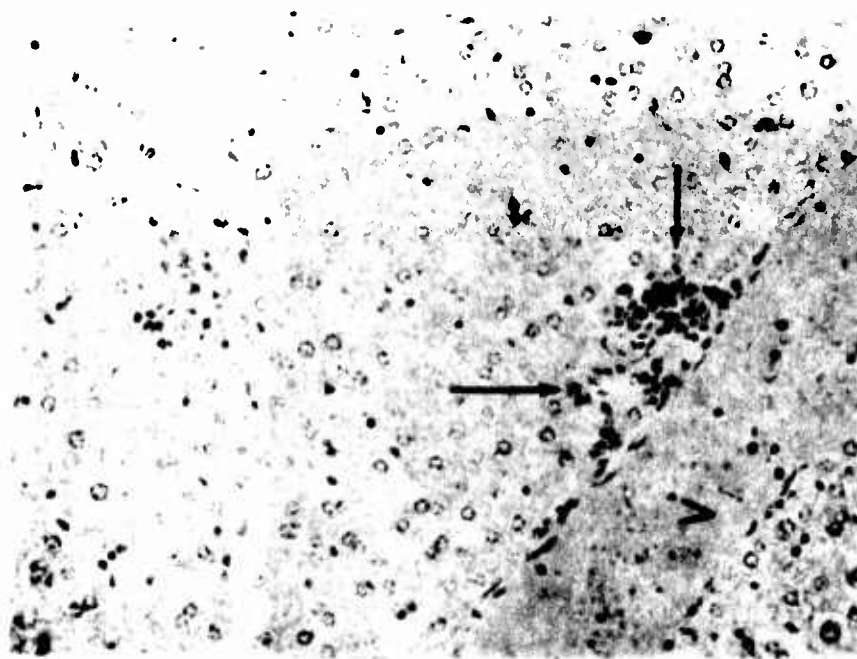


FIG. 9 Liver 9 days after Gilliam infection. Arrows indicate relationship of "granuloma" to large hepatic vein (V). (Hematoxylin and eosin, X250)

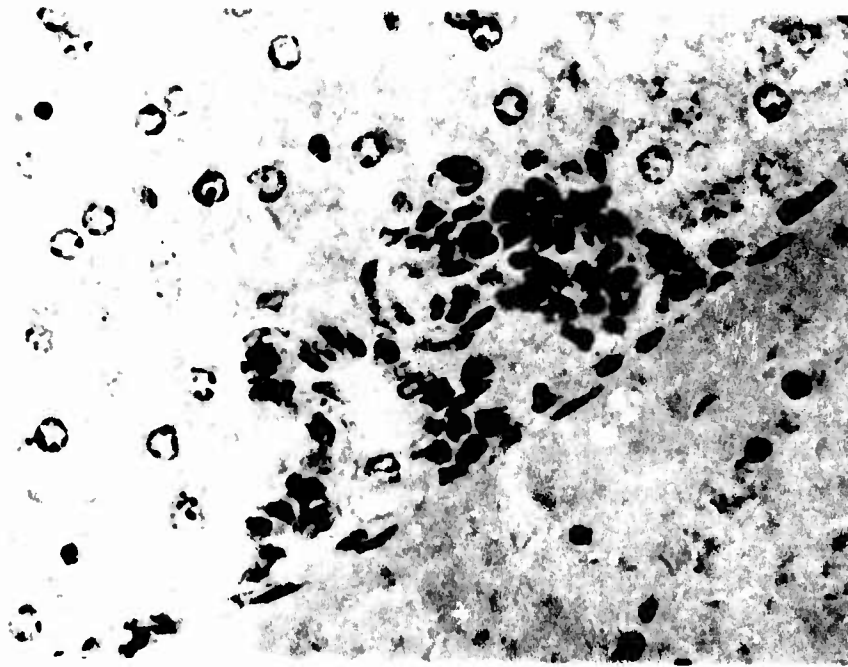


FIG. 10 Higher-power view of Fig. 9. Granuloma consists of mononuclear cells, many of which appear to be Kupffer cells. Organisms were not seen in such lesions. (Hematoxylin and eosin, X620)

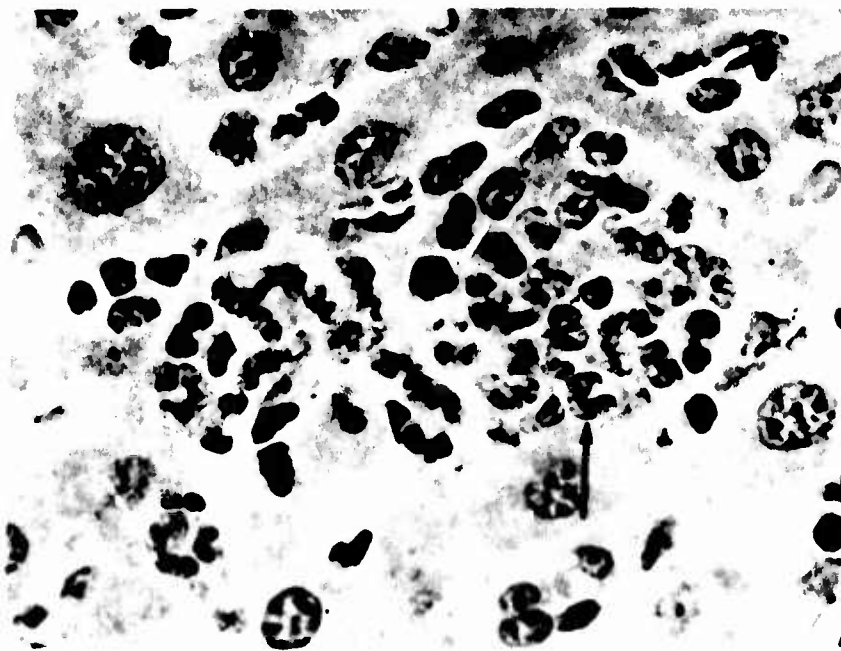


FIG. 11 High-power view of typical hepatic granuloma 9 days after Karp infection. Note the conspicuous absence of PMNs. Some of the cells appear to be Kupffer cells, whereas others appear to be typical monocytoid macrophages (arrow). Organisms could not be demonstrated histologically. (Hematoxylin and eosin, X1,020)

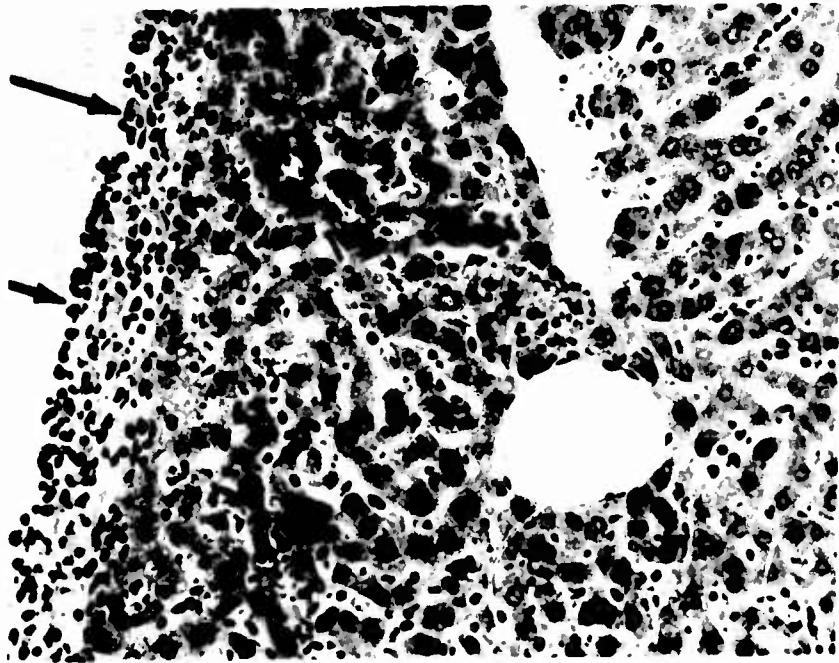


FIG. 12 Peritonitis (arrows) is quite prominent on the surface of the liver 13 days after Karp infection. Such peritonitis is absent from animals infected with Gilliam and those immunized with Gilliam and subsequently challenged with Karp. (Hematoxylin and eosin, X250)

Since chemically inactivated immunogens elicited little heterologous protection and only moderate homologous protection in laboratory animals, we explored inactivation by a physical process. Ultraviolet light has been employed to inactivate rickettsiae, but the necessity of preparing thin films of rickettsial suspensions for exposure to a light source detracted from the usefulness of this technique. Gamma radiation has been used previously for preparation of immunogens from protozoan parasites and is known to inactivate bacteria. Since its penetrating capacity facilitates preparation of large volumes of immunogen, it was chosen for use in this study.

Immunogens were prepared from infected yolk-sac suspensions of R. tsutsugamushi either by exposure to lethal doses of γ -radiation or, for comparison, by addition of formalin and merthiolate. We chose to duplicate the formalinization techniques and the vaccination and challenge schedules of Smadel *et al.* (1946) to insure that comparisons were valid with respect to the capabilities of the formalinized immunogens and to provide a sense of historical continuity to the research. The relative immunizing capacities of the two types of immunogens were contrasted by vaccination of mice followed by homologous and heterologous challenge with highly virulent strains of scrub typhus rickettsiae. These protection tests showed that radiation inactivated preparations were far superior to those containing formalin-killed rickettsiae.

Mouse protection tests showed that formalinized immunogens prepared from two different suspensions of the Karp strain of R. tsutsugamushi provided vaccinated mice with similar levels of protection against homologous challenge, although the preparation containing greater numbers of rickettsiae evidenced a higher immunity index (Table 3). Absolute protection was never achieved, and most surviving mice showed signs of distress during the challenge period. The suspension providing the greatest protection against homologous challenge afforded insignificant protection against challenge with the heterologous Kato strain.

The effect of increasing doses of γ -radiation on the lethality of two different Karp preparations is shown in Table 4. Regression analysis (Table 5) indicates that the radiation-inactivation response is linear, with a slope that is independent of the Karp suspension tested. Linearity is important because mouse mortality due to injection of rickettsial suspensions that had received radiation doses above 100 Krad was too low to permit estimation of surviving rickettsiae. Since the responses were linear and the correlation coefficients similar, the data was used to approximate the numbers of viable rickettsiae remaining after exposure of suspensions to high doses of radiation.

Table 3. Protection of ICR mice vaccinated with formalinized Karp immunogens

Number of MLD ₅₀ of formalinized rickettsiae/injection	Challenge strain	Approximate challenge dose (MLD ₅₀)				Log ₁₀ MLD ₅₀ in vaccinated mice	Log ₁₀ MLD ₅₀ in control mice	Immunity index	
		10,000	1,000	100	10				
3.0 x 10 ⁷	Karp	1/10 ^a	7/10	6/9	9/9	8/10	-5.4 ^b	-8.3	2.9
		5/10	10/10	10/10	10/10	9/10	-4.5	-8.0	3.5
1.8 x 10 ⁸	Kato	0/5	1/10	1/10	1/10	1/10	≤ -7.6	-8.1	≤ 0.5

151

^a Ratio of survivors to total number of vaccinated mice challenged.

^b Values based on exact challenge doses, which were determined from titration of inoculum in control mice and dilution factors used to achieve the approximate challenge doses noted.

Table 4. Effect of γ -radiation on lethality of Karp suspensions

γ -radiation dose (Krad)	Suspension	
	1	2
0	-8.3 ^a	-8.3
1	-8.0	
5	-8.0	
10	-7.2	
25		-7.3
50		-7.2
100	-3.7	-3.8
150		\geq -0.7
200	\geq -0.7	\geq -0.7

^a Values expressed as $\log_{10} \text{MLD}_{50} / \text{g yolk sac}$.

Table 5. Regression analysis of radiation dose - Karp lethality curve

Calculated value	Suspension	
	1	2
$\log_{10} \text{MLD}_{50}$ unirradiated suspension/g yolk sac	-8.1	-8.6
100% lethal γ -radiation dose (Krad) ^a	180	190
Slope ($\log_{10} \text{MLD}_{50} / \text{g yolk}$ sac/Krad)	-4.4×10^{-2}	-4.4×10^{-2}
Correlation coefficient	0.99	0.96

^a Point at which there will be ≤ 1 surviving rickettsia/g irradiated yolk sac.

The calculated 100% lethal dose was 180-190 Krad (Table 5), but on one occasion 4 mice inoculated with a suspension receiving 200 Krad died. The application of a radiation dose in excess of 200 Krad was obviously desirable to provide a safety factor, but it was also important to know the effect of higher doses on the immunogenicity of the rickettsiae. The effects of increased radiation doses on immunogenicity (Table 6) were comparable for the two suspensions and indicated a reciprocal relation between γ -radiation dose and immunogenicity (i.e., an increase in quantity of rickettsiae required for protection indicates a decrease in immunogenicity). However, the decrease in immunogenicity seen with increasing radiation dose was small in comparison to the corresponding decrease in MLD₅₀ of the suspension. Referring to Tables 4 and 6, it can be seen that a 200 Krad dose caused a decrease of approximately 1×10^8 MLD₅₀ while application of an additional 200 Krad dose caused only a 10-fold reduction in the 50% protective dose (PD₅₀). Since an increase of 100 Krad over the calculated 100% lethal dose had relatively little effect on immunogenicity, it was used to provide the necessary safety factor, resulting in the 300 Krad dose which was employed for the remainder of these studies.

Previous experiments (Table 6) established that one injection of 4×10^6 MLD₅₀ of rickettsiae irradiated with the selected dosage afforded mice protection against homologous challenge of 1,000 MLD₅₀. Approximately the same level of protection, evidenced by an immunity index of 2.9 (Table 3), required the injection of a total of 9×10^7 MLD₅₀ of formalinized rickettsiae. This indicated that the irradiated rickettsiae

Table 6. Effect of lethal doses of γ -radiation on immunogenicity of Karp suspensions

γ -radiation dose (Krad)	Suspension	
	1	2
200	1.1×10^{6a}	
250		2.5×10^6
300	4.0×10^6	3.8×10^6
400	1.5×10^7	7.9×10^6

^a PD₅₀ = 50% protective dose, the number of MLD₅₀ of irradiated rickettsiae required to protect 50% of vaccinated mice from a 1,000 MLD₅₀ homologous challenge.

were considerably more immunogenic than the formalized organisms. Studies were initiated to determine if increasing the number of irradiated rickettsiae and modifying the injection regimen would result in further enhancement of protection. Homologous protection was studied after vaccination with 1 and 3 injections of either 10^6 or 10^7 radiation-inactivated rickettsiae (Table 7). Increase in either concentration of rickettsiae or number of injections resulted in increased protection. The immunity indices indicate that protection was heightened 100-fold by increasing the regimen from 1 injection of 10^6 rickettsiae to either 3 injections of 10^6 or 1 injection of 10^7 organisms. Since challenges calculated to contain greater than 10,000 MLD₅₀ were not employed, immunity indices do not clearly differentiate the further increase in protection that may have been achieved by use of 3 injections of 10^7 irradiated Karp. However, from Table 7 it can be seen that this latter regimen provided absolute protection against homologous challenge with at least 10,000 MLD₅₀ of Karp. In addition, mice showed no signs of illness throughout the observation period following challenge.

As a result of the excellent homologous protection demonstrated in this experiment, additional tests were performed to evaluate the protective effect of a two-injection regimen against homologous challenge and the effectiveness of all three regimens against heterologous challenge. As can be seen from the immunity indices in Table 7, all regimens provided substantial protection against heterologous challenge, but a large increase in protection was seen when more injections of immunogen were administered. Within the limits of the experiment, both multiple injection regimens appear to provide approximately the same level of protection. Both schedules protected vaccinated mice against homologous and heterologous challenges of 10,000 MLD₅₀. Homologous protection was absolute with no morbidity observed at any time after challenge. Mice challenged with the heterologous Kato strain showed signs of distress and generally some died at each challenge level, although the immunity indices were of similar magnitude to those seen after homologous challenge.

Studies previously reported by this laboratory indicated that spleen cells obtained from mice surviving infection with small doses of Gilliam strain are able to protect normal recipient mice against heterologous challenge with Karp. Considering these observations, experiments were performed to determine if differences in immunogenicity observed between formalized and irradiated rickettsiae were due to their capacities to activate a cell mediated immune response. Neither homologous nor heterologous protection was observed in mice that received cells from

Table 7. Protection of ICR mice vaccinated with irradiated Karp immunogens

Number of MLD ₅₀ of irradiated rickettsiae/injection	Number of injections	Challenge strain	Approximate challenge dose (MLD ₅₀)		Log ₁₀ MLD ₅₀ in vaccinated mice	Log ₁₀ MLD ₅₀ in control mice	Immunity index
			10,000	1,000			
1.6 x 10 ⁶	1	Karp	2/10 ^a	3/10	2/8	-8.8	2.6
	3		7/10	9/10	8/8	-8.8	> 4.6
1.6 x 10 ⁷	1	Karp	7/10	9/10	10/10	-8.8	> 4.6
	3		10/10	10/10	11/11	-8.8	> 4.9
1.6 x 10 ⁷ 1520	2	Karp	10/10	9/9	10/10	-8.2	> 4.3
	1		2/8	1/10	5/10	-7.7	2.7
	2	Kato	8/8	9/10	7/10	-7.9	> 4.7
			7/10	8/10	5/10	-7.7	4.1

^a Ratio of number of survivors to total number of vaccinated mice challenged.

^b Values based on exact challenge doses, which were determined from titration of inoculum in control mice and dilution factors used to achieve the approximate challenge doses noted.

donors vaccinated with formalinized organisms (Table 8). On the other hand, 80% of the mice receiving cells from donors vaccinated with irradiated immunogens survived subsequent homologous challenge of 1,000 MLD₅₀, and 100% of the recipients resisted homologous challenge of 100 MLD₅₀. Spleen cell recipients challenged with the heterologous Kato strain showed little resistance, with only 20% surviving 100 MLD₅₀ challenge.

The results from serological studies on mice vaccinated using the 3 injection regimen are shown in Table 9. Neither type of immunogen proved to be a potent stimulator of antibody production as assayed by the complement fixation test. On the other hand, mice were capable of responding against the challenge strain and showed excellent antibody titers when tested 4-6 weeks post challenge.

Our results show that the lethality of scrub typhus rickettsiae can be abolished by application of γ -radiation doses in excess of 200 Krad, producing immunogens that are markedly superior to formalinized rickettsiae in protection of mice against both homologous and heterologous challenge. Not only are the mice resistant to larger challenge doses, but the protection is achieved with fewer rickettsiae. Resistance to homologous challenge of 1,000 MLD₅₀ required 9×10^7 MLD₅₀ of formalinized rickettsiae, while a similar level of protection was achieved with 4×10^6 MLD₅₀ of irradiated organisms. When multiple-injection regimens were used with the irradiated immunogens, they routinely elicited homologous protection levels higher than those achieved with formalinized suspensions, but it has not yet been determined if this enhanced protection is due to the temporal regimen employed or is simply the result of accumulating a larger amount of immunogen in the host. The homologous protection with irradiated immunogens was absolute to at least 10,000 MLD₅₀, while formalin-killed rickettsiae were unable to routinely induce absolute immunity at any challenge level. The differences in protection levels induced by the two types of immunogens were even more striking when heterologous challenge was employed. Vaccination with formalinized rickettsiae provided negligible protection against heterologous challenge, the results being quite similar to those reported by Jackson and Smadel (1951). On the other hand, although absolute immunity was not observed, use of multiple-injection regimens with radiation-inactivated organisms resulted in protection of the majority of mice against the heterologous Kato strain.

The mechanism responsible for the heightened protection observed after vaccination with radiation-inactivated scrub typhus immunogens remain undefined. It is possible that radiation inactivation simply

Table 8. Survival of BALB/c mice receiving spleen cells from donors vaccinated with Karp immunogens

Type of immunogen	Number of MLD ₅₀ of inactivated rickettsiae/injection	Challenge strain	Mouse status ^a	Approximate challenge dose (MLD ₅₀)	
				10,000	1,000
Formalinized	1.8 x 10 ⁸ ^b	Karp	Donor	0/5 ^c	2/5
			Recipient	0/5	0/5
	None	Kato	Donor	0/5	0/5
			Recipient	0/5	0/5
γ-irradiated	7.9 x 10 ⁷	Karp	Donor	5/5	5/5
			Recipient	0/5	4/5
	None	Kato	Donor	4/5	5/5
			Recipient	0/5	0/5

^a Donor = vaccinated mouse. Recipient = normal mouse receiving i.p. injection of one mouse-equivalent of spleen cells from donor mouse. Control = normal mouse.

^b Values expressed as MLD₅₀ based on titration of suspensions before inactivation. All mice received 3 i.p. injections of immunogen.

^c Ratio of number of survivors to total number of animals challenged.

Table 9. Complement fixation titers of mice vaccinated with Karp immunogens and of protected mice surviving subsequent challenge

Type of immunogen	Mouse strain	Titer on day of challenge		Challenge strain	Titer of survivors 4-6 weeks post challenge	
		Karp ^a	Kato		Karp ^a	Kato
Formalinized ^b	ICR	10 ^c	< 10	Karp	640	20
				Kato	80	160
	BALB/c	10 ^d	< 10 ^d	Karp	640 ^e	10 ^e
Irradiated ^h	ICR	10	< 10	Karp	320	20
				Kato	20	80
	BALB/c	20 ^d	< 10 ^d	Karp	320 ^e	< 10 ^e

^a Strain of *R. tsutsugamushi* used to make CF antigen.

^b All mice received 3 i.p. injections.

^c Titers expressed as reciprocal of highest dilution showing hemolysis \leq 50%. Lowest dilution tested was 1:10.

^d The antibody titer reported for BALB/c mice on day of challenge represents the humoral response of donor mice at the time their spleens were removed and transferred to recipient mice, who were challenged 8 hrs later with Karp.

^e The antibody titer reported for BALB/c mice 4-6 weeks post challenge represents the humoral response of surviving mice that received one spleen - equivalent of cells from an immunized BALB/c donor followed 8 hrs later by Karp challenge.

causes less structural damage to peripheral macromolecules than dose chemical inactivation, thereby allowing a more effective response by the immune system. Alternatively, it is possible that scrub typhus rickettsiae inactivated by exposure to ionizing radiation retain some physiological capabilities important in stimulating host defenses. Numerous studies have shown that mice surviving active infection are solidly immune to homologous challenge for extended periods of time and to heterologous challenge for shorter periods of time. Recent studies performed in this laboratory have shown that cell-mediated immunity plays an important role in the heterologous protection. Our spleen-cell transfer data revealed that significant levels of homologous protection could be provided to recipients by injection of cells from mice vaccinated with irradiated suspensions, although the failure to achieve substantial heterologous protection required consideration of the possibility that some transferred cells produced antibody that aided in protection against homologous challenge. No protection was observed in mice receiving cells from donors vaccinated with formalinized suspensions nor is cell-mediated immunity elicited by killed preparations of other bacteria capable of intracellular infection (Mitsuhashi *et al.*, 1961; Blanden *et al.*, 1966). These observations, taken in conjunction with the low antibody titers observed after vaccination with either γ -irradiated or formalinized rickettsiae, suggest that differences in immunogenicity are related to stimulation of cell mediated immunity and that properties shared by both infectious and irradiated rickettsiae enhance this type of host response.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 075 Rickettsial Diseases of Military Personnel

Literature Cited.

References:

1. Buhles, W.C., Jr., Huxsoll, D.L., and Elisberg, B.L. Isolation of Rickettsia rickettsii in primary bone marrow cell and circulating monocyte cultures derived from experimentally infected guinea pigs. Infect. Immun. 7:1003-1005, 1973.
2. Gambrill, M.R., and Wisseman, C.L., Jr. Mechanisms of immunity in typhus infections. II. Multiplication of typhus rickettsiae in human macrophage cell cultures in the nonimmune system: influence of virulence of rickettsial strains and of chloramphenicol. Infect. Immun. 8:519-527, 1973.
3. Badger, L.F. An infection of the Rocky Mountain spotted fever type. Public Health Reports 48:463-470, 1933.
4. Saslaw, S., Carlisle, H.N., Wolf, G.L., and Cole, C.R. Rocky Mountain spotted fever: Clinical and laboratory observations of monkeys after respiratory exposure. J. Infect. Dis. 116:243-255, 1966.
5. Nyindo, M.B.A., Ristic, M., Huxsoll, D.L., and Smith, A.R. Tropical canine pancytopenia: In vitro cultivation of the causative agent - Ehrlichia canis. Am. J. Vet. Res. 32:1651-1658, 1971.
6. Blanden, R.V., Mackaness, G.B., and Collins, F.M. Mechanisms of acquired resistance in mouse typhoid. J. Exp. Med. 124:585-600, 1966.
7. Jackson, E.B., and Smadel, J.E. Immunization against scrub typhus. II. Preparation of lyophilized living vaccine. Am. J. Hyg. 53: 326-331, 1951.
8. Mitsuhashi, S., Sato, I., and Tanaka, T. Experimental salmonellosis. Intracellular growth of Salmonella enteritidis ingested in mononuclear phagocytes of mice, and cellular basis of immunity. J. Bacteriol. 81: 863-868, 1961.

9. Smadel, J.E., Rights, F.L., and Jackson, E.B. Studies on scrub typhus. II. Preparation of formalized vaccines from tissues of infected mice and rats. Proc. Soc. Exp. Biol. Med. 61:308-313, 1946.

Publications:

1. Shirai, Akira, Dietel, J.W., and Osterman, J.V. Indirect hemagglutination test for human antibody to typhus and spotted fever group rickettsiae. J. Clin. Microbiol. 2:430-437, 1975.

2. Catanzaro, P.J., Shirai, A., Hildebrandt, P.K., and Osterman, J.V. Host defenses in experimental scrub typhus: Histo-pathologic correlates. Infect. Immun. 13:861-875, 1976.

3. deShazo, R.D., Boyce, J.R., Osterman, J.V., and Stephenson, E.H. Early diagnosis of Rocky Mountain spotted fever using the primary monocyte culture technique. JAMA 235:1353-1355, 1976.

4. Eisenberg, G.H.G., Jr., and Osterman, J.V. Scrub typhus immunogens. Abstracts of the Annual Meeting of the American Society for Microbiology, p. 85, 1976 (May).

Presentations:

1. Eisenberg, G.H.G., Jr., and Osterman, J.V. Scrub typhus immunogens. Annual Meeting of the American Society for Microbiology, Atlantic City, N.J., 1976 (May).

2. Eisenberg, G.H.G., Jr., and Osterman, J.V. Experimental scrub typhus immunogens. Army Science Conference, West Point, N.Y., 1976 (June).

3. Osterman, J.V., Shirai, A., and Catanzaro, P.J. Mechanisms of immunity in scrub typhus. Second International Symposium on Rickettsiae and Rickettsial Diseases, Bratislava, Czechoslovakia, 1976 (June).

Project 3A762758A833
BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00
Biomedical Factors in Drug Abuse

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD DR&E(AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A DRG'S INSTR ^a	8B SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
75 07 01		U	U	NA	NL	9 LEVEL OF SUM A. WORK UNIT	
10 NO CODES ^a		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		62758A	3A762758A833	00	101		
B. CONTRIBUTING							
11 TITLE (Precede with Security Classification Code) ^a (U) Assay Methodology for Drugs of Abuse							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 002300 Biochemistry							
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 ^a				75		8	
C. TYPE				CURRENT		280	
D. KIND OF AWARD				76		6	
E. AMOUNT						150	
F. CUM. AMT.							
20 RESPONSIBLE OOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, DC 20012				ADDRESS ^a Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME ^a 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: Bass, B.G., MS			
				NAME: Kazyak, L., BS			
				DA			
22 KEYWORDS (Precede EACH with Security Classification Code) (U) Mass Spectrometry; (U) Drugs of Abuse; (U) Toxicology; (U) ESR; (U) Chromatography; (U) Immunoassay							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text with security Classification Code.)							
23. (U) The technical objective of this work unit is to develop and evaluate analytical procedures for the assay of drugs and their metabolites and to assess the applicability of these methods to drug abuse detection and treatment programs in the armed forces.							
24. (U) Efforts will be concentrated on the development and evaluation of simple, rapid and accurate systems of drug detection for the operational laboratory, on the establishment of appropriate standardization techniques for the research laboratory as well as the drug screening laboratory, and on the design of sophisticated, highly sensitive and specific analytical methods for investigating the pharmacokinetics of drugs of abuse.							
25. (U) 75 07 - 76 06 The study of methaqualone metabolism in humans was completed. These data reveal a wide individual variation in the patterns and rates of metabolism which appears to be independent of age or previous drug use. These results affirm the validity of using the chromatographic patterns of several methaqualone metabolites for confirmation of screening results. The RIA for cocaine metabolite was evaluated and was found to be remarkably sensitive and remarkably specific for benzoyl ecgonine, the major metabolite. The reagent was not as reactive with cocaine, ecgonine, norecgonine or nor benzoyl ecgonine. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

PII Redacted

DD FORM 1498
1 MAR 68

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

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 101 Assay methodology for drugs of abuse

Investigators.

Principal: LTC Gale E. Demaree, MSC

Associate: Billy G. Bass, M.S.; Ann R. Berman, B.S.; CPT James A. Cella, MSC; James E. Doolittle, A.A.; SP5 Richard E. Droege, B.S.; MAJ Thomas P. Gibson, MC; SP5 Piyush K. Gandhi, B.S.; Leo Kazyak, M.A.; CPT James A. Kelley, MSC; SSG Frank E. Johnson, B.S.; SP5 John T. McLennon; Edward J. Matusik, B.S.; SP5 Joseph K. Stafford; Robert C. Permisohn, M.S.

The technical objectives of this work unit are to develop and evaluate analytical methods for the detection, identification and quantification of drugs of abuse, pharmaceutical compounds and their metabolites in biological fluids and to exploit these techniques for application to the study of the pharmacokinetics of these drugs, to mass screening, rehabilitation and chemotherapy management. Technical efforts were focused on the following principal areas of interest:

1. Metabolism of methaqualone

Seven volunteers received doses of methaqualone according to the following schedule:

- 3 persons received a single 300 mg. dose of methaqualone base
- 2 persons received 300 mg. doses of methaqualone base on two consecutive days (600 mg. total)
- 2 persons received 150 mg. doses of methaqualone base on four consecutive days (600 mg. total)

All urine specimens were collected (casual voiding) beginning with the day prior to the dose and continuing until 48 hours after the final dose. The specimens were analyzed according to the procedure given by Permisohn, Hilpert, and Kazyak (J. of Forensic Sciences, 21:1, 98-107) with the exception that enzymatic hydrolysis was employed instead of acid hydrolysis to convert the glucuronide conjugate to the hydroxylated metabolites. Capillary column gas chromatography was utilized throughout to separate and quantify the metabolite isomers.

Consistent with earlier observations on the initial studies, the 2-methylhydroxy-3-O-tolyl-4(3H) quinazolinone decreased twenty-four hours after the last dose to a concentration that was of the same magnitude as the methaqualone, i.e., less than 0.1 mcg./ml., in marked contrast to much larger amounts of the other four metabolites under

consideration. (First indications were that the 2-methylhydroxy... metabolite decreased within the first ten hours to a barely detectable level, but some of this metabolite may have been destroyed in the acid hydrolysis employed for these analyses).

The cumulative effect of the drug was most evident in both the physiological response and the urinary excretion of the metabolite for the individuals who were given 150 mg. of methaqualone on the four consecutive days. In these individuals a slight incoordination and tiredness and malaise were the only effects after the dose on the first two days. Metabolites were excreted, but the concentration was not as high as this time as evidenced in those persons who received a 300 mg. dose. However, on the fourth day and following the dose on that day, the physiological response was similar to that of a 300 mg. dose, and the drug metabolite concentrations in the urine were significantly higher. For example, the peak concentration of the 2-methylhydroxy... metabolite was equivalent to that of the individual who had received a second 300 mg. dose of methaqualone.

All analyses of specimens from the volunteers have been completed, and the methaqualone metabolite data are being evaluated and prepared for publication.

2. Analysis of N-acetylprocainamide in patients with end-stage renal failure.

When a therapeutic drug or its metabolites can be eliminated only by the kidneys, patients with renal failure may become toxic. Using an analytical procedure developed in this laboratory, serum from patients on maintenance hemodialysis was examined after a single oral dose of procainamide. Levels of N-acetylprocainamide in the renal failure patients were from 0.6 to 3.0 $\mu\text{g}/\text{ml}$ and persisted for periods of over 100 hours. Since this metabolite is as active as the parent compound levels of N-acetylprocainamide must be monitored in renal failure patients to avoid drug toxicity.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 101 Assay methodology for drugs of abuse

Literature Cited.

Publications:

1. Permisohn, R.C., Hilpert, L.R., and Kazyak, L.: Determination of methaqualone in urine by metabolite detection via gas chromatography. J. of Forensic Sciences. 21:(6), 98-107, 1976.
2. Matusik, Edward J. and Gibson, Thomas P.: Fluorometric assay for N-Acetylprocainamide. Clin. Chem. 21(13): 1899-1902 (December, 1975).
3. Gibson, Thomas P., Matusik, Edward J. and Briggs, William R.: N-Acetylprocainamide levels in patients with end-stage renal failure. Clin. Pharmacol. Therap. 19(2): 206-212 (February 1976).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL ³	
				DA OB 6492	76 06 30	DD DR&E(AR)636	
3 DATE PREV SUMMARY ⁴	4 KIND OF SUMMARY ⁵	5 SUMMARY SCTY ⁶	6 WORK SECURITY ⁷	8 REGRADING ⁸	9A DISC N HIST'N ⁹	9B SPECIFIC DATA CONTRACTOR ACCESS ¹⁰	9C LEVEL OF SUB A WORK UNIT ¹¹
75 07 01		U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10 NO CODES ¹²	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
	62758A	3A762758A833		00	102		
b. PRIMARY							
c. CONTRIBUTING							
c. CONTRIBUTING				CARDS 114F			
11 TITLE (Precede with Security Classification Code) ¹³							
(U) Military Performance and Drug Abuse							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ¹⁴							
013400 Psychology 012600 Pharmacology 012900 Physiology 003500 Clinical Medicine							
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				PRECEDE		b. FUNDS (in thousands)	
b. NUMBER ¹⁷				FISCAL		75	
c. TYPE				YEAR		CURRENT	
d. KIND OF AWARD				76		2	
e. CUM. AMT.						258	
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME *Walter Reed Army Institute of Research				NAME * Walter Reed Army Institute of Research			
ADDRESS * Washington, D.C. 20012				Division of Neuropsychiatry			
				ADDRESS * Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DDAR if U.S. Academic Institution)			
NAME JOY, COL R. J. T.				NAME * MANNING, CPT F. J.			
TELEPHONE (202) 576-3551				TELEPHONE (202) 576-2483			
21 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: ELSMORE, T. F., Ph.D.			
				NAME: BOREN, M.C. Ph.D.			
22 KEYWORDS (Precede each with Security Classification Code) ¹⁵							
(U) Drug Abuse (U) Military Performance (U) Drug Tolerance (U) Psychology							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRAM (Furnish individual paragraphs identified by number. Precede rest of each with Security Classification Code) ¹⁶							
23. (U) Specific objective of studies managed within the work unit is to specify the probable impact of drug abuse on military performance using laboratory models and performance measures logically equivalent to critical aspects of military performance. In addition, data are obtained from studies managed within related work units. These data are used to establish the significance of changes in endocrine function, physiology and social environment brought about by the abuse of drugs in terms of their implications for the performance of military personnel.							
24. (U) The techniques of experimental psychology and behavioral pharmacology are applied to the assessment of performance decrements associated with the abuse of drugs. Important data are derived from related studies employing epidemiological, neuroendocrinological and psychophysiological methods for use in constructing an integrated concept of how social, pharmacological, and physiological consequences of drug abuse may interact to alter the performance of military personnel.							
25. (U) 75 07 - 76 06 Studies of self-administration of drugs by baboons indicate that heroin is preferred over morphine infusions even when the unit dose of morphine is 64X that of heroin. Saline infusions may develop conditioned reinforcing properties, and even serve as partial substitutes for heroin. Some behavioral effects of delta-9-THC were greatly potentiated by ethanol, even in ethanol-tolerant subjects. Ethanol effects were also potentiated by THC, even in THC-tolerant animals. Further work by this unit precluded by deletion of drug abuse research funds from DA budget. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

PII Redacted

DD FORM 1498

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

1531

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 102 Military performances and drug abuse

Investigators.

Principal: CPT Frederick J. Manning, MSC
Associate: CPT Steven R. Hursh, MSC; MAJ Frank J. Sodetz, MSC; James Bonbright, M.S.; Mary Carol P. Boren, Ph.D.; Donald Conrad, M.S.; Timothy F. Elsmore, Ph.D.; Gordon V. Fletcher; Deborah M. Rhodus, M.A.

Description.

During FY 76 funds for research on drug abuse were deleted from the DA budget. The experiments reported here are thus to a large extent incomplete. Specific objectives of this work unit have been to specify the probable impact of drug abuse on military performance by means of laboratory models and performance measures logically equivalent to critical aspects of military performance. Research of this type to be reported here may be considered in two general categories: drug-induced decrements in health or performance; and causes of drug self-administration. Both types of work are related to, and have been coordinated with, research being conducted in Work Units 103, 110, and 111, as well as certain MRDC contracts.

DRUG-INDUCED DECREMENTS IN HEALTH OR PERFORMANCE

The role of experience in acquisition and loss of tolerance to the effect of W-9-THC on spaced responding.

Albino rats were given extensive training in spaced responding, using a DRL 30-sec schedule of food reinforcement (only lever presses occurring 30 or more seconds apart produce food). All rats then went 12 days without behavioral testing. During this period half the rats received daily intragastric doses of delta-9-tetrahydrocannabinol (THC, the major active constituent of marijuana) and the rest equal volumes of the THC vehicle. On day 13, all rats receiving THC

3 hours before behavioral testing showed a sharp increase in lever-press rate, over baseline levels and the rates of control rats receiving only vehicle 3 hrs before the session. The rats with 12 prior THC doses were no less affected than those with no previous drug history. Continued testing resulted in recovery of baseline performance by both groups, within 5 sessions, again with no group differences. Similar results were obtained with doses of 4 mg/kg and 16 mg/kg, though the drug's effects were more pronounced at the higher dose. The results demonstrate that performance in the drug state can be a far more important determinant of tolerance than mere exposure to THC. Drug administration was then suspended for one week. One group of rats continued to receive doses of vehicle and DRL sessions, a second received DRL sessions without vehicle, and one group received neither vehicle nor DRL sessions for this week. Subsequent DRL testing after THC administration showed only the groups receiving DRL sessions in the intervening week lost their previously acquired tolerance. Experience thus appears to play an important role in loss of tolerance to THC as well as in acquisition of tolerance.

Effects of delta-9-THC on Sidman avoidance performance in the rat

Rats trained to avoid electric shock to the feet by lever pressing under a temporal schedule of shock presentation (Sidman avoidance) were exposed to delta-9-THC prior to each of at least 14 consecutive daily sessions. The initial effect of the drug was to elevate shock rates in every rat, but with continued drug administration some shock rates remained elevated, some returned to pre-drug levels, and some dropped below pre-drug levels. The degree and direction of this chronic effect was related to the pre-drug performances of the rats, though not in a simple manner. Subjects emitting a high proportion of their presses in the 2 sec immediately following shocks were little effect by chronic THC; those emitting a very low proportion of their responses in this interval showed improved performance under chronic THC conditions; and those with intermediate proportions were impaired by the drug.

Some behavioral effects in rats of administering delta-9-THC and ethanol in combination.

Albino rats were trained to lever-press under a complex schedule of food reinforcement in which different fixed-interval schedules were associated with each of two levers, but no stimuli were provided to indicate to the rat which schedule (and which lever) was in effect at any given time. Both ethanol and THC suppressed responding over a wide dose range, but the effect of THC was much larger when given in combination with ethanol, even when the subjects were made tolerant to the dose of ethanol used in the combination (2.5 g/kg). The converse was also true: THC greatly potentiated the effects of ethanol even though all rats were tolerant to the dose of THC that was used (16 mg/kg). After rats became tolerant to the ethanol-THC combination, drug administration was suspended for 60 days. After this period rats were still tolerant to the THC and ethanol when administered separately, but were no longer tolerant to the combination.

Chronic administration of delta-9-THC: ovarian function in the rat.

A number of recent reports have called attention to the possibility of detrimental effects of THC or chronic marijuana use on the reproductive system. Lowered plasma testosterone in humans has been reported, as well as pathology of the secondary sexual organs of the male rat. Surprisingly, there has been little published on possible effects in females, although it is known that a single 10 mg/kg dose of THC on the afternoon of proestrus will inhibit the normal surge of LH which triggers ovulation some 6-8 hrs later. We have given female rats 10 mg/kg THC each day for 30 days while monitoring their locomotor activity. The latter is a highly reliable, non-invasive, non-obtrusive means of checking the estrus cycle, for female rats increase their activity by as much as 100x during the estrus portion of their cycles. This rhythmicity disappears after ovariectomy and can be restored only by estrogen injections every fourth day. THC produced large drops in the activity levels of all four rats we tested, and in no case was any tolerance to this effect noted in the thirty days. However, a 4-day cycle was still evident in this diminished activity, suggesting that THC had not disturbed the basic mechanisms underlying the estrus cycle. Microscopic examination of the subjects' reproductive systems is now underway.

DRUG SELF-ADMINISTRATION

All of the research to be described below has employed male baboons (*Papio anubis*) prepared with chronic venous catheters to permit the infusion of heroin solutions under a variety of contingencies without handling or interacting with the subject in any way. One such contingency has been the depression of a small lighted disk by the baboon himself. The principal thrust of these experiments has been an examination of variables controlling the acquisition and maintenance of this heroin self-administration by the baboons. It is thus drug-taking itself which is the target of these experiments rather than the disruption of some model of performance. The final stage of this program, which was to be directed at the prevention or elimination of heroin-taking in an environment in which heroin is freely available, has been postponed indefinitely due to Congressional funding decisions.

Ad libitum self-administration of heroin; effects of heroin dose.

This experiment is a continuation of an earlier completed study on the acquisition of heroin self-administration under conditions of free access. In this study, it was found that the rate of acquisition and overall frequency of intravenous heroin self-administration was relatively insensitive to the unit dose of the drug. The present study, then, was an attempt to further explore effects of unit dose. Two baboons were allowed to infuse heroin for 20 to 22 hours daily. A single press on the leftmost of three response keys produced a 0.1 ml infusion of heroin dissolved in saline. The concentration of heroin was adjusted to provide unit doses (micrograms/kg/infusion) ranging from 0.0064 to 500 mcg/kg/inf. Each unit dose was in effect for a minimum of 20 consecutive days. An inverted "U" shaped dose-response curve was obtained, with the maximum infusion frequency occurring between .8 and 4 mcg/kg/infusion. The three doses used in the prior study, 20, 100 and 500 mcg/kg/inf. were all shown to lie at the upper end of the dose-response curve, perhaps accounting for the apparent lack of sensitivity to unit dose found in that study. Even the lowest doses maintained key-pressing rates higher than that for saline. Thus heroin was shown to maintain operant responding even at unit doses of less than 1/78,000 of the largest employed in this study. This unit dose resulted in a total daily heroin intake of less than .01 mg. These results suggest that physical dependence on heroin is not a necessary condition for it to function as a reinforcer.

The acquisition of heroin preference.

The first experiments conducted in-house with baboons self-administering freely available heroin suggested that the rate of "drug" key pressing for heroin did not surpass the rate of "drug" key pressing for saline until after about 30 days of exposure to heroin. This could indicate that heroin does not immediately reinforce responding but requires a period of low level exposure before it becomes an effective reinforcer. This observation seems to square well with informal accounts of heroin use with humans. However, there remained alternative explanations of the 30 day period of baseline responding for heroin. First, the responding on the "drug" key was usually above zero so that some drug was infused immediately after it became available. This was usually reflected in an immediate drop in food intake. It is plausible that heroin intake above this low level was not reinforcing but that the few infusions that were taken were reinforcers. Since every response produced an infusion, it was impossible for responding to increase (demonstrating reinforcement) above baseline without also approaching the subject's physiological limit for heroin. This ceiling could have "masked" the observation of reinforcement. Second, the baseline level of saline infusions may have been unusually high because of the sterility of the environment or superstitious association with simultaneously available food and water.

In order to observe heroin reinforcement without these potential confounding variables, a procedure was developed that permitted two measures of reinforcement that do not require the subject to infuse additional drug. The first measure was obtained by scheduling infusions for responses after a variable interval of time from the onset of a trial; only the first response after that unpredictable interval produced an infusion; earlier responses had no effect. This procedure allowed the rate of responding to increase during the interval without increasing the number of infusions, which was fixed on a random time schedule. Experiments with monkeys working for food scheduled by this procedure show that as a reinforcer increases in value more responses occur for it during the intervals between scheduled deliveries of the reinforcer. This occurs even when the responses have no effect as far as increasing the frequency of reinforcers. The second measure of reinforcement was obtained by providing, simultaneously with the drug key,

a second key that delivered saline on the same schedule. Pressing either the drug key or the saline key would occasionally produce an infusion and then provide the subject access to food. If the subject was indifferent between drug and saline it would press the two keys equally often in order to get the earliest scheduled infusion and turn on the food condition. If heroin was preferred to saline, the subject would distribute proportionally more responses to the heroin key. This preference would indicate reinforcement even if the total number of responses did not exceed baseline.

Last year the results of the first subject were reported. Evidence for a small preference for heroin appeared within two weeks after exposure to the drug (the exact point within this period that heroin demonstrated reinforcement was obscured by a variable baseline preference). After thirty-two days of exposure to heroin, preference increased again from a stable 60% level to 70%. Analysis of absolute response rates for heroin and saline indicate that during the first month of heroin intake, responding for both saline and heroin tended to vary together even though more responses occurred for heroin. After thirty-two days, saline response rate went into a steady decline while heroin response rate remained stable. It appeared that during the early period of heroin intake the subject did not discriminate well between the heroin infusions and the placebo saline infusions. This poor discrimination may have acted to artificially dampen the observation of strong heroin preference during the first thirty-two days of exposure.

This experiment was replicated in a second baboon, with the provision that the alternative to heroin, while still functional in leading to food, did not provide any infusion or other external stimulation similar to an infusion. During the first fifteen days after introduction of heroin no evidence of heroin preference was observed nor was there any general increase in drug or placebo key responding. Between the 16th and 24th sessions, the "placebo" key response rate dropped 62% and the heroin key response rate increased 10%. As a consequence, by the 24th day of heroin availability, heroin preference was nearly 80%. This preference was stable for twelve sessions before the animal was removed from the experiment. This result confirms the conjecture based on the results of the first subject that the initial increase in placebo reinforcers was based upon similarity with

the heroin infusions. When the placebo alternative was made more distinct no increase in placebo responding occurred during the acquisition of heroin preference. In addition, the acquisition of a strong preference for heroin (80%) appeared much sooner, after 24 days for this subject compared to 48 days for the first subject. Thus, the speed of acquisition of heroin preference is in part a function of the nature of the alternative reinforcers. The more similar the alternatives to actual heroin infusions the slower the progress of acquisition. It is not at this time clear whether the mechanism involved is stimulus generalization, the stimulus properties of the "placebo" alternative being confused with the actual heroin reinforcer, or the mechanism may be conditioned reinforcement, with the "placebo" alternative acting as a reinforcer owing to its external similarity to heroin. In either case, increasing the difference between the stimulus properties of the alternatives reduces the amount of responding for the "placebo".

These above experiments confirmed that a reliable preference for heroin requires a period of time to develop and that this "incubation" period varies between subjects and appears related to the nature of alternative consequences for responding. The delay in acquisition thus appeared to be a learning phenomenon-i.e. acquisition of a heroin seeking response required the development of a discrimination between that response and other responses which yield similar immediate but dissimilar delayed consequences (the pharmacological effect). An alternative account might rely on the phenomenon of tolerance-i.e., the value of our heroin dose was low, if not aversive, early in the experiment, until the subjects developed tolerance to some of the aversive effects of the drug. At this point the rewarding properties predominated and the behavioral preference emerged. One strategy to separate these two accounts would be to reverse the consequences of the previous heroin and placebo responses thus requiring reacquisition of the discrimination between these two responses. If this learning process is the basis for the first acquisition period, than reacquisition of this reversed discrimination should be slow also. If pharmacological tolerance was responsible, then reacquisition in a drug experienced animal should be rapid. Reacquisition with the one animal receiving saline placebo infusions versus heroin infusions

was slow, requiring approximately 40 days to reach the previous 80% level of preference. A replication with the second animal was attempted repeatedly but in every case failed because of difficulties with his catheters. On the basis of one subject, we would make the tentative judgement that the acquisition of heroin preference is a learning or behavioral phenomenon, not a pharmacological phenomenon.

The maintenance of heroin preference.

The control of heroin preference once established became the focus of our next set of manipulations. First, it was important to verify the strength of heroin as a reward independent of the other consequence for responding, attainment of access to food. Although food was available after either choice, placebo or heroin, it was not clear to what extent the food consequence controlled the magnitude of preference. This problem was investigated by removing the relationship between drug key responding and onset of food sessions. In this new experiment, each odd numbered hour provided a single choice between heroin and saline while each even numbered hour provided access to food independent of the amount or distribution of drug responding in the previous hour. As a result of this change, the experience subject continued to show a strong 90% preference for heroin, about the same level as prior to the change.

This result established that our dose of heroin (0.1 mg/kg) was a strong motivator independent of other consequences for responding. The next question was the relationship between this level of preference and the size of the dose. We reported above that animals with continuous or free access to heroin emitted more responses per day, taking more infusions, for a 0.02 mg/kg dose than for the 0.1 mg/kg dose used in the preference experiment. One account of this increased responding for a lower dose assumes that the subjects adjust their responding to maintain, as far as possible, some ideal level of heroin concentration in the body. When the dose is lowered, more infusions are required to maintain this concentration, hence the increased responding. In the preference experiment, this factor could not operate because a fixed number of heroin infusions were available per day independent of the rate of heroin responding or

the magnitude of heroin preference. This factor, by the forgoing account, should alter the dose-response rate relationship.

The dose of heroin offered for choice was lowered to 0.02 mg/kg, then to 0.0004 mg/kg. Preference (and response rate) for heroin dropped from 90% to 82% to 70%. A further reduction from 0.004 mg/kg to 0.0008 mg/kg did not produce any appreciable change in heroin preference but further reduced both heroin and placebo responding. A further reduction ending in complete withdrawal from heroin is in progress. These results suggest that the value of heroin as a reinforcer is a direct function of dose, at least up to our maximum of 0.1 mg/kg. This finding also lends support to the general account of free access responding in terms of a behavioral adjustment mechanism motivated by defense of the physiological economy of drug concentration.

Conditioned reinforcement in the maintenance of heroin self-administration in baboons.

Two adult baboons were surgically prepared with chronic venous catheters and afforded an opportunity to infuse heroin (.1 mg/kg). Heroin infusion was associated with a compound auditory and visual stimulus. Through a second catheter inserted in the same vein, each animal could also infuse an equal volume of saline and institute the same stimulus conditions associated with heroin infusion. Heroin infusions were limited to 10 each day, but no limit was placed on the number of saline infusions. After about 12 days of exposure to this procedure heroin infusions stabilized at their limit of 10 infusions/day. Over about 30 days, the distribution of heroin infusions had gradually shifted until all 10 occurred within 5 min of the onset of each daily 20 hr period during which drug and saline were available. Concurrently, the number of saline infusions began to rise markedly, but with a distribution that concentrated most of the saline infusions nearer the end of the session. Thus the longer the animal had been without heroin, the more probable a saline infusion. During withdrawal (substitution of saline for heroin) the number of saline infusions increased to several hundred each day and then gradually fell to basal levels. Responding on the key that had produced heroin was sustained longer than responding on the key that had only produced saline infusions. These data further

exemplify the role of conditioning in the maintenance of drug-self administration behavior. The patterning of saline infusions (with their associated stimuli) strongly suggest that they serve as partial substitutes for infusions.

Three additional baboons have been trained under experimental conditions designed to permit parametric study of the control exerted by environmental stimuli associated with self-administration of heroin. During 20-hour daily periods, 4 sets of stimulus-conditions, associated with food or water delivery, drug or saline infusion respectively, were available for the baboon to select by pressing an illuminated button. Repeated button presses changed the stimulus-conditions sequentially and failure to press the condition-selecting button left the animal in a neutral, non-reinforcing, situation. When a given reinforcement-associated stimulus the reinforcement could be obtained by pressing a second button illuminated with one of the associated stimuli. After acquisition training to establish baseline performances, the availability of stimulus-conditions associated with self-infusion of heroin was manipulated, while the other conditions continued to be available. The heroin-infusions were limited to a maximum of 10 per day for a period of time, eliminated completely to observe the effects of withdrawal, and then reintroduced after a number of days during which the heroin-related stimuli were unavailable. For example, the 10 heroin infusions might be made available only after 1, 2, 4, 8, or 16 days enforced abstinence. Food and water-associated performance is typically suppressed on days heroin is available, and saline infusions, although unlimited, are few. During intervening days food and water performances occur in relatively stable baseline ranges while the frequency of saline infusions gradually increase day by day until heroin is available again.

Choice between heroin and alternative reinforcers.

These experiments are based on the premise that heroin (or drugs in general) is merely one of many alternative reinforcers available in the natural environment. The question being asked is, to what extent can the frequency of heroin self-administration

be manipulated by varying the context in which the choice of heroin occurs. A related series of experiments examined several of the many possible variables controlling choice between food and heroin. In all of these experiments baboons were provided the opportunity to choose between food, and 100 mcg/kg of heroin. On each choice opportunity (trial) two response keys were illuminated. One was always associated with food, and the other with heroin delivery. A press on one key darkened the other, and changed the key that was pressed to red if it was the heroin key, or green, if it was the food key. Completion of an additional number of presses (the completion ratio) on the colored key produced the reinforcer associated with that key. Choice trials were presented for 20 hours each day.

In the first experiment, the amount of food delivered per reinforcer was manipulated in the range of one to four pellets. The interval between choices was always two minutes, and the completion ratio was one response. As the amount of food delivered between reinforcers was increased, the total number of food choices per day decreased, suggesting that since the animals required fewer food choices per day to maintain their daily food intake at a suitable level, more time was available to infuse drugs.

In a second experiment, the interval between choice opportunities was manipulated, with the amount of food per food choice held constant at 4 pellets. Both of the animals studied under these circumstances decreased total daily drug intake more than total daily food intake as the interval between choices was increased. These results, and those of the first experiment can be analyzed in economic terms. The two commodities, food and drug, compete for the available time and behavior of the animal. As constraints are placed on the amount of behavior the animals can emit (analogous to the amount of money available in an economy) the elasticity of the commodities becomes important in determining how the behavior is partitioned. In this particular case, it would appear that heroin is the more elastic of the two commodities, since greater proportional decreases were seen in heroin intake when available choices were restricted.

Preliminary data are available from a third experiment, in which the amount of work required to obtain both reinforcers (the completion ratio) was simultaneously manipulated for both of the alternatives, over the range

from 1 to 80. As the ratio was increased, making it more difficult to obtain either of the reinforcers, intake of both decreased approximately by the same amount.

What these experiments collectively suggest is that heroin intake can be effectively manipulated by altering the context within which choices of heroin occur. The experiments are, however, only suggestive, and further work is required to more clearly delineate the circumstances and the extent to which heroin intake can be altered by changing the context within which the choice of the drug occurs. Particularly, in light of the dose-response experiment cited earlier, it would be important to investigate different unit doses of heroin as the alternative to food.

Choice between heroin and morphine.

The drug literature contains relatively few direct comparisons between heroin and morphine, these being largely comparisons of analgesic potency, and pharmacodynamic properties relating to absorption, distribution, and elimination of the compounds. No direct comparisons of the reinforcing properties of the two compounds exist. Thus, in light of the finding that heroin penetrates the blood-brain barrier much more quickly than morphine (due to its greater lipid solubility), and the well-known psychological fact that immediacy of reinforcement is one of its most important dimensions, the present study was designed to directly compare the reinforcing properties of morphine and heroin.

Two baboons were each prepared with two venous catheters, one for infusion of heroin, and the other for infusion of morphine. Trials were provided four times each hour for 20 hours of the day. Choice trials consisted of the illumination of two keys, one red, and one green, with the position of the colors randomized. A single press on one of the keys turned the other key off, and five additional presses produced an infusion of the drug associated with that key color. Following a choice, the next trial was a forced choice of the non-chosen drug, thus equalizing the total number of infusions of each drug each day. Initial training was begun with equimolar doses of the two drugs

for both animals (0.1 umoles/kg/infusion). Both animals showed a clear preference for the heroin solution within 30 days, choosing the heroin on greater than 90% of all free choice trials. Several control manipulations, switching the catheters, and switching the colors associated with the drugs, failed to disrupt the heroin preference. Within two weeks following each manipulation, the preference for heroin was always reestablished. With one animal, it was possible to conduct a series of manipulations of morphine dose, with the heroin dose held constant. For this animal, the morphine dose was quadrupled three times (0.4, 1.6, and 6.4 umoles/kg/inf.) with no disruption of the preference for heroin, even when the unit dose of morphine was 64 times that of heroin. Catheter failures and physical problems with the animals precluded further data acquisition in this experiment, though continuation of similar work is clearly warranted.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 102 Military Performance and drug abuse

Literature Cited.

Publications:

1. Elsmore, T.F. Differential tolerance to delta-9-THC effect on performance by monkeys on FI and DRL reinforcement schedules. Bulletin of the Psychonomic Society, 1975, 6, 420.

2. Manning, F.J. Chronic Delta-9-Tetrahydrocannabinol. Transient and Lasting Effects of Avoidance Behavior. Pharmacology, Biochemistry, & Behavior, 1976, 4, 17-21.

3. Manning, F.J. Possible serotonergic involvement in morphine-seeking behavior in rats. Bulletin of Psychonomic Society, 1975, 6, 419-420.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OB 6493	76 06 30	DD-DR&E(AK)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ³	6. WORK SECURITY ⁴	7. REGRADING ⁵	8. DRG'S INSTR ⁶	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM
75 07 01	R. Comp.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES ⁷	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62758A	3A762758A833	00	103			
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ⁸							
(U) Drug Abuse Prevention in Military Psychiatry							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹							
013400 Psychology 003500 Clinical Medicine 012900 Physiology 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. DATE/EFFECTIVE: NA				PRECEDING		F FUNDS (In thousands)	
B. NUMBER: NA				FISCAL YEAR		75 6 230	
C. TYPE:				CONCURRENT		76 8 401	
D. KIND OF AWARD:				F. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				ADDRESS: Division of Neuropsychiatry Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish DDAR if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME: Marlowe, D.H., Ph.D.			
TELEPHONE: (202)576-3551				TELEPHONE: (301)593-1996			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Ingraham, CPT L.H.			
				NAME: Sodetz, MAJ F.J.			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Military Adjustment; (U) Psychiatric Illness; (U) Stress Performance; (U) Deviant Behavior							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) This unit examines social, environmental, psychological, and organizational factors that influence the spread of drug abuse. The impact of drug abuse on unit health and the performance of soldiers has also been studied. This study has military relevance for the development of future prevention and treatment programs.							
24. (U) The methods of clinical psychiatry, social psychology, experimental analysis of behavior, anthropology, epidemiology, physiology, and toxicology are used to identify and modify factors which contribute to drug abuse in the military.							
25. (U) 75 07 - 76 06 This work unit has been terminated following the withdrawal of funds for drug research and the reorganization of the Department of Psychiatry. The field phase of the study of the epidemiology of drug and alcohol abuse at a large Army post has been completed. This study is an attempt to determine the environmental and social factors which can be modified to decrease the likelihood of the initiation of drug abuse, disrupt its maintenance, or treat its consequences. The analysis and publication of the materials gathered through urine screening, individual and group interview questionnaires, studies of demography and population dynamics, participant observation and survey is nearly complete. Work was coordinated with experimental psychology, observing primate behavior. The analysis of choice behavior in the presence of available heroin is being studied in primates in an environment characterized by qualitatively different reinforcers. A commander's handbook on Drug and Alcohol Abuse and Soldier's Behavior is being prepared as per tasking by DCSPER. Monographs and scientific articles are being prepared for the professional literature. The analysis data and materials gathered in this work are utilized within the work units 032 and 037. For technical report see WRAIR Annual Report 1 July 75 - 30 June 76.							

PII Redacted

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

1546

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug Abuse Prevention in Military Personnel

Investigators.

Principal: COL Harry C. Holloway, MC
Associate: David H. Marlowe, Ph.D.; CPT George D. Bishop, MSC;
MAJ T. Peter Bridge, MC; E5 Joseph Bruer; MAJ
Rodney V. Burbach, MC; Linda J. Cunningham, BA;
Rosemary A. Diliberto, MSW; MAJ Steven P. Gilbert,
MC; CPT Eugene E. Grossman, MSC; Glenn T. Gurley,
BA; E6 Nelson S. Henry, MAJ William Hollinshead,
MC; MAJ Larry H. Ingraham, MSC; Ellen N. Levine,
MS; E4 James M. Maedke; MAJ John H. Newby, Jr.,
MSC; William G. Palm, MA; MAJ David W. Pearson,
MC; E4 Stephen A. Perkins; E5 Alberto J. Rivas;
Joseph M. Rothberg, Ph.D.; CPT Robert J. Schneider,
MSC; Boyd Stephenson, SSG Charles I. Taylor

Description

This work unit has been terminated due to withdrawal of funds. This work unit consisted of a series of field studies of the epidemiology of drug and alcohol use at an army post in CONUS. The purpose of these studies was to determine individual, environmental, and social factors that contribute to the initiation and maintenance of drug and alcohol abuse. Quantitative data has been coded, and transferred to magnetic tape for analysis that is essentially completed. Qualitative data has been reviewed, indexed, abstracted and analyzed.

Progress

1. Repeated interviews with both illicit drug users and non-users were used to trace the social networks and contacts critical to the transmission and maintenance of drug and alcohol use among individuals in social groups. Tape recordings of the unstructured interviews have been reviewed, indexed, and abstracted. Working papers have been written, and a final report of this phase of the project is being prepared.

2. Participant observers collected data concerning the formal organization of company-sized units; the sets of informal relations within the companies; the interactions between the formal and informal systems; and the performance of both individuals and units in mission-relevant tasks. Abstracts of the field notes and working

papers have been prepared. A draft of the final report has been prepared, and is currently being revised for publication.

3. Observers examined treatment, rehabilitation, and service systems at the post in both the alcohol and drug treatment facilities. The observers' field notes have been reviewed, indexed, and abstracted. Working papers have been prepared.

4. A health diary study using a random sample of soldiers examined health problems and patterns of consultation and therapy that do not come to the attention of the Army medical system. This data has been coded and transferred to magnetic tape, and their analysis is essentially complete. Working papers have been prepared, and a final report of this study is in the final stages of preparation for publication.

5. A random sample of 700 soldiers completed survey instruments designed to map cognitive and organizational factors, attitudes, behavioral repetoires, and patterns of drug and alcohol use. Analysis of these data is essentially complete, and a final report is currently being prepared.

6. Certain conclusions have been drawn from our data and are presented here.^{I-10} Our field studies have found that the relationships between young soldiers are determined by organizational factors such as barracks of residence, platoons and company. Barracks residence or residence with fellow soldiers in an off-post situation seem to provide optimal conditions for peer group relations to influence an individual's behavior, including behaviors relating to illicit use of drugs. The data suggest that these basic living arrangements consistently affect such behavior. Thus, married men living with their wives are a low risk group for drug abuse, while married men living in barracks behave very much like their single barracks mates and are at high risk.

Such barracks living arrangements have considerable influence upon the ease with which drugs are obtained and distributed. Men acquire their drugs largely from their barracks mates, platoon mates or from fellow members of their companies. Soldiers who can be considered pushers or who specialize as dealers in drugs are rare. Rather, members of the group informally provide access to drugs depending upon the individual supplies that exist at a given moment. The drugs which they distribute are often obtained in their home towns while on pass or leave, in the surrounding civilian community, and from other individuals on post. Opportunities for drug acquisition and for the introduction of new drugs

are maximized in this environment. When one man's source of supply disappears one can almost be certain that friends will be able to supply his regular and casual requirements for drugs.

It is important to note that the same groups in which drugs are used also support and encourage their fellows to perform as good soldiers. They encourage them to show up for duty on time and teach them various ways to improve performance as acceptable soldiers. Most of the activities of such groups do not center around drugs but around more mundane everyday social activities. Since these activities do not draw attention to those individuals who illicitly use drugs on a regular basis during off-duty time, they provide a mantle of invisibility for endemic drug using groups. The satisfactory performance of men in such groups makes them invisible to their commanders, who often underestimate the extent of drug use in their companies and battalions. A given individual who uses drugs in an overly blatant manner or performs so poorly as to draw attention to himself is frequently excluded from such groups. Occasionally, such drug users are identified to authorities by their fellow users because they are seen as a danger to the general well-being of the barracks living group. Informants or undercover agents are identified by such groups and then isolated or threatened into silence.

Prevalence data demonstrate that the Army, at present, has an endemic drug problem. There are a large number of men versed in the use and acquisition of drugs, positively disposed to drugs as a normal aspect of their recreational life, and skilled in the discreet use of drugs in a fashion that does not interfere with their routine behavior in garrison. Since they are not addicted, they do not have a fixed physiological requirement for the drugs. They can, therefore, modify their drug usage in response to other social and routine military demands as long as they and their social organization are not stressed by a sudden deployment for operations. Soldiers using drugs in this manner are rarely identified by their superiors as drug users. They regulate the timing of their drug use and choose drugs which minimize the possibility of urine detection. A study of soldiers referred for treatment demonstrates that these men, unlike those just described, have poor work habits, absent themselves from duty, or make a flagrant point of their use of drugs. Thus, in our experience, those soldiers who actually reach treatment facilities represent a population of relatively poor soldiers who also happen to use drugs. ¹¹⁻¹⁴

7. The Behavioral Mathematics section has continued efforts to secure an adequate data base for alcohol and drug related dys-

function. Tabulation of IDPS records is in progress,¹⁵⁻¹⁶ and¹⁷⁻¹⁹ new predictive and analytic techniques are being explored.

8. In anticipation of continued drug and alcohol funding, a series of life history interviews was conducted with residents of the U.S. Soldiers' and Airmens' Home in Washington, DC in an attempt to survey drinking patterns as they relate to life in the Army under various conditions during the period 1900 to 1975. The initial interviews included Army careers that extended from 1900 to the end of WWII. The data from this preliminary effort has been indexed and abstracted, and suggest, impressionistically to be sure, that despite the radical changes in formal structure and operations following WWII that the patterns of alcohol use among lower ranking enlisted men have remained remarkably consistent. This project has also been abandoned.

9. Progress reports of the drug abuse research effort have been communicated to appropriate army agencies, DCSPER has tasked the Division of NP, WRAIR to provide a training manual suitable for company commanders informing them of drug and alcohol use patterns as they relate to company organization and administration. This outline is currently being revised for submission in compliance with the request.

10. As the final reports are completed the results will be published in a series of articles in the professional literature. A projected volume detailing the history, scope, methods and results of the comprehensive project has been abandoned because of drastic changes in personnel that resulted from the loss of drug and alcohol research fund for FY77. For the same reasons, projected studies of the military community with a continuing focus on drug and alcohol use have also been abandoned.

11. Elements of the data and techniques developed in the course of this terminated work unit will be carried forward in the new preventive psychiatry and psychiatric epidemiology work units of the department.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug Abuse Prevention in Military Personnel

Literature Cited

References:

1. Diliberto, R.A. Specialist Caesar: A Case Study of Drug Use and Successful Military Performance. Working paper, Psychiatry Department, WRAIR, 1976
2. Newby, J. Specialist Topman: A Case Study of Drug Use and Outstanding Military Performance. Working paper, Psychiatry Department, WRAIR, 1975.
3. Newby, Jr. and Diliberto, R.A. Sickleside: Behavior Themes and Drug Use Variations. A Study of Social Network Relationships at an Army Post. Working paper, Psychiatry Department, WRAIR, 1976.
4. Ingraham, L.H. The Boys in the Barracks: An Ethnography of Common American Soldiers. Working paper, Psychiatry Department, WRAIR, 1976.
5. Marlowe, D.H., Holloway, H.C. and Hegge, F.W. The Military Environment and Drug Abuse. Working paper, Psychiatry Department, WRAIR, 1975, prepared for the U.S. House of Representatives Appropriations Committee.
6. Way, S.W. Mary Jane: A Case Study of Civilian/Military Interaction in a Military "Strip" Milieu. Working paper, Psychiatry Department, WRAIR, 1975.
7. Way, S.W. Notes from the Inferno: Experiences of a Participant Observer. Paper delivered at the American Psychological Association Convention, Chicago, Illinois, 1975.
8. Ingraham, L.H. Idols of the Experimental Mind. Paper delivered at the American Psychological Association Convention, Chicago, Illinois, 1975.
9. Grossman, E.E. Nonexperimental Field Research: Science or Solism? Paper presented at the American Psychological Association Convention, Chicago, Illinois, 1975.

10. Matthews, R.W. Coming in From the Cold: Graduate Study Following Field Experience. Paper delivered at the American Psychological Association Convention, Chicago, Illinois, 1975.
11. Cunningham, L. Background and History of an Alcohol Rehabilitation Center at an Army Post. Working paper, Psychiatry Department, WRAIR, 1975.
12. Palm, W. The Acceptance of the Sick Role at an Army Drug Rehabilitation Program. Working paper, Psychiatry Department, WRAIR, 1975.
13. Palm, W. The Processes of Social Incorporation and Extrusion in an Army Drug Rehabilitation Program. Working paper, Psychiatry Department, WRAIR, 1975.
14. Palm, W. Notes on the Organization and Operation of an Army Drug Rehabilitation Center. Working paper, Psychiatry Department, WRAIR, 1975.
15. Rothberg, J.M. Alcohol, Drug and Psychiatric Data Summary. Working paper, Psychiatry Department, WRAIR, 1975.
16. Rothberg, J.M. Drug/Alcohol Disease/Symptom Data Summary. Working paper, Psychiatry Department, WRAIR, 1976.
17. Rothberg, J.M. A Small Sample Application of Discriminant Analysis. Presented at the Advanced Seminar on Classification and Clustering, Madison, Wisconsin, University of Wisconsin Mathematics Research Center, 1976.
18. Rothberg, J.M.; Holloway, H.C.; Nace, E.P.; Meyers, A.L.: An Application of Stepwise Discriminant Analysis to the Characterization of Military Heroin Dependent, Illicit Drug Users, and Psychiatric Patients. In press, International Journal of the Addictions, Vol 11, No. 5, 1975.
19. Schneider, R.J. and Rothberg, J.M. Predicting Involvement with Illicit Drugs, Part I: Development of the Instrument. In press, Educational and Psychological Measurements, Fall, 1976 Biannual Test Development issue.

Publications

1. Rothberg, J.M. Alcoholism and the U.S. Army (cont.) Correspondence New England Journal of Medicine, 294: 343, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
				DA OB 6498	76 06 30	DD-DR&E(AR)6.16	
3. DATE PREV. SUMMARY ^c	4. KIND OF SUMMARY ^d	5. SUMMARY SCTY. ^e	6. WORK SECURITY ^f	7. REGRADING ^g	8A. DMS'S INSTR' ^h	8B. SPECIFIC DATA - CONTRACTOR ACCESS ⁱ	8. LEVEL OF DOW ^j
75 07 01	R. Comp.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES ^k	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62758A	3A762758A833	00	105			
b. CONTRIBUTING							
c. NONCONFORMING X	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ^l							
(U) Cellular Aspects of the Metabolism of Drugs of Abuse							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^m							
012600 Pharmacology 012900 Physiology 016800 Toxicology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 10		76 06		DA		C. In-House	
17. CONTRACT/GRANT				19. RESOURCES ESTIMATE		18. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PREVIOUS		b. FUNDS (in thousands)	
EXPIRATION:				75		2.0	
b. NUMBER:				FISCAL YEAR			
c. TYPE:				CURRENT			
d. KIND OF AWARD:				76		2.0	
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: Div. of Medicine Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME: Glinos, A.D. MD			
TELEPHONE: 202-576-3551				TELEPHONE: 202-427-5284			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Bartos, E.M. PhD			
				NAME: Robinson, R.C. DA			
22. KEYWORDS (Precede EACH with Security Classification Code) ⁿ							
(U) Cholinergic fibroblasts, (U) Cultured cells acetylcholine, (U) Cultured cells acetylcholinesterase, (U) Morphine tolerance.							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To investigate the alterations of cellular metabolism underlying the development of tolerance to and dependence on drugs of abuse, an important military problems.</p> <p>24. (U) The possibility that non-neuronal cells may develop tolerance to opiates is investigated by using WRL-10A mouse fibroblasts.</p> <p>25. (U) 75 07 - 76 06 We have previously reported the development of a cell culture system with demonstrable tolerance to morphine both in terms of respiratory activity and of survival. We have further reported that although of connective tissue origin the phenotype of the cells capable of developing tolerance expresses acetylcholinesterase (AChE) activity, in all respects similar to the activity exhibited by cells of neural origin, provided that the cells are maintained as high density growth inhibited populations. We are now reporting that under these conditions in addition to AChE these cells contain also acetylcholine (ACh) in concentrations approximately 20 percent of the concentration of this neurotransmitter in the brain. These results were obtained during the first quarter of the year and established the phenotype of WRL-10A cells as truly unique, because under conditions of exponential growth these cells have the properties of fibroblasts while in the high density growth inhibited phase they express neuronal characteristics. Plans to exploit these exceptional properties for studying further pharmacological responses of interest to the Army were abandoned when commitment of funds for this project was ordered discontinued. (cf. Memo for Record from Deputy Director, WRAIR, dated 24 Sep 75). For technical reports see Walter Reed Army Institute of Research Annual progress Report 1 Jul 75 - 30 Jun 76.</p>							

PII Redacted

DD FORM 1498
1 MAR 68

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

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 105 Cellular aspects of the metabolism of drugs of abuse

Investigators.

Principal: Andre D. Glinos, M.D.

Associate: Edwin M. Bartos, Ph.D.; Richard C. Robinson, B.A.

Description

Attempts to reproduce the phenomena of drug tolerance and dependence in cultured cells thus paving the way for the eventual uncovering of the metabolic processes involved in drug abuse are not new. Beginning with the prewar heroic period of tissue culture¹ and up to the present time² a sizeable number of reports on the subject have accumulated with an approximately equal distribution of positive and negative results. Thus, it is characteristic that recently one group of investigators reported that levorphanol prevented the induction of acetylcholinesterase (AChE) in cultures of mouse neuroblastoma cells without development of either tolerance or dependence², a second group found that in a human neuroblastoma cell line acute exposure to morphine decreased acetylcholine (ACh) levels with no effects on choline acetylase (ChAc) or AChE while chronic exposure resulted in a significant decrease of ChAc and an increase in AChE^{3,4}, and a third obtained elevation of the activity of both ChAc and ChE by adding morphine to 7-day chick embryo neurons in culture but failed to do so if either the embryo or the cultures were pre-exposed to the drug; on the other hand, when the same experiment was performed with mouse neuroblastoma cells exactly the opposite effect was obtained, i.e., morphine had no effect on the enzymes of naive cells but did increase both ChAc and AChE activities when the cells were obtained from tumours born by mice treated with the drug^{5,6,7}. The multitude of cell types, culture methods, treatment schedules and observational criteria used as well as the rather large variance inherent in long-term linear tissue culture experiments are undoubtedly responsible for these inconsistencies. It follows that to answer unequivocally the question as to whether it is possible to reproduce the phenomena of drug tolerance and dependence *in vitro* there is an urgent need to use a well characterized cell culture system in conjunction with a rigorously standardized methodology. At this point, the system does not need to be neuronal as opiate tolerance and dependence have been reported in other cell types with no greater inconsistency than described above for neuronal cells^{8,9}. Accordingly, we undertook a study of tolerance to morphine using WRL-10A cells, a subline of L-929 mouse fibroblasts, developed in our laboratory¹⁰.

It was found that continuous culture of WRL-10A cells in progressively increasing concentrations of morphine resulted in the development of a

cell population which after 15 months of exposure to 1.0 mM morphine remains fully viable, has a slightly depressed growth rate and exhibits only minor cytopathology. Control cells cultured in the same concentration of the drug without pre-exposure, die within 3 weeks. Tolerance to morphine extended also to the respiratory activity of the cells which upon addition of 1.0 mM morphine to the control cells was depressed by 30 - 40% but remained unchanged in cells pre-exposed to 0.75 mM of the drug. As with neuronal tissues, morphine had no effect on basal respiratory rates such as exhibited by high density growth inhibited populations. In terms of survival, however, such populations exhibited considerably greater sensitivity than low density growing cultures. High density populations are characterized by the presence of AChE located on the external surface of the cell membrane and also released into the media. It was further found that addition of morphine inhibits AChE activity in the media while cellular synthesis of AChE is enhanced. These findings are interpreted in terms of a homeostatic mechanism whereby synthesis and release of AChE would be secondary to synthesis and release of ACh and would represent an adaptive process preventing the accumulation of toxic levels of ACh with increasing cell density. By inhibiting AChE activity, morphine in high doses it would induce a compensatory increase of the rate of AChE synthesis.

Progress and Results

The concept outlined above is based on the postulate that WRL-10A cells of fibroblastic origin are capable of synthesizing not only AChE but also ACh. To test the validity of this postulate suspension cultures of WRL-10A cells were sampled at different time intervals during their progression from low density exponentially growing populations through the transition phase to high density stationary populations as previously described¹¹. Since the density of the cultures varied, sample volume was adjusted to yield at all times 5×10^6 cells/ml to which paraoxon, a potent anticholinesterase agent was added at a final concentration of $10^{-6}M$ to prevent the hydrolysis of any ACh present by AChE previously shown to be released by these cells in their media(1). The samples were then centrifuged for 5 min. at 300g in room temperature, the cells separated from the media and washed 3 times with Earle's balanced salt solution also containing $10^{-6} M$ paraoxon. To the cell pellet remaining after the last centrifugation were added 0.5 ml of formic acid (1N,15 vol)-acetone (85 vol) mixture. The cells were then sonicated in a salt ice bath using 5 sec bursts with a 15-20 sec cooling period between bursts. The sonicate was then centrifuged at 500g for 15 min and measured 0.95 ml aliquots of the supernatant removed and assayed for ACh according to the method described by Goldberg and McCaman¹². The results obtained are shown in the following table.

Acetylcholine (ACh) content and acetylcholinesterase (AChE) activity
during the development of high density stationary suspension
cultures of WRL-10A cells

Growth phase	Culture age (days)	Cell density (cells/ml X 10 ⁻⁵)		ACh	AChE
		Initial	Final	nmol/gm of cells (wet wt)	nmol product/min /mg protein
Log	1-3	5.0	21.2	0	Traces
Early Transition	4-6	53.2	98.0	0	Traces
Late Transition	7-10	107.3	110.2	0	.5-1.8
Stationary	11-18	100.1	100.8	4.0-8.5	2.0-6.0

The phenotype reprogramming of WRL-10A cells previously described in regard to only one component of the cholinergic system, i.e. AChE¹³, is thus demonstrated to extend to a second, ACh which is synthesized by these cells in amounts comparable to the brain since the latter contains an average of 18 nmol/g of ACh. In turn, this implies that the cells are capable of synthesizing the enzyme choline acetyl-transferase since its presence is an absolute requirement for the synthesis of ACh. It is of particular interest that the synthesis of AChE appears to precede the synthesis of ACh.

The table also shows that the synthesis of both components of the cholinergic system takes place only after growth has ceased and is therefore an expression of the maturation of the cells. Control experiments carried out with the parent L-929 cell line, from which WRL-10A cells were derived, showed absence of ACh and AChE in both low density exponentially growing cultures as well as in high density growth inhibited populations and the same negative results were obtained in attached cultures of 3T3 and Wi-38 fibroblasts. These results therefore establish the WRL-10A phenotype as unique among fibroblastic lines in its capacity to express components of the cholinergic system when quiescent in high density populations, while behaving as typical cultured fibroblasts of a long established line when growing exponentially in low density cultures. Plans to exploit these exceptional characteristics in investigating further pharmacological responses of military interest were cancelled when, at the end of the first quarter of this year, further commitment of funds for this project was terminated (cf. Memo for Record from Deputy Director, WRAIR, dated 24 Sep 75).

Summary and Conclusions

We have previously reported the development of a cell culture system with demonstrable tolerance to morphine both in terms of respiratory

activity and of survival. We have further reported that although of connective tissue origin the phenotype of the cells capable of developing tolerance expresses AChE activity, in all respects similar to the activity exhibited by cells of neuronal origin, provided that the cells are maintained as high density growth inhibited populations. We are now reporting that under these conditions in addition to AChE these cells contain also ACh in concentrations approximately 20% of the concentration of this neurotransmitter in the brain. These results were obtained during the first quarter of the year and established the phenotype of WRL-10A cells as truly unique, because under conditions of exponential growth these cells have the properties of fibroblasts while in the high density growth inhibited phase they express neuronal characteristics. Plans to exploit these exceptional properties for studying further pharmacological responses of interest to the Army were abandoned when commitment of funds for the project was ordered discontinued.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 105 Cellular aspects of the metabolism of drugs of abuse

Literature Cited.

References:

¹Sasaki, M.: Studies on phenomena of morphine abstinence of cultures *in vitro* of fibroblasts and on curative effect of morphine and its derivatives on them. Arch. Exp. Zellforsch. 21: 289, 1938.

²Hiller, J.M. and Simon, E.J.: Effect of levorphanol on the induction of acetylcholinesterase in a mouse neuroblastoma cell line. Fed. Proc. 31: 757, 1973.

³Manner, G., Foldes, F.F., Kuleba, M. and Deery, A.M.: Morphine tolerance in a human neuroblastoma line: Changes in choline acetylase and cholinesterase activities. Experientia 15: 137, 1974.

⁴Manner, G., Foldes, F.F., Kuleba, M. and Deery, A.M.: Opiate agonist and antagonist effects on acetylcholine metabolism in cultured neurogenic cells. In Vitro 10: 365, 1974.

⁵Peterson, G.R. and Shuster, L.: Effects of morphine on choline acetyltransferase and acetylcholinesterase in cultured mouse neuroblastoma. Proc. West. Pharmacol. Soc. 16: 129, 1973.

⁶Peterson, G.R. and Shuster, L.: Effects of narcotic analgesics on enzymes of acetylcholine metabolism in cultured chick brain cells. Fed. Proc. 32: 757, 1973.

⁷Peterson, G.R., Webster, G.W. and Shuster, L.: Effect of narcotics on enzymes of acetylcholine metabolism in cultured cells from embryonic chick brains. Neuropharmacology 13: 365, 1974.

⁸Corssen, G. and Skora, I.A.: "Addiction" reactions in cultured human cells. J. Amer. Med. Assoc. 187: 328, 1964.

⁹Ruffin, N.E., Reed, B.L. and Finnin, B.C.: Effects of morphine withdrawal on cells in continuous culture. Life Sciences 8 (II): 671, 1969.

¹⁰Glinos, A.D. and Hargrove, D.D.: Interrelations among chromosome number, type and size in L strain cells. Exp. Cell Res. 39: 249, 1965.

¹¹Glinos, A.D. and Werrlein, R.J.: Density dependent regulation of growth in suspension cultures of L-929 cells. *J. Cell Phys.* 79: 79, 1972.

¹²Goldberg, A.M. and McCaman, R.E.: An enzymatic method for the determination of picomole amounts of choline and acetylcholine. In *Choline and Acetylcholine: Handbook of Chemical Assay Methods*, I. Hanin, ed., Raven Press, New York, p. 47, 1974.

¹³Glinos, A.D. and Bartos, E.M.: Density dependent regulation of growth in L cell suspension cultures III. Elevation of specific activity of acetylcholinesterase. *J. Cell. Physiol.* 83: 478, 1974.

Publications:

1. Bartos, E.M. and Glinos, A.D.: Properties of growth related acetylcholinesterase in a cell line of fibroblastic origin. *J. Cell Biol.*, 69: 638, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ³	6 WORK SECURITY ⁴	7 REGRADING ⁵	8A DR&E INSTR ⁶	8B SPECIFIC DATA - CONTRACTOR ACCESS	9 LEVEL OF SUM
75 07 01		U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
10 NO CODES ⁷	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	62758A	3A762758A833		00	106		
B. CONTRIBUTING							
C. CONTRIBUTING	Cards 114F						
11 TITLE (Precede with Security Classification Code) ⁸							
(U) Clinical and Demographic Studies of Military Drug Abusers							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹							
003500 Clinical Medicine 012600 Pharmacology 016800 Toxicology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
73 07		76 06		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 FUNDS (in thousands)	
A. DATES/EFFECTIVE N/A				B. PRECEDING		C. PROFESSIONAL MAN YRS	
D. NUMBER *				FISCAL YEAR		70	
E. TYPE				G. CUM. AMT.		8	
F. KIND OF AWARD				H. YEAR		76	
I. AMOUNT				J. CUM. AMT.		.5	
20 RESPONSIBLE OGD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME * Walter Reed Army Institute of Research				NAME * Walter Reed Army Institute of Research			
ADDRESS Washington, D.C. 20012				ADDRESS Division of Neuropsychiatry Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME if U.S. Academic Institution)			
NAME Joy, COL R.J.T.				NAME * Marlowe, D.H., Ph.D.			
TELEPHONE (202)576-3551				TELEPHONE (301)427-5210			
22 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Ream, LTC N.W.			
				NAME: Blanck, LTC R.R.			
23 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Hepatitis B Antigen and Antibody; (U) Pulmonary Functions; (U) Immunoglobulins; (U) Demographic Variables							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede rest of each with Security Classification Code.)							
23. (U) Drug abuse has recently been a major problem in military populations. The objectives of these studies are to obtain information on medical problems related to drug abuse and to examine demographic variables and drug use histories of a military population.							
24. (U) Clinical studies evaluated medical complications of drug abuse by extracting data from clinical records. A review of drug associated death in USARV was done. Clinical laboratory studies done and/or results analyzed in heroin and non-heroin using military populations (USARV) include: biochemical studies by procedures according to Hycel; hepatitis B antigen and antibody determination by radioimmunoassay; determination of immunoglobulins by the immunodiffusion technique; pulmonary functions by means of an electric spirometer. Analysis of demographic variables and drug use histories of heroin and non-heroin using military (USARV) populations is in progress by frequency analysis and cross-tabulation.							
25. (U) 75 07 - 76 06 This work unit has been terminated following the withdrawal of funds for drug research and the reorganization of the Department of Psychiatry. A study of clinical aspects of acute abstinence in 320 heroin dependent USARV soldiers and a review of drug associated deaths in USARV has been completed. Data from laboratory studies in heroin and non-heroin using soldiers (USARV) were analyzed. Frequency analyses and cross tabs have been completed on demographic variables and drug use histories in 3484 non-heroin using soldiers (USARV). Equivalent data on users is being card punched. The analysis data and materials gathered in this work are utilized within the work units 032 and 079. For technical reports see WRAIR Annual Report 1 July 75 - 30 June 76.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) OBSOLETE

1560

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 106 Clinical and Demographic Studies of Military Drug Abusers

Investigators.

Principal: David H. Marlowe, Ph.D.
Associate: LTC R. R. Blanck, MAJ Willima Hollinshead,
MC; LTC Norman W. Ream, MC

Description

These studies involved demographic analyses of drug users and non-users in Viet Nam.

Progress

These studies have now been terminated. At present, following the transfer of the principal investigator during the past year, the materials have been put on punch cards and further analysis will take place on those segments of the data pertinent to Psychiatric Epidemiology and Preventive Psychiatry.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A DISB INSTR ^a	8B SPECIFIC DATA CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9 LEVEL OF SUM A WORK UNIT
74 07 01		U	U	NA	NL		
10 NO CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62758A	3A762758A833	00	109			
B. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11 TITLE (Precede with Security Classification Code) ^a							
(U) Neurophysiological Localization of Sites of Action of Drugs of Abuse							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
012600 Pharmacology 012900 Physiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
73 07		76 06		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE N/A				PRECEDING		B. FUNDS (In Resources)	
B. NUMBER ^a				FISCAL YEAR		C. CURRENT	
C. TYPE				75		2	
D. KIND OF AWARD				76		71	
E. AMOUNT							
F. CUM. AMT.							
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, D.C. 20012				ADDRESS ^a Div of Neuropsychiatry Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (FURNISH NAME IF U.S. Academic Institution)			
NAME JOY, COL R.J.T.				NAME ^a Spector, N.H., Ph.D.			
TELEPHONE 202-576-3551				TELEPHONE 202-576-3457			
22 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME Koob, G.F., CPT			
				NAME Martin, G.E., Ph.D.			
23 KEYWORDS (Precede EACH with Security Classification Code) ^a (U) Heroin; (U) Temperature Regulation; (U) Feeding Behavior; (U) Intracranial Self-Stimulation; (U) Catecholamines; (U) Morphine							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) This research attempts to identify areas of the central nervous system which are most affected by drugs of abuse. These physiological studies may provide a basic understanding of the mechanisms of drug-induced alterations in the functioning of the nervous system and eventually lead to effective treatment of military personnel using and misusing drugs.							
24. (U) The techniques of neurophysiology, neuropharmacology and physiological psychology are used.							
25. (U) 75 07 - 76 06 Effects of restraint on the alterations in body temperature in the rat caused by systemic and intracerebral injection of morphine and heroin have been examined: these opiates were found to cause increased body temperature in free-moving rats but a temperature decrease in restrained rats. Microinjection of morphine into the rat hypothalamus, and related areas, made to localize the anatomic site of the effects, also showed dissimilar results dependent on the rat's mobility: marked temperature increases accompany injections in a subsequently free-moving animal while lesser temperature rises were seen after injections in subsequently restrained animals. Both effects were blocked by microinjection of morphine antagonist in the same brain regions. Further work in this unit precluded by deletion of drug abuse research funds from DA budget. For technical report see Walter Reed Army Institute of Research Annual Progress Report 01 July 75 - 30 June 76.							

PII Redacted

DD FORM 1498
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 109 Neurophysiological localization of sites of action of drugs of abuse

Investigators

Principal: N.H. Spector, Ph.D.
George F. Koob, CPT, MSC; Gregory E. Martin, Ph.D.

DESCRIPTION

This research has continued to pursue the objective of a neurophysiological localization of sites of action of drugs of abuse and related compounds using behavioral and physiological techniques.

PROGRESS

Effects of Opiates and Restraint on Body Temperature

Two studies have been completed concerning the effects of restraint on the body temperature changes which occur following administration of morphine sulfate (MS) or heroin hydrochloride (H) to the rat. In the first, the effect of an intraperitoneal (IP) injection of MS or H on body temperature in restrained and free-moving rats was compared; this work involved development of a chronically implanted temperature measuring system which does not impair the rat's movement (A method for the continuous chronic measurement of core temperature in small animals - Pryzbylik, A.T., G.E. Martin, and N.H. Spector, 1976, submitted to J. App. Physiol.) Results show that 30 mg/kg of MS or 5 mg/kg of H cause severe hypothermia in restrained rats but hyperthermia in free-moving rats. Furthermore, the hyperthermic response in the free-moving rats is relatively difficult to suppress with Naloxone and shows no development of tolerance with repeated MS injections, while the hypothermic response of the restrained rats is more easily suppressed with Naloxone and on repeated injections becomes a hyperthermic response.

In the second study, the effect of restraint on these body temperature changes was examined after microinjection of MS directly into the hypothalamic areas implicated in temperature control. In free-moving rats, a wide range of directly-injected MS doses caused subsequent hyperthermia, but in restrained rats the same doses produced attenuated hyperthermia or hypothermia. In collaboration with the Department of Experimental Psychology, an attempt was made to determine whether the

hyperthermic responses could be secondary to generally increased motor activity. Using standard behavioral techniques for monitoring activity, experiments showed that the initial temperature rise following ip injections of MS is accompanied by a period of increased activity, but that a second episode of hyperthermia which starts about 3 hours post-injection is not. When MS is injected directly into the hypothalamus, an immediate body temperature rise is seen in company with an immediate increase in body activity.

These studies indicate the importance of assessing general body activity or inactivity, including restraint, when examining the effects of drugs of abuse on behavior and body physiology.

Effects of Clonidine on Systemic Blood Pressure

Earlier studies in this Department have examined the effects of clonidine -- a potent agonist of central adrenergic receptors -- on several behavioral measures (food and water intake, lever-pressing to obtain rewarding intra-cranial stimulation, locomotor activity) and on body temperature. Those studies indicated that low doses of the drug produce increased food and water intake and lever pressing, plus a slight rise in body temperature; but that higher doses of the drug cause a fall in body temperature and inhibition of the behavioral variables noted. In an effort to determine whether the reduced behavior following high doses is a specific inhibition of some adrenergic system, or a general depression of the brain and body, the blood pressure of unanesthetized rats was measured following administration of several different doses of the drug. The results corroborate the earlier findings concerning body temperature: high doses of clonidine produce dramatic decreases in systemic blood pressure. These experiments indicate that the sharp fall in body temperature and blood pressure underlie the reduction of behavior following high doses of the drug.

Research in this work unit was terminated 30 June 1976.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 109 Neurophysiological localization of sites of action of drugs of abuse

Literature Cited.

Publications:

1. Koob, G.F., Spector, N.H., and Meyerhoff, J.L.: Effects of heroin on lever pressing for intracranial self-stimulation, food and water in the rat. *Psychopharmacologia* 42: 231-234, 1975.
2. Martin, G.E. and Myers, R.D.: Evoked release of [¹⁴C] norepinephrine from the rat hypothalamus during feeding. *Am. J. Physiol.* 229: 1547-1555, 1975.
3. Martin, G.E., Myers, R.D., and Newberg, D.C.: Catecholamine release by intracerebral perfusion of 6-hydroxydopamine and desipramine. *European J. Pharmacol.* 36: 299-311, 1976.
4. Martin, G.E. and Myers, R.D.: Dopamine efflux from the brain stem of the rat during feeding, drinking, and lever-pressing for food. *Pharmacol. Biochem. Behav.* 4: 551-560, 1976.
5. Martin, G.E., Pryzbylik, A.T., and Spector, N.H.: Morphine: rise or fall in core temperature as a function of restraint. *Fed. Proc.* 35: 530, 1976.
6. Meyerhoff, J.L., Martin, G.E., and Koob, G.F.: Effects of Clonidine on self-stimulation, eating, drinking and body temperature in the rat. *Proc. Soc. Neuroscience* 1: 479, 1975.
7. Waller, M.B., Myers, R.D., and Martin, G.E.: Thermoregulatory deficits in the monkey produced by 5, 6-dehydroxytryptamine injected into the hypothalamus. *Neuropharmacology* 15: 61-68, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ¹	2 DATE OF SUMMARY ²	REPORT CONTROL SYMBOL DD FORM (AR) 1498	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY S.C.T.Y. ³	6 WORK SECURITY ⁴	7 REGRADING	8a DISTR INSTR ⁵	8b SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM A WORK UNIT
75 07 01		U	U	NA	NL	<input type="checkbox"/> YES <input type="checkbox"/> NO	
10 NO CODES ⁶	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62758A	3A762758A833	00	110			
B. CONTRIBUTING							
C. CONTRIBUTING	ORDS 114F						
11 TITLE (Precede with Security Classification Code) ⁷							
(U) Biorhythm Studies in Drug Abuse							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ⁸							
013400 Psychology 012900 Physiology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
73 07		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 ⁹				75		4	
C. TYPE				CURRENT		240	
D. KIND OF AWARD				76		4	
E. CUM. AMT.						279	
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME ¹⁰ Walter Reed Army Institute of Research				NAME ¹¹ Walter Reed Army Institute of Research			
ADDRESS ¹² Washington, DC 20012				ADDRESS ¹³ Division of Neuropsychiatry Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. academic institution)			
NAME ¹⁴ Joy, COL R. J. T.				NAME ¹⁵ Hegge, F. W. Ph.D.			
TELEPHONE ¹⁶ 202-576-3551				TELEPHONE ¹⁷ 202-427-5521			
22 GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME ¹⁸ Ream, LTC N. W.			
				NAME ¹⁹ Tahmoush, LTC A. J.			
23 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Drug Abuse; (U) Biorhythms; (U) Heroin; (U) Abstinence Syndrome							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number Precede text of each with Security Classification Code.)							
23. (U) Achievement of an understanding of changes in the temporal organization of biological functions attendant upon sustained use of drugs of abuse and upon cessation of drug use in military personnel. Information developed serves to explicate mechanisms of drug action, biomedical consequences of sustained abuse, and post-detoxification readdiction liability. Therapeutic implications are explored.							
24. (U) Sophisticated electronic monitoring and bioproduct sampling techniques are employed to generate long, nearly continuous electrophysiological, behavioral, and biochemical measures of biologic functioning during periods of sustained drug use and abstinence. Time series analysis techniques are applied to these data to achieve a full characterization of similarities and differences between normal and drug abusing individuals.							
25. (U) 75 07 - 76 06 This work unit has been terminated as a result of the re-organization of the Division of Neuropsychiatry. The analysis of electrophysiological, biochemical, and behavioral data derived from heroin users undergoing complete abstinence continued. The abstinence metric previously developed was improved and techniques for quantifying abnormalities in biorhythmic functions related to sleep, activity, and heart rate were further developed and applied. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 75 - 30 JUN 76.							

PII Redacted

DD FORM 1498

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

1566

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 110 Biorhythm studies in drug abuse

Investigators.

Principal: Frederick W. Hegge, Ph.D.
Associate: LTC Norman W. Ream, MC; LTC Albert J. Tahmoush,
MC; CPT John G. Varni, MSC; Paul Kasper, B.S.;
Jeanne C. Stringfellow, B.A.

Description

This work unit is directed at the understanding of changes in the temporal organization of biological functions attendant upon sustained use of drugs of abuse. To date, principal emphasis has been focused on the sequelae of heroin abstinence. The information developed serves to explicate mechanisms of drug action, to delineate the functional consequences of sustained abuse, and to assist in the assessment of post-detoxification readdiction liability. The technology employed involves a variety of techniques for continuously monitoring electrophysiological variables and for sampling behaviors, clinical parameters, and biological fluids. Data are analyzed using a variety of time series statistical procedures.

Progress

As a result of the non-appropriation of funds in support of drug abuse research, this research was terminated at the end of the first quarter of the fiscal year. Personnel associated with this research were reassigned to the Follow-up Study of Soldiers Given LSD and to studies of military stress.

In order to save portions of the technology developed under this work unit, an interagency agreement was reached with the National Institute of Drug Abuse. Work on the development and application of a scaling metric for opiate abstinence has been transferred to this Agency. Other efforts, dealing with the assessment of chronobiological factors in human psychophysiology has been applied to the development of a realistic simulation of military stress using an artillery fire direction center model.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OB 6509	76 06 30	DD-DR&E(AR)636	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ³	6. WORK SECURITY ⁴	7. REGRADING ⁵	8. DES'N INSTR ⁶	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM
75 07 01	NA - Comp.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES ⁷	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62758A	3A762758A833	00	111			
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ⁸							
(U) Neuroendocrinology and Neurochemistry of Drugs of Abuse							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹							
003500 Clinical Medicine 012900 Physiology 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
73 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)	
EXPIRATION:				FISCAL YEAR		75	
b. NUMBER:				CURRENCY		1.5	
c. TYPE:				76		4	
d. AMOUNT:						105	
e. CUM. AMT.						380	
20. RESPONSIBLE OOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution)			
NAME: Joy, COL, RJT				NAME: Meyerhoff, JL, MD			
TELEPHONE: (202) 576-3551				TELEPHONE: (202) 576-3559			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Lenox MAJ RH			
				NAME: Kant GJ			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Drug Abuse; (U) Psychophysiology; (U) Treatment/Rehabilitation; (U) Neuroendocrinology; (U) Stress							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Principal objective is to establish the neurochemical and endocrinological consequences of the abuse of psychoactive compounds, including such phenomena as tolerance and withdrawal. The over-all objective is to better specify the impact of drug abuse on the integrated functions of the organism including military performance.							
24. (U) Methods include measurements of urinary and plasma hormone levels in humans and non-human primates. Animal models are utilized to substantiate and interpret data obtained from humans and the techniques of experimental psychology are applied to produce critical features of the behavior of drug abuse.							
25. (U) 75 07 - 76 06 Levels of cGMP are one-fourth of control levels in cerebellum of rats following chronic administration of sodium barbital. Comparable changes occur in nine of fifteen brain regions studied. Levels of GABA do not change. Project terminated due to termination of the drug abuse mission by congressional action. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 111 Neuroendocrinology and Neurochemistry of drugs of abuse

Investigators.

Principal: James L. Meyerhoff, M.D.

Associate: G. Jean Kant, Ph.D.; John W. Holaday, M.S.;
MAJ Robert H. Lenox, MC.

I. DRUGS OF ABUSE ON BRAIN CHEMISTRY AND ON BEHAVIOR

Studies are in progress on the mechanism of action of various drugs of abuse on brain chemistry and on behavior. These include the acute and chronic effects of opiates, stimulants, alcohol, and barbiturates on neurotransmitter chemicals in the brain. Research methods employed include neurochemistry, neuroanatomy, pharmacology, neuroendocrinology and physiological psychology. Specific approaches include:

1. Effects of drugs of abuse on intracranial self-stimulation: neuroanatomical and neurochemical substrate.
2. Release of endogenous catecholamine from brain.
3. Effects of drugs of abuse on cyclic nucleotides, amino acids and neurotransmitters as measured in brain regions following microwave fixation.
 - a. chronic drug administration.
 - b. abrupt withdrawal following chronic administration.

Effects of amphetamine on intracranial self-stimulation: neuro-anatomical and neurochemical substrates.

Previously reported work from this laboratory (Koob, 1974) has employed the intracranial self-stimulation (ICS) model to assess the neurochemical and neuroanatomical substrates of amphetamine effects on behavior. Postulated central mechanisms of action of amphetamine have included facilitation of dopaminergic and noradrenergic neurotransmission. Because stimulation of central alpha noradrenergic receptors has been termed critical in mediating ICS (Wise, 1973) and because of reports of similar effects of amphetamine and clonidine on firing rates of locus coeruleus (Graham, 1971; Svensson, 1975), it was decided to examine the effects on ICS of clonidine, a potent centrally-acting alpha noradrenergic receptor stimulator.

Clonidine facilitates ICS at one-hundredth of the dose of amphetamine typically used. High doses of clonidine disrupt ICS as do high doses of amphetamine. There are important differences:

ICS-facilitating doses of amphetamine (1-5 mg/kg) cause increased locomotor activity and anorexia; ICS-facilitating doses of clonidine (12.5-50 μ g/kg) stimulate feeding, drinking, and cause elevations in body temperature but do not significantly affect locomotor activity.

Doses of clonidine greater than 100 μ g/kg produce prostration and markedly depress locomotor activity, body temperature, blood pressure, feeding, drinking and ICS.

Release of endogenous catecholamine from brain: the effect of amphetamine.

Previously this laboratory has reported enhanced release of endogenous norepinephrine and dopamine with amphetamine. Recent experiments have determined that the release of endogenous dopamine from corpus striatum is not calcium-dependent. The measurement of release of endogenous (rather than of exogenous, radioactively-labelled transmitter) represents a significant technical advance which has become feasible through modification of an extremely sensitive enzymatic-isotopic assay (Coyle, 1973).

Effects of drugs of abuse on cyclic nucleotides, amino acids and neurotransmitters as measured in brain regions following microwave fixation.

A number of projects have been initiated to test the effects of drugs of abuse on neurotransmitters and cyclic nucleotides in specific brain regions. A method has been established which permits assay of gamma-aminobutyric acid (GABA), glutamic acid (GLU), cyclic adenosine 3'5', monophosphate (cAMP), and cyclic guanosine 3'5' monophosphate (cGMP) in the same sample of brain tissue after microwave inactivation of enzymes, thereby increasing the amount of information obtainable from a single experiment. The assays employed are the radioimmunoassay of Steiner (1969, 1972) for cyclic nucleotides and the enzymatic method of Graham and Aprison (1966) for GABA and GLU. It is thought that cGMP is responsive to cholinergic transmission (Ferendelli, 1972) and under various conditions, brain tissue cAMP is stimulated by norepinephrine, dopamine, serotonin and histamine (Huang, 1972; Brown, 1971; Keabian, 1972). Emphasis in the field has shifted to cAMP/cGMP ratios, and the capacity to study both is essential. GABA and GLU are considered to be, respectively, inhibitory and excitatory transmitters (Krnjevic, 1966). The tentative implication (Schumann, 1962) of GABA deficiency in convulsions suggests that it is an important variable to monitor in studies involving administration of or withdrawal from ethanol or barbiturates (Patel, 1973).

In conjunction with the foregoing, previous studies have demonstrated that the technique of using high-intensity microwave irradiation for enzyme inactivation as indispensable for determining levels of cAMP, cGMP and GABA in brain regions (Lenox, Meyerhoff and Wray, 1974); (Balcom, Lenox and Meyerhoff, 1975). The elimination of artifact has permitted the establishment of new levels of these substances in the regions studied previously. The previously reported preliminary study of effects of chronic barbiturate administration was replicated with the addition of a tolerant-dependent group withdrawn from drug. Measurements were made of a time independently determined to correspond to maximum sensitivity to audiogenic seizure in barbiturate-withdrawn subjects. In barbiturate-dependent subjects, a marked depression of cGMP values in 9 brain regions was confirmed, the effects being most striking in brainstem, cerebellum and midbrain. Values were unchanged in four brain regions. During withdrawal, cGMP levels in all brain regions were equal to or higher than control values. These data suggest a possible relationship between the state of excitability in the CNS and cGMP levels in specific brain regions.

It is known that amphetamine has a "paradoxical" effect in certain conditions associated with hyperactivity. We decided to compare the neurochemical responses to amphetamine in hyperactive vs normal subjects. Data from a preliminary study show a high degree of variability between subjects that may be due to stress interactions.

Several laboratories have attempted to use in-vivo incorporation of radioactive precursors into amino-acid pools as a means of studying mechanism of action of drugs of abuse. A number of amino acids are rapidly metabolized, however, and are closely linked metabolically with pyruvate or Krebs cycle intermediates. Studies using liquid nitrogen immersion have reported post-mortem changes occurring in amino acid levels in rat brain. We decided to examine the effect of microwave fixation on levels of a range of amino acids in mouse brain. Swiss Webster Walter Reed strain mice weighing approximately 25 grams each were sacrificed by decapitation into liquid nitrogen, by decapitation at room temperature or by exposure to microwave irradiation at a frequency of 2450 MHz, at 2.5 KW for 1 second. Samples were sonicated in 10% trichloroacetic acid and analyzed for amino acids by auto analyzer in collaboration with the Division of Biochemistry, WRAIR. The following amino acids were measured: alanine, taurine, aspartic acid, threonine, serine, GABA, glutamine, lysine, histidine, arginine, glutamic acid, glycine, valine, leucine, tyrosine and phenylalanine. There was a very marked post-mortem increase in levels of alanine in brains of mice decapitated at room temperature. The significant post-mortem elevation in GABA as well as absence of change in glutamate previously demonstrated in rats was also observed in this study.

Glycine levels were not affected by method of sacrifice. It would appear that the microwave technique has applicability to the study of amino acids in brain, and may have important implications for studies of effects of drugs on in-vivo incorporation of labelled precursors into brain amino acid pools.

Studies of neurochemical mechanisms of action of drugs of abuse are terminated as of 30 June 1976, by congressional action.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 111 Neuroendocrinology and Neurochemistry of drugs of abuse

Literature Cited.

References:

1. Koob, G.G., Winger, G.D., Meyerhoff, J.L., and Annau, Z.: Concurrent self-stimulation in the medial forebrain bundle ventral tegmentum and locus coeruleus: the effects of d-amphetamine. Proceedings of the Society for Neurosciences, 1974.
2. Wise, C.D., Berger, B.D., and Stein, L.: Evidence of α -noradrenergic reward receptors and serotonergic punishment receptors in the rat brain. Biol. Psychiatry 6:3-21, 1973.
3. Graham, A.W., and Aghajanian, G.K.: Effects of amphetamine on single cell activity in a catecholamine nucleus, the locus coeruleus. Nature (Lond) 234:100-102, 1971.
4. Svensson, T.H., Bunney, B.S., and Aghajanian, G.K.: Inhibition of both noradrenergic and serotonergic neurons in brain by the α -adrenergic agonist clonidine. Brain Research 92:291-306, 1975.
5. Coyle, J.T., and Henry, D.: Catecholamines in fetal and newborn rat brain. J. Neurochem. 21:61-68, 1973.
6. Steiner, A.L., Kipnis, D.M., Utiger, R., and Parker, C.W.: Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. Proceedings of the National Academy of Sciences 64: 367 (1969).
7. Steiner, A.L., Wehmann, R.E., Parker, C.W., and Kipnis, D.M.: Radioimmunoassay for the measurement of cyclic nucleotides. In Advances in Cyclic Nucleotide Research, Vol. 12, Edited by P. Greengard, G. Alan Robison and R. Paoletti. Raven Press, New York, 1972.
8. Graham, L.T., and Aprison, M.H.: Fluorometric determination of aspartate glutamate and gamma-amino butyrate in nerve tissue using enzymatic methods. Analytical Biochem. 15:487-497, 1966.

9. Ferrendelli, D.A., et al: The effect of oxotremorine and atropine on cGMP and cAMP in mouse cerebral cortex and cerebellum. *Biochemical and Biophysical Resch Communications* 41(4):1061-1067, 1970.
10. Huang, M.J.: Accumulation of cyclic adenosine monophosphate in incubated slices of brain tissue. *Medicinal Chem.* 15:458, 1972.
11. Brown, J.H.: Stimulation by dopamine of adenylate cyclase in retinal homogenates and of adenosine 3'5' cyclic monophosphate formation in intact retina. *Proc. Nat. Acad. Sci.* 69(3):539-543, March 1971.
12. Keabian, J.W., Petzold, G.L., and Greengard, P.: Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain and its similarity to the "dopamine receptor." *Proc. of the Nat. Acad. of Sci.* 69:2145-2149, 1972.
13. Krnjevic, K.: An inhibitory process in the cerebral cortex. *J. Physiol. (London)*, 184:16-48, 1966.
14. Schumann, E.L.: The synthesis and gamma aminobutyric acid transaminase inhibition of aminoxy acids and related compounds. *J. Med. Pharm. Chem.* 5:464-477, 1962.
15. Patel, G.J., and Lal, H.: Reduction in brain gamma aminobutyric acid and in barbital narcosis during ethanol withdrawal. *J. Pharm. Exp. Ther.* 186:625-629, 1973.
16. Lenox, R.H., Meyerhoff, J.L., and Wray, H.L.: Regional distribution of cyclic nucleotide in rat brain as determined after microwave fixation technique. *Proceedings of the Soc. for Neurosciences Fourth Annual Meeting*, 1974.
17. Balcom, G.J., Lenox, R.L., and Meyerhoff, J.L.: Regional gamma-aminobutyric acid levels in rat brain determined after microwave fixation. *J. Neurochem.* 24:609-613, 1975.

Publications:

1. Koob, G.F., Spector, N.H., and Meyerhoff, J.L.: Effects of heroin on lever pressing for intracranial self-stimulation of food and water in the rat. *Psychopharmacologia* 42:231-234, 1975.
2. Koob, G.F., Balcom, G.J., and Meyerhoff, J.L.: Dopamine and norepinephrine levels in the nucleus accumbens, olfactory tubercle and corpus striatum following lesions in the ventral tegmental area. *Brain Research* 94:45-55, 1975.

3. Koob, G.F., Balcom, G.J., and Meyerhoff, J.L.: Increases in intracranial self-stimulation in the posterior hypothalamus following unilateral lesions in the locus coeruleus. *Brain Research* 101:554-560, 1976.

4. Balcom, G.J., Lenox, R.H., and Meyerhoff, J.L.: Regional glutamate levels in rat brain determined after microwave fixation. *J. Neurochem.* 26:423-425, 1976.

5. Lenox, R.H., Wray, H.L., Balcom, G.J., Hawkins, T.D., and Meyerhoff, J.L.: Regional brain levels of cyclic nucleotides, gamma aminobutyric acid (GABA) and glutamate during chronic barbiturate ingestion and withdrawal. *Proceedings of the Soc. for Neurosciences Fifth Annual Meeting*, 1975.

6. Meyerhoff, J.L., Martin, G.E., and Koob, G.F.: Effects of clonidine on self-stimulation, eating, drinking and body temperature in the rat. *Proceedings of the Soc. for Neurosciences Fifth Annual Meeting*, 1975.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ¹	2. DATE OF SUMMARY ²	REPORT CONTROL SYMBOL	
				DA OB 6515	76 06 30	DD-DR&E(AR)636	
3. DATE PREV. SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY ³	6. WORK SECURITY ⁴	7. REGRADING ⁵	8A. DMB'S INSTR ⁶	8B. SPECIFIC DATA - CONTRACTOR ACCESS	
75 07 01	R. Tech.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
9. NO / CODES ⁷		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY		62758A		3A762758A833		00 113	
b. CONTRIBUTING							
c. XREFS/REFERENCES		CARDS 114F					
11. TITLE (Precede with Security Classification Code) ⁸							
(U) Metabolism of Drugs of Abuse							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ⁹							
002300 Biochemistry 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
73 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)	
EXPIRATION:				FISCAL		3 125	
c. NUMBER:				YEAR		76 95	
d. TYPE:				CURRENT			
e. KIND OF AWARD:				f. CUM. AMT.			
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Punish DDA if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME: Demaree, LTC G.F.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2211			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Sleeman, H.K., Ph.D.			
				NAME: Lofberg, R.T., Ph.D. DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Drug Metabolism; (U) Drugs of Abuse; (U) Enzyme Induction; (U) Microsomal Enzymes							
23. TECHNICAL OBJECTIVE, ¹⁰ 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede rest of each with Security Classification Code.)							
<p>23. (U) The technical objective of this work unit is to determine sites, modes and mechanisms of biotransformation of the principal drugs of abuse in man and animal and to develop a suitable animal model to study the influence of other drugs on the biotransformation of drugs of abuse in order to evaluate the impact of these interactions on drug abuse detection and on toxicity of drugs of military importance.</p> <p>24. (U) Analytical and chromatographic methods will be used to study quantitatively and qualitatively the effects of environmental changes on the metabolism of drugs of abuse in intact animals and isolated organ preparations. Human body fluids will be studied for presence of drug metabolites and other substances which interfere with drug abuse detection methods.</p> <p>25. (U) 75 07 - 76 06 Rats receiving high doses of pyridoxine and pyridoxal showed less severe withdrawal from morphine dependence than animals on normal diets. A condensation product of pyridoxal and dopamine given just before naloxone challenge in dependent rats also decreased the severity of withdrawal. The significance of these findings should be investigated further. The levels of benzoyl ecognine were measured in plasma of patients receiving cocaine for surgical anesthesia. Peak levels were reached in about one hour and decayed with a half-life in plasma of about 5 hours. This indicates that plasma levels of this metabolite are not correlated with anesthetic action of cocaine. For technical report, see the Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.</p>							

PII Redacted

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.

1576

Project 3A762758A833 BIOCHEMICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 113 Metabolism of drugs of abuse

Investigators.

Principal: Gale E. Demaree, MSC

Associate: Jeffrey A. Lyon, Ph.D; SP5 Ronald W. Hill; Earl C. Richardson, M.A.; Donald E. Mahan, Ph.D; Mary A. Sodd, M.S.; B.P. Doctor, Ph.D.

During this period the objective of the work was to provide a rapid, sensitive, specific, precise and inexpensive assay for the detection of opiates and their metabolites in human urine and serum. To this end a method was established based upon the following hypothesis:

Nitrocellulose filters, bearing a net negative charge, will bind a limited amount of protein quantitatively. Using this property, a radioimmunoassay was developed which allowed a rapid separation of free from bound ligand, consequently permitting the detection of morphine at concentration less than 2 pmoles/ml of serum (0.7 ng/ml) and less than 1 pmole/ml of urine. The methodology can also be used to detect morphine-specific antibody in either crude serum or fractionated IgG at concentration less than 0.01 pmoles morphine antibody per mg of protein (0.5 pmoles/ml serum). It is possible to apply 1.5 mg of serum protein to the filter with no loss of antibody-hapten complex binding by the filter. Increasing the concentration of total protein above this threshold invalidates this assay. It is possible to process 100 samples in a three hour period. Due to the minimal requirement of reagent (1 to 20 μ l crude serum) the assay is useful in following the day to day antibody titer of an individual animal; and finally, the assay is sensitive enough to be useful in detecting antibody produced in lymphocyte culture as well as that translated from mRNA to cell-free protein synthesizing systems. The summary of accomplishments are as follows:

1. 6-succinyl morphine - BSA conjugate was synthesized and the free derivative was separated from the conjugate by gel filtration using Sephadex G-100 column.

The rabbits were immunized with 750 μ g of conjugate in Freund's complete adjuvant (multiple sites). The animals were tested for 12 weeks and were then boosted with 10 μ g of conjugate in Freund's incomplete adjuvant. The immune sera thus obtained were of high titer. The serum was subjected to 0-52% $(\text{NH}_4)_2 \text{SO}_4$ to precipitate

the immunoglobulins. The precipitates were dissolved and subjected to Sephadex G-200 column gel filtration. IgG essentially free of other immunoglobulins was thus obtained. Using either the immune sera or the purified IgG fraction and the following procedure for the hapten-antibody interaction characterization was carried out.

Thirty μ l of ^3H -Morphine (500 nCi/ml; 30 Ci/mole), was mixed with 20 μ l of urine or serum sample. To this, 50 μ l antibody diluted in 40 mMolar PO_4 , 300 mMolar KCl at pH 6.5. (The antibody should be diluted to such an extent that 50 μ l of diluted antibody solution binds approximately 6000 cpm or (36% of ^3H -Morphine). The reaction mixture was incubated for 30 minutes at 4°C in the dark. The samples were diluted with 3 ml of cold 20 mMolar PO_4 pH 6.5 and 300 mM KCl and filtered through a 0.22 μ millipore filter. The sample container was washed twice with the same amount of buffer and applied to the filter. The filter was dissolved in instabray and counted. A standard curve was generated with each set of samples analyzed.

2. Titration of the morphine antibody with ^3H -Morphine.

Using the following equations, the amount of IgG produced by immunizing the animals was calculated.

$$\text{a. } \frac{\text{pmoles } ^3\text{H-Morphine}}{(\mu\text{l serum}) (2 \text{ binding sites})} = \frac{150 \text{ pmoles IgG}}{\text{ml serum}}$$

$$\text{b. } (150 \text{ pmoles IgG/ml}) (150,000 \text{ mg}) = \frac{\text{mg}}{\text{antibody ml. in serum}}$$

The most significant attribute of this assay procedure is that the non-specific binding of ^3H -Morphine accounts for less than 10% of the total binding.

3. Effect of concentration of reagents on extent of complex formation.

The morphine antibody complex formation, as expected is dependent on the concentration of reagents. Optimal binding in 50 μ l reaction mixtures and minimal binding in 200 μ l was found. For practical purposes, 100 μ l reaction mixture was found to be acceptable for this assay.

4. Saturation of 0.22 μ and 0.45 μ millipore filters with serum proteins.

The serum protein retention capacity of 0.22 μ millipore filter is approximately 1.5 mg whereas that of 0.45 μ filter is about 60% of

the former. The IgG retention capacity of 0.22 μ filter is approximately 0.8 mg, the amount is twice as much as the IgG present in 1.5 ml serum. Thus the proteins other than IgG in serum appear to compete with IgG for binding to filters. The morphine-antibody complex does not appear to change upon application to the filter, because the filtered complex (from a saturated filter) binds upon refiltration of another filter. The results indicate that 25 μ l of immune serum or 0.8 mg IgG can be used in the reaction mixture for quantitative results. More than these amounts of proteins invalidates the results by this method.

5. Effect of pH, ionic strength, temperature and time dependence on morphine-antibody complex formation.

Altering pH of the reaction mixture between 6 and 7.5 indicated that, within this range, changes only the background binding of morphine and not the complex formation. For this reason pH 6.5 was selected as an ideal one for this assay procedure.

The reaction is relatively independent of ionic strength as judged by binding of complex to filters at concentrations of KCl up to 500 mM. However, it is dependent on temperature and time of incubation. At 0-4°C the reaction is complete in 20 minutes whereas at 25°C, 5 minutes incubation is sufficient to complete the reaction. Also the reaction mixture at 0-4°C after completion can be diluted up to 40 fold without any dissociation of complex for up to 30 minutes.

Systematic investigation of all variable parameters related to hapten-antibody interaction was essential for not only developing a highly sensitive, reliable and rapid method of measurement of hapten concentration in biological and clinical specimen but also for measurement of trace amounts of antibody synthesized by lymphocytes in response to antigen. The assay procedure is being employed for serum of the patients undergoing open heart and other surgery. These patients are given morphine in addition to other anesthetics such as nitrous oxide or halothane. The application of this assay procedure for the measurement of De Novo synthesized immunoglobulins by lymphocytes is described elsewhere in this report (see Work Unit 075).

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 113 Metabolism of drugs of abuse

Literature Cited.

Publications:

1. Lyon, J.A., Hill, R.W., Mahan, D.E., Richardson, E.C., Sodd, M.A., and Doctor, B.P.: A novel RIA for the detection of hapten antibody complexes using nitrocellulose filters. Fed. Proc. 35: 1651, 1976.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OB 6516	76 06 30	DD-DR&E(AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^b	6 WORK SECURITY ^b	7 REGRADING ^c	8A DNR INSTR ^d	8B SPECIFIC DATA - CONTRACTOR ACCESS	9 LEVEL OF SUM
75 07 01	A. C. R. I.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10 NO CODES ^e		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		62758A	3A762758A833	00	114		
B. CONTRIBUTING							
C. CONTRIBUTING							
KXKXKXKXKXKX		CARDS 114F					
11 TITLE (Precede with Security Classification Code) ^f							
(U) Pharmacokinetics of Drugs of Abuse							
12 SCIENTIFIC AND TECHNOLOGICAL AREA ^g							
002300 Biochemistry							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
73 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 ^h				75		2	
C. TYPE				CURRENT		75	
D. KIND OF AWARD				76		6	
E. AMOUNT:							
F. CUM. AMT.						212	
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 Biochemistry			
				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			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Lofberg, R.T., Ph.D.			
				NAME: Demaree, LTC G.E. DA			
23 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Drug Excretion; (U) Pharmacodynamics;							
(U) Pharmacokinetics; (U) Drugs of Abuse; (U) Drug Absorption; (U) Drug Distribution							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The technical objective of this work unit is to determine the rates and modes of absorption, distribution, biotransformation, binding and excretion of principal drugs of abuse in animals to predict these parameters in military personnel.							
24. (U) Biochemical analytical and pharmacological techniques are employed to assess the influences of diet, environment and chemicals on the pharmacokinetics of drugs of abuse. Correlations of binding and distribution with pharmacodynamic effects will be used to study mechanisms of dependence and tolerance.							
25. (U) 75 07 - 76 06 Treatment of morphine dependent rats with large excess of pyridoxine or pyridoxal alone or in combination reduced naloxone-induced withdrawal symptoms. An isolated product which resulted from the condensation reaction of pyridoxal and dopamine also reduced naloxone induced withdrawal symptoms in morphine dependent rats. Investigations were continued on the role of the plasticizer, diethylhexylphthalate and its metabolites, in producing atherosclerosis in patients undergoing hemodialysis. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75 - 30 Jun 76.							

PII Redacted

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

1581

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 114 Pharmacokinetics of drugs of abuse

Investigators.

Principal: LTC Gale E. Demaree, MSC

Associate: Ann R. Berman, B.S.; Clarence E. Emery, B.S.; CPT James A. Cella, Ph.D.; Seymour Garson, Ph.D.; SP4 James W. Nitchkey; SP4 Steven E. Engelsen; SP5 James P. McGrath; SP5 El-Soukkary; Patrick M.L. Siu, Ph.D.; Nesbitt D. Brown, M.S.; SFC Mary J. Dowery; SP4 Edward J. Michalski; H. Kenneth Sleeman, Ph.D.

The technical objectives of this work unit are to develop and evaluate analytical methods for the detection, identification, and quantitation of drugs of abuse, pharmaceutical compounds and their metabolites in biological fluids and tissues and to exploit these techniques for application to rehabilitation and chemotherapy management; to determine sites, modes and mechanism of biotransformation and to determine the rates and modes of absorption and excretion. Efforts were concentrated in the areas of:

1. Radioimmunoassay for cocaine: Evaluation
2. Cocaine levels in biological fluid (Joint with ENT, WRAMC).
3. Latex agglutination - inhibition test for barbiturates.
4. Radioimmunoassay for morphine (Technology).
5. Studies on morphine dependency.
6. HPLC method for p-aminobenzoic acid and metabolites.
7. Hydrolysis of N-acetyl-p-aminohippuric acid.

1. Radioimmunoassay for cocaine: Evaluation.

The Roche Diagnostics radioimmunoassay (RIA) kit for cocaine determination was evaluated for use in the Tri-Service drug screening program. Since cocaine is rapidly metabolized in the body, the test is based on the detection of one of the major metabolites of cocaine, benzoylecgonine, in biological specimens. The assay was evaluated with respect to technical variation, specificity, sensitivity, and application.

Modifications of the methodology described in the company's brochure were evaluated before sample analysis. Changes in incubation, precipitation, and centrifugation times showed no significant effects and separate pipetting or premixing of test reagents produced no effects on the assay. The standard curves prepared in the laboratory from benzoylecgonine showed excellent agreement with those prepared from kit reagents.

The specificity of the assay for the cocaine metabolite, benzoylecgonine was good. When cocaine and some of its metabolites were compared to benzoylecgonine, cocaine reacted slightly at 500 ng/ml and gave approximately 63% of the reactivity of benzoylecgonine at 1000 ng/ml. Ecgonine reacted slightly at 1000 ng/ml, nor-benzoylecgonine reacted very slightly, and nor-ecgonine did not react. Urine specimens from persons receiving no medication or receiving known medications other than cocaine were negative in the assay. Normal urine with added other drugs of abuse (morphine, codeine, amphetamines, barbiturates) also showed no cross reactivity. The sensitivity of the assay for benzoylecgonine in urine v.s 50 ng/ml.

The assay was applied to urine samples collected from 5 monkeys for 72 hours after the administration of cocaine (1 mg/IV). All specimens were positive by RIA. The peak concentration occurred between 4 and 7 hours after drug administration, and evidence of drug excretion was still present at 72 hours after drug administration. Urine collected from surgical patients for 24 hours following the topical application of cocaine for local anesthesia also gave positive results.

The RIA for cocaine is highly specific and sensitive for routine screening for cocaine usage. The test is technically simple to perform, and adaptable to large volume drug screening. There appears to be no cross reactivity with other drugs of abuse or selected chemotherapeutic agents.

2. Radioimmunoassay for cocaine in serum of ENT surgical patients.

This project is a collaborative study with the ENT service, WRAMC. The cocaine RIA was used to determine if the anesthetic dose and mode of application of cocaine to the nasal mucosa during surgery results in different blood levels, clearance time and whether these levels correlated with observed side effects. The objective of the study was to establish a safe, effective anesthetic dose of cocaine and to acquire basic data on cocaine metabolism in humans.

The RIA for cocaine, which was originally designed for urine analyses, was adapted to the detection of benzoylecgonine in serum. A total of 13 patients have been studied. Serial blood samples have been collected for baseline and from 15 minutes to 24 hours after the topical

administration of 200 ng of cocaine to the nasal mucosa. Analyses of the samples showed peak serum levels of benzoylecgonine at 4 to 6 hours after the administration of cocaine. The half-time for plasma levels was 5.5 ± 0.30 hours. The 24 hour sample still contained an appreciable amount of benzoylecgonine (89.4 ± 14.2 ng/ml).

This project is being continued using different methods of application and different amounts of cocaine. Gas-liquid chromatography will be used as a confirmatory analytical procedure.

3. Latex agglutination - inhibition test barbiturates: Evaluation:

The Roche Diagnostics Latex flocculation test for barbiturates was evaluated for its efficacy in the detection of barbiturates in urine. Flocculation of a latex-barbiturate complex occurs with the addition of barbiturates free urine; the reaction is inhibited by urine containing barbiturates. The test was evaluated with respect to sensitivity, specificity, and ease of performance.

Urine samples from various sources was assayed in duplicate. These included normal urine (120) with added barbiturates and other drugs of abuse and urines (54) from laboratory personnel and hospital patients. Urines with added barbiturates showed that secobarbital gave a positive test at 2.5, 5, and 10 μ /ml, apobarbital and sodium pentobarbital were positive at 10 μ g/ml, Phenobarbital and amobarbital gave a negative response at 10 μ g/ml. Other drugs of abuse gave negative tests. The 54 urines from laboratory personnel and hospital patients gave 13 positive reactions. All of the positive reactions were from patients who had received either secobarbital or pentobarbital. The 54 urines were tested also by another laboratory with identical results.

The latex test was found to be applicable for the assay of barbiturates in serum. Sera from surgical patients (81) were assayed. Positive results were found only in the sera of those patients (64) who had received barbiturates. No cross reactivity was found with patients receiving other drugs of abuse of selected medication.

Conclusions: The test appeared to be good for secobarbital (2 μ g/ml), pentobarbital (10 μ g/ml) and apobarbital (10 μ g/ml). The test is simple to perform, fast (3 hrs.), relatively low in cost, and has a long shelf-life. The fact that serum reacts equally as well as urine makes the test applicable in emergency room situations. No evidence of cross reactivity with other drugs was found.

4. Radioimmunoassay for morphine: Technical Evaluation.

The double antibody technique used in the radioimmunoassay for morphine

is accurate and precise but tedious and very time consuming. A study was initiated in an attempt to shorten the assay time by using polyethylene glycol (PEG) as a co-precipitant with the second antibody. The addition of a cold 4% aqueous solution of PEG, immediately following the second antibody, produced a rapid, complete precipitation of the globulin proteins. This was in contrast with the 18 hour incubation required without PEG. The addition of PEG reduced also the non-specific binding. The standard curves for the two procedures were essentially the same.

The within run precision on three different days (50 tubes of 200 pg morphine) was, expressed as percent ^{125}I bound \pm SEM:

38.264 \pm 0.149	CV = 2.7%
38.414 \pm 0.131	CV = 2.2%
37.775 \pm 0.184	CV = 3.4%

The optimum size of each run with this method is 50 samples. If over 50 tubes are used, an error will be introduced due to the slow dissolving of the precipitate while decanting the supernatant solution.

5. Studies on morphine dependency.

Studies were continued on the biochemistry of morphine dependency. Rats were made morphine dependent by implanting morphine pellets. Morphine dependency was measured by counting the number of wet dog shake episodes that occurred the first 10 minutes after naloxone injection 3 days after morphine pellet implantation.

Rats placed on a Vitamin B₆ deficient diet and then made morphine dependent had fewer episodes of naloxone-induced wet dog shakes than a similar group of morphine dependent rats on a normal Purina chow diet. A group of rats on a Vitamin B₆ repleted diet, treated in the same manner, were comparable to the normal Purina chow group. Also, the injection of Vitamin B₆ (4 to 100 mg/Kg body wt.) into morphine dependent rats maintained on a normal Purina chow diet had no effect on the number of wet dog shake episodes during naloxone-induced withdrawal. However, when morphine dependent rats on a Purina chow diet, were given a large excess of Vitamin B₆ (200 mg/Kg) the frequency of the wet dog shake episodes was reduced during naloxone-induced withdrawal.

Further studies showed that, when morphine dependent rats were given 40 or 60 mg/Kg of pyridoxal, the wet dog shake episodes were reduced 60% to 78% when compared to morphine dependent rats receiving only saline. The combination of 40 or 60 mg/Kg of pyridoxal and 100 mg/Kg of Vitamin B₆ when given to morphine dependent rats, produced no wet

dog shake episodes during naloxone-induced withdrawal, while 100 mg/Kg of Vitamin B₆ or saline had no observable effect on decreasing the number of wet dog shake episodes.

The mechanism involved in the reduction of the wet dog shake episodes in morphine dependent rats by pyridoxal was investigated. Preliminary studies showed that the injection of a condensation product of pyridoxal and dopamine reduced the frequency of the wet dog shake episodes in morphine dependent rats subjected to naloxone withdrawal. Further studies are required on the identification of this condensation product and its mechanism of action.

6. HPLC method for p-Aminobenzoic acid and metabolites.

An improved high performance liquid chromatographic (HPLC) method for determining p-aminobenzoic acid and its metabolites in physiologic fluids was developed. The new method is simple and specific and offers increased sensitivity, higher resolution, and shorter analysis time. A Varian LC series 4100 liquid chromatograph with a 10 μ m-size Partisil-10-SAX (anion) column was used for all separations. The buffer system for elution was 0.1 M sodium formate-formic acid, pH 3.50, at a flow rate of 40 ml/hr.

The separation of p-aminobenzoic acid, p-aminohippuric acid, n-acetyl-p-aminobenzoic acid, and n-acetyl-p-aminohippuric acid was accomplished in 10 minutes. Serum or urine specimens (2 μ l) were analyzed directly without pretreatment. The detection limit for each compound was 1 ng on column, and the peak height produced for standards was proportional to the quantity on column.

The n-acetyl-p-aminohippuric acid was prepared and characterized by the organic chemistry section of the Department of Physiological Chemistry.

7. Hydrolysis of N-acetyl-p-aminohippuric acid.

Most analytical methods for conjugated aromatic amines require hydrolysis to free the amine group. The rate of conversion of N-acetyl-p-aminohippuric (PAAHA) acid to its deacetylated and deglycinated derivatives is directly related to the hydrolysis reaction time and the concentration of hydrochloric acid used in the reaction. Variations in methodology involving either of these hydrolysis parameters produced artifacts which are not detectable by standard colorimetric procedures.

High performance liquid chromatography offers an improved tool for detecting and quantitating the change produced during hydrolysis.

The optimum conditions for hydrolyzing N-acetyl-p-aminohippuric acid

in urine was with 0.8N HCl for 30 minutes. The formation of p-amino-hippuric acid (PAHA) began within 5 minutes and was completed within 25 minutes. Approximately 97% of the PAAHA was converted to PAHA during this time period and only 1 to 2% p-aminobenzoic acid (degradation product) was formed. The hydrolyzed sample was chromatographed on a 1-meter X 2 mm ID 316 stainless steel column, dry-packed with Whatman AS-Pellionex-SAX anion exchanger. The eluting solution was 0.005 M formate buffer, pH 3.50 + .05. Excellent separation was obtained at a flow rate of 40 ml/hr and PABA and PAHA were eluted within 10 minutes. The detection limit (ultraviolet, 254nm) was 5 nanograms on column. The relationship of peak height to quantity of compounds was linear from 5 nanograms to 5 micrograms on column. The method was superior in both sensitivity and specificity to the commonly used colorimeter procedures. These studies revealed that artifacts are introduced into the analyses of PAH when hydrolysis conditions are excessive.

Project 3A762758A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 114 Pharmacokinetics of drugs of abuse

Literature Cited.

Publications:

1. Siu, P.M.L., West, S., and Bogdanove, A.J.: Morphine dependency in rats on a pridoxine-deficient, a pridoxine-repleted, and a normal chow diet plus pyridoxine injections. *Jour. Neurochemistry.* 26: 633, 1976.
2. Berman, A.R., McGrath, J.P., Permisohn, R.C., and Cella, J.A.: Radioimmunoassay of methaqualone and its monohydroxy metabolites in urine. *Clin. Chem.* 21: 1878, 1975.
3. Gibson, T.P., Briggs, W.A., Boone, B.J.: Delivery of di-2-ethylhexyl phthalate to patients during hemodialysis. *J. Clin. Lab. Med.* 87: 519, 1976.

Project 3A762760A837
MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00
Military Animal Resources Development

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
				DA OB6441	76 0 (3)	DD DR&E(AR)636	
3 DATE PREV SUMRY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^c	6 WORK SECURITY ^d	7 REGRADING ^e	8A OMB'S INSTN ^f	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM A. WORK UNIT
75 07 01		U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10 NO. CODES ^g	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
	62110A	3A762760A837	00	055			
a. PRIMARY							
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114E						
11 TITLE (Precede with Security Classification Code) ^h							
(U) Development and Evaluation of Improved Biological Sensor Systems							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ⁱ							
001700 Animal Husbandry 01180 Operations							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
67 09		76 06		DA		C, In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE		b. EXPIRATION		PRECEDING		c. FUNDS (In thousands)	
NA				75		265	
d. NUMBER ^j				FISCAL YEAR			
c. TYPE				76		4	
e. KIND OF AWARD				CURRENCY		280	
f. CUM. AMT.							
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME * Walter Reed Army Institute of Research				NAME * Walter Reed Army Institute of Research			
ADDRESS * Washington, DC 20012				Div of Bio Sensor Research			
				ADDRESS * Aberdeen Proving Ground, MD 21010			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Joy, COL R.J.T.				NAME * Castleberry, COL M.W.			
TELEPHONE: 202-576-3551				TELEPHONE 301-671-3312			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Linn, MAJ, J.M.; Nyland, CPT, T.G.;			
				NAME: Leighton, CPT, F.A.			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Detector dog; (U) Selective breeding; (U) Mines; (U) Explosives, drugs; (U) Ambush							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) To better protect the combat soldier by genetic development of a more intelligent and sensually acute detector dog that was free of hip dysplasia and was temperamentally better suited for detecting the presence of the enemy than was then generally available.							
24. (U) Critically evaluated AKC registered German Shepherd Dogs were purchased as foundation stock. The progeny of these and subsequent generations were closely evaluated by recognized tests designed to reveal the superior individuals. These were in turn used as breeders.							
25. (U) 75 07 - 76 06 During the past two years the breeding colony of thirty-one animals was replaced with late third and early fourth generation offspring of the original stock. Almost without exception these were proven, progeny tested breeders which produced 229 fourth and early fifth generation animals. With the establishment of a stable gene pool producing consistently successful military working dogs, the project passed beyond the primary development stage and was ordered to transfer its breeding stock and their progeny to other organizations for production purposes. This was accomplished with distribution of 322 dogs during the past year. The final shipment, made on 22 June, consisted of 15 breeding dogs to Lackland Air Force Base, Texas. This organization was deactivated 30 June 1976. Two papers were written. These were "A Genetic Study of Canine Hip Dysplasia", and "The Use of Frozen Semen in Artificial Insemination of the German Shepherd Dog". A third paper, "Efficacy of Breeding Phenotypically Hip Dysplasia-free Dogs", is in manuscript. 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							

PII Redacted

DD FORM 1498

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

1589

Project 3A762760A837 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 055 Development and evaluation of improved biological sensor systems

INVESTIGATORS.

Principal - COL Merida W. Castleberry, VC
Associates - MAJ Jeffrey M. Linn, VC
CPT Thomas G. Nyland, VC
CPT Eldin A. Leighton, MSC

OBJECTIVE. To better protect the combat soldier by genetic development of a more intelligent and sensually acute detector dog that is free of hip dysplasia and is temperamentally better suited for detecting the presence of the enemy than is now generally available.

BACKGROUND. Despite the large number of pet dogs in the nation, the acquisition of suitable working dogs for military purposes is always a problem. This is especially true during wartime because of the greatly increased demand. This project was authorized during the Vietnam conflict for the purpose of developing a line of more proficient military working dogs and to assist in relieving the shortage of acceptable dogs

APPROACH. Critically evaluated AKC registered breeding stock purchased especially for this purpose was selectively bred to produce superior progeny. These were in turn closely evaluated by recognized tests designed to reveal the superior individual. Linebreeding combined with progeny testing of each generation was used to accomplish the objective.

PROGRESS.

A. Breeding Program.

1. Twenty-three litters produced 125 puppies.
2. Present kennel population is 0.
3. Disposition of 322 dogs was made as follows:

DOD Dog Training Center, Lackland AFB, TX	
Military working dogs 8 months or older	115
Puppies less than 8 months	33
Breeding stock	15
Walter Reed Army Institute of Research	109

US Customs Service	
Puppies less than 8 months of age	10
Breeders	6
Working Dogs	1
The Seeing Eye, Inc.	
Breeders	11
Working Dogs	5
US Army Scout Dog Platoon, Ft. Benning, GA	4
US Park Police	3
Civilian Police Departments	10

B. Special Projects.

1. Continuing Projects

a. No new developments occurred in the puppy diarrhea study conducted in conjunction with Veterinary Division, WRAIR.

b. Surgical repair approach to canine hip dysplasia did not prove to be of significant practical value. The one most promising of this series of six operated dogs was sent to the Air Force for training and, to date, is a completely functional sentry dog in Korea.

2. New Projects. To identify those puppies with exceptionally keen noses and to maximize their explosive detection ability, 116 puppies from 21 litters were "pre-scented" to dynamite twice weekly from their eighth week to their fourteenth week of life. These were short sessions lasting from five to ten minutes in which the puppy was rewarded with a piece of semi-moist dog food for successfully locating a small amount of commercial dynamite. This procedure provided a high degree of motivation despite the puppies having access to dry dog food ad libitum. Twenty-three of these puppies were identified as being exceptional. The Air Force and Customs dog training sections were alerted to such of these young dogs as they received. None of these is yet old enough to demonstrate their capability as trained explosive detectors.

C. Veterinary Medicine. Previously reported immunization and husbandry programs remained unchanged. Quarterly testing of breeding stock for B. canis and D. immitis continued. The kennels remained brucella-free but four D. immitis cases were diagnosed and treated.

Major surgical procedures during the past fiscal year included 55 ovario-hysterectomies, one caesarean section, one femoral fracture repair, one abdominal hernia and two umbilical hernia repairs, eight patent ductus

arteriosus surgeries and five persistent fourth right aortic arch surgeries.

D. Genetics.

1. Data derived from the pelvic radiographs of 1186 German Shepherd Dogs born in these kennels places the heritability of canine hip dysplasia in this colony at 22.0% -- a moderately heritable condition. Progeny testing to identify superior replacement breeders with final selections being limited, as far as possible, to individuals from dysplasia-free litters gave the best promise for breeding dysplasia-free dogs. During the last twelve months of this organization's operations, of the 72 fourth generation dogs receiving at least their eighth month radiograph, only two were dysplastic. Although not too comparative because of age difference, the 63.4% dysplasia rate encountered in 230 one to three year old civilian dogs radiographed for procurement by the Air Force at Lackland AFB, TX is at least indicative of the problem. (1)

The exceptionally low rate experienced by this kennel in the past 12 months is attributed to the fact that the sire or dam of 15 of the 17 litters producing these dogs were themselves from two late third generation almost dysplasia-free litters (one of the 15 dogs in those two litters became dysplastic). Prior to this, and almost without exception, the few previous dysplasia-free litters of earlier generations were not of breeding quality because of undesirable temperament, heritable physical defects, or combinations of these qualities.

2. Analyses of unpublished data examined the degree of both the phenotypic and genotypic relationships between temperament (measured by intermediate evaluation) and hip dysplasia. The phenotypic correlation between temperament and hip dysplasia was approximately -0.25. The genotypic correlation was approximately -0.35. The significance of the size and direction of the genotypic correlation became apparent when the selection of new breeders was made. As selection pressure was applied to improve the overall temperament of the dogs, there was a corresponding increase in the rate of hip dysplasia among the offspring. The reverse also occurred when selection pressure was applied to reduce hip dysplasia. Because of this correlation, overall progress in simultaneously improving both traits in this colony of German Shepherd Dogs was made more difficult.

3. A review was completed of this organization's progress in the abatement of canine hip dysplasia by mating phenotypically dysplasia-free dogs through four generations. This was based upon radiographic examination of the progeny of each succeeding generation as initiated by the original breeding stock. The rate of dysplasia experienced by each generation was as shown below.

Rates of Dysplasia Experienced by Generations

Generation	Number normal at shipment	Number Dysplastic	Total	Rate
1	257	84	341	24.6%
2	337	120	457	26.3%
3	207	74	281	26.3%
4*	139	32	171	18.7%

*During the final twelve months of this organization's operations, only two of the 72 dogs radiographed during their eighth month or beyond were dysplastic. A third case occurred in the final 33 pups receiving their initial five month radiograph. None of these 33 are included in the above figures.

Several debatable factors surround the above figures. Questions concerning the number of puppies rejected prior to being radiographed that might subsequently have become dysplastic and the number of dogs that became dysplastic following training as military working dogs are examples. Had the mission of this project been concerned solely with dysplasia eradication, these and other questions would have been answered. As can be seen no noticeable progress was accomplished during the first three generations because of the interplay of unacceptable temperament, hip dysplasia, and other heritable defects. Two third generation litters, the S₁₁ consisting of 8 pups, and the V₁₁ consisting of 7 pups, more nearly possessed the desired qualities than any previous litters. With but one exception, all 15 of these animals possessed excellent hip conformation. One male and two bitches from each litter were introduced to the breeding line in April 1975. These animals were subsequently the sire or dam of 19 litters consisting of 92 progeny. Twelve of these litters and their 59 progeny received at least their eighth month radiograph prior to this organization's cessation of operations. Only one of these individuals proved dysplastic. Since approximately 80% of the dysplasia rate experienced in this kennel occurred by the eighth month of age, this drop was most encouraging. Establishment of pedigree depth of freedom from hip dysplasia, progeny testing, and breeding only from dysplasia-free litters will, apparently, greatly abate or perhaps eliminate canine hip dysplasia.

E. Visits.

1. Sixty-two visitors toured these facilities during the past year.
2. Consultant visits were made by Dr. W.H. Riser, (hip dysplasia).

DISCUSSION. With the development of a gene pool producing consistently successful military working dogs, the decision was made to transfer the stock to using organizations for production purposes. Per a formal Memorandum of Understanding between US Army Medical Research and Development Command and the US Customs Service six mature breeders and five young potential breeding canines were transferred to the US Customs Service at Front Royal, VA. With the concurrence of the Commanding General, US Army Medical Research and Development Command, eleven breeders were donated via contract to The Seeing Eye, Inc., for use in their breeding program. As the major gaining agency with fifteen breeders, the Air Force will be admirably situated to implement the establishment of a canine remount service at selected military installations.

PUBLICATIONS.

Three papers were prepared during the period of this report they are:

1. "A Genetic Study of Canine Hip Dysplasia" has been cleared for publication by Walter Reed Army Institute of Research Board of Review and tentatively accepted for publication by the American Journal of Veterinary Research. Principle author is CPT E. A. Leighton, MSC.

2. "The Use of Frozen Semen in Artificial Insemination of the German Shepherd Dog". The author is G. E. Lees, DVM.

3. "Efficacy of Breeding Phenotypically Hip Dysplasia-free Dogs" in manuscript. Principle author is MAJ J. M. Linn, VC.

CONCLUSION. Man's ability to breed animals for a desired genetic result was repeated with the establishment of a gene pool of the German Shepherd Dog which produced consistently successful military working dogs.

Project 3A762760A837 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 055 Development and evaluation of improved biological sensor systems

LITERATURE CITED.

References.

(1) Townsend, L., Hip Dysplasia in Canine Military Candidates. In Proceedings: Canine Hip Dysplasia Symposium and Workshop. Orth. Foun. Anim., (1972): 127-130.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL	
				DA OB 6444	76 07 01	DD DR&E (AR) 636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^c	6 WORK SECURITY ^d	7 REGRADING ^e	8A ORG'S INSTR ^f	8B SPECIFIC DATA CONTRACTOR ACCESS ^g	9 LEVEL OF SUP ^h
75 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
10 NO CODES ⁱ		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY		162760A	3A762760A837	00	056		
B. CONTRIBUTING							
C. CONTRIBUTING		LABS 114E					
11 TITLE (Precede with Security Classification Code) ^j							
(U) Diseases of Military Animals							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^k							
010100 Microbiology 005900 Environmental Biology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
68 07		CONT		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA		EXPIRATION		PRECEDING			
B. NUMBER ^l				FISCAL YEAR		FUND\$ (In thousands)	
C. TYPE		D. AMOUNT		76		3 262	
E. KIND OF AWARD		F. CUM. AMT.		77		3 276	
20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME ^m Walter Reed Army Institute of Research				NAME ⁿ Walter Reed Army Institute of Research			
ADDRESS ^o Washington, DC 20012				ADDRESS ^p Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME // U.S. Academic Institution)			
NAME JOY, Robert J. T., COL, MC				NAME ^q STEPHENSON, Edward H. LIC, DVM			
TELEPHONE 202-576-3551				TELEPHONE 202-428-5389			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
22 GENERAL USE				23 ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: KEEPL, Thomas J., LTC, MC			
				NAME: BINN, L. N., Ph.D.			
24 KEYWORDS (Precede EACH with Security Classification Code) ^r (U) Military Dogs; (U) Ehrlichiae; (U) Parainfluenza SV-5; (U) Amyolytic Intestinal Enzymes; (U) Random-Source Dogs; (U) Viral Respiratory Disease; (U) Monkeys; (U) Hamsters; (U) Enterotoxigenic E. coli							
25 TECHNICAL OBJECTIVE ^s 26 APPROACH ^t 27 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
25. (U) To investigate diseases and/or conditions affecting or associated with the military dog; to enhance disease diagnosis, treatment and control, and to investigate diseases and/or conditions affecting laboratory animals used for military research, to enhance production quality and health management to provide research animals free of pathogens.							
24. (U) Conventional epidemiological, pathological, and microbiological methods are employed; special procedures are developed as needed.							
25. (U) 75 07 - 76 06 Inocula of Ehrlichia canis, including those sonically disrupted, failed to infect cell cultures of 4 separate canine and 5 other species cell lines. In field trials of a new SV-5 vaccine in recruit military dogs, 90 per cent developed low serum neutralizing antibody titers after two doses given subcutaneously. Vaccine virus did not infect unvaccinated dogs; untoward reactions were not observed. Wild strains of SV-5 could not be evaluated. An optimal standard procedure for preparation and assay of canine digestive enzymes was presented. 76 03 - 76 06 Severe respiratory disease (mostly canine distemper) affected more than half of the random-source dogs during conditioning, with 40 per cent mortality. SV-5 and Toronto A26/61 caused most of the URD in CD immune dogs, and contributed to the severity of CD. Hamsters were raised free of Enterobacteriaceae to evaluate the role played in antibiotic-induced diarrhea and death; their absence from the intestinal tract had no sparing effect. E. coli strains from 116 monkeys and 25 hamsters with diarrhea failed to show heat labile enterotoxin. E. coli from 4 of 52 monkeys showed heat stable enterotoxin. Shigella sp was isolated from 27 percent of the monkeys. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 75-30 Jun 76. New work initiated with mission and title change 76 03, will be separated under work unit 057 in FY 77. Support in the amount of \$31,000 from FY 77 funds is programmed for the period 1 Jul-30 Sep 76.							

PII Redacted

DD FORM 1498

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

1596

Project 3A762760A837 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 056 Diseases of Military Animals Section I

Investigators:

Principal: LTC E. H. Stephenson, VC
Associate: L. N. Binn, Ph.D.; LTC T. J. Keefe, VC; CPT J. R. Boyce, VC; E. S. Windham, M.S.; K. Noon, B.S.; R. L. Marchwicki, B.S.; I. E. Hemelt, A.B.; SP5 A. Hussain; SP5 D. White; SP4 R. Federman; SP4 E. K. Vinson; SP4 L. R. Nornhold; PFC A. Kanthack; and PFC R. Thomas

Description:

To define, study, diagnose, and control known and potential infectious diseases of military dogs. A major effort is directed toward developing an alternate in vitro method for cultivating Ehrlichia canis rather than the primary canine macrophage. Additional studies concern the epidemiology, diagnosis, treatment and control of other disease agents or conditions affecting the military dog.

During the reporting period, research activities have included:

(1) attempts at cultivation of E. canis in cells other than primary canine macrophages, (2) evaluation of parainfluenza SV5 vaccine in military dogs, and (3) development of a standardized method for preparation and assay of canine intestinal carbohydrases.

1. Propagation of Ehrlichia canis in cells other than canine peripheral monocytes.

Ehrlichia canis, the causative agent of canine ehrlichiosis, is the type species of a group of rickettsia-like organisms that form intracytoplasmic inclusions in circulating leucocytes of susceptible mammalian hosts. Until the development of the primary monocyte cell culture system, ehrlichial investigations were limited to in vivo studies. Use of the peripheral monocyte culture technique afforded a means for developing the indirect immunofluorescence technique and conducting limited studies on comparative morphogenesis and pathogenesis. The morphogenetic and pathogenetic studies of E. canis, however, are hindered by 2 technical barriers: the inability to propagate ehrlichiae in large numbers and the inability to quantitate the organisms. Canine peripheral blood monocyte cultures, as currently prepared, do not support the growth of sufficient organisms to be experimentally useful. Therefore, another cell and/or host must be defined in which ehrlichiae will replicate in large numbers and

retain all the properties of the agents propagated in vivo. This study was designed to determine the susceptibility of different available cell types and embryonated chicken eggs to infection with E. canis.

Ehrlichia canis propagated in cultures of primary canine peripheral blood monocytes was used as the source of inocula when greater than 90% of the detached monocytes contained one or more morula. Inocula for the different experiments were: procedure 1, detached cells and media from infected cultures; procedure 2, attached, infected cells which were trypsin-EDTA dispersed, then pelleted by centrifugation and resuspended; and procedure 3, infected cells treated as (2) above, then exposed to 60W of ultrasound treatment for 15 sec using a Branson sonifier and the free agents were concentrated by centrifugation. Following sonication, the infected cells were completely disrupted leaving numerous intact ehrlichiae, as determined by fluorescent microscopy. Sonicated inocula were used to infect canine peripheral blood monocyte cultures. Seventy to 80% of the cells contained 1 or more agents at 24 hr post inoculation (PI), and morulae developed by 7 days PI. The percent of cells infected approached 100% by 10 days PI. By contrast, when canine peripheral blood monocytes were inoculated with ehrlichial suspensions prepared as described in procedures 1 and 2, approximately 30 to 40% of the cells contained 1 or more agents at 24 hr PI. The number of cells infected gradually increased until greater than 90% of the cells contained morulae by 10 to 14 days PI. Thus, use of ehrlichial suspensions that were sonically treated resulted in enhanced synchrony of the infection and in a reduction by 3 to 7 days of the time required to achieve maximum infection of canine peripheral blood monocytes.

Both primary cells and cell lines were inoculated with E. canis. Primary cells used were canine spleen, canine kidney, canine thyroid, bovine fetal spleen, and mouse macrophage cells. Cell lines included the Walter Reed canine continuous line, A-72 canine tumor continuous line, L929 mouse fibroblast, J-111 human monocytic leukemia, and LLC-MK2 rhesus monkey kidney cells. Coverslip cultures of each cell type were prepared in Leighton tubes. Culture medium was decanted, the cultures were washed twice with Hank's balanced salt solution (HBSS), and the inoculum was added. When inocula were prepared by procedures 1 and 2 above, a total of 1.0 ml was transferred to each culture tube. After 24 hr, the inoculum was decanted, the cultures were washed twice with HBSS, and 1.0 ml of medium (EMEM + 2 μ M glutamine + 20% heat inactivated canine serum) was added. When the sonicated inoculum was used, the 24 hr incubation period was replaced by a 1 hr adsorption period. All cultures were incubated at 35 C.

Ehrlichiae were detected in coverslip cultures of infected cells by the Giemsa stain method and by direct fluorescent microscopy.

In one series of experiments, irradiation was used to arrest multiplication of all the cell types, except mouse macrophages, in the G-1 phase of the cell cycle. The reasons for employing this procedure were 4-fold: (1) the canine monocyte is a non-replicating cell,² (2) chlamydiae replicate in cells in the G-1 phase of the cell cycle,² (3) the susceptibility of McCoy cells to infection by Chlamydia trachomatis and C. psittaci is significantly increased when the cells are irradiated with 4000 to 5000 r prior to inoculation,³ and (4) rickettsiae replicating in non-irradiated L929 mouse fibroblasts become undetectable due to the cells replicating more rapidly than the rickettsiae, which results in a smaller percent of the total cell population being infected with each doubling of the host cells.⁴ Suspensions of cells were exposed to gamma radiation from a ⁶⁰Co source. The radiation dose received by each cell type was predetermined as the amount necessary to stop replication and varied from 3000 to 5000 r. The integrity of monolayers of irradiated cells was maintained in excess of 21 days after exposure if the medium was changed every 3 to 4 days. Irradiated cells were inoculated with ehrlichial suspensions described in procedures 2 and 3.

Six- to 8-day-old embryonated chicken eggs were inoculated via the yolk sac, chorioallantoic, and amniotic routes using inoculum prepared by procedure 2. Three eggs each were inoculated with 0.5 ml of the undiluted inoculum and the next 2 serial 10-fold dilutions. Inoculated eggs were incubated at 35 C and 55 to 60% relative humidity, and were candled daily for 18 days for evidence of embryonic death. Impression smears obtained from tissues of embryos surviving 3 days⁵ PI were examined for rickettsia-like organisms by the Gimenez method⁵. Two blind subpassages were made before the ability of E. canis to replicate in embryonated eggs was considered to be negative.

The initial attempts to infect other cell types with E. canis were made using inocula prepared by procedures 1 and 2. Only canine spleen and bovine fetal spleen cells supported the growth of ehrlichiae. Less than 0.5% of the cell population of these cultures has small intracytoplasmic inclusions that stained specifically with conjugated anti-E. canis serum. Subpassage of such cultures did not result in increased numbers of infected cells, but resulted in the loss of the agent. If ehrlichiae entered any of the other cell types, destruction occurred within 24 hr, which was the time selected for the initial examination. Co-cultivation of the cell types with infected canine peripheral blood monocytes also was tried. Replication of ehrlichiae was detected in a rare canine spleen and bovine fetal spleen cell. Again, the result of subpassage of the canine spleen and bovine fetal spleen cultures was loss of the agent. Ehrlichiae were detected only in a few cells of canine spleen and

bovine fetal spleen cultures when sonically treated ehrlichial preparations were used as inocula. Irradiated cells failed to support the growth of ehrlichiae, with the exception of about 0.5% of the canine spleen and bovine fetal spleen cells. The agents were lost upon subpassage to irradiated cultures of the same type.

Ehrlichia canis would not replicate in the yolk sac, on the chorio-allantoic membrane, or in the amniotic cavity of embryonated chicken eggs, even after 2 blind subpassages. The few embryo deaths that occurred were not attributable to ehrlichial infection and a specific death pattern was not observed. Rickettsia-like organisms were not seen in Gimenez-stained impression smears of the appropriate tissues.

Failure to propagate E. canis in host cells other than the primary canine monocyte suggested a specific genetic and physiologic relationship between ehrlichiae and canine monocytes that does not exist for any of the cell types tested to date. This relationship appears to be species specific because the mouse macrophage, which is ontogenetically similar to the canine monocyte, did not support the growth of ehrlichiae. A search for a more efficient cell and/or host for propagation of large numbers of ehrlichiae is still in progress.

Cultures of canine spleen, canine kidney, canine thyroid, bovine fetal spleen, mouse macrophage, Walter Reed canine continuous line, A-72 canine tumor continuous line, L929 mouse fibroblast, J-111 human monocytic leukemia, and LLC-MK2 rhesus monkey kidney cells were inoculated with E. canis that had been propagated in primary canine peripheral blood monocytes. Ehrlichiae did not replicate in any of the cultures, except canine spleen and bovine fetal spleen cells. Small intracytoplasmic inclusions were detected in less than 0.5% of these cells. Identical results were obtained when the host cells were irradiated with 3000 to 5000 r prior to inoculation and when co-cultivation techniques were used. Ehrlichiae could not be propagated in the yolk sac, on the chorioallantoic membrane, or in the amniotic cavity of embryonated chicken eggs, even after 2 blind subpassages.

2. Evaluation of a parainfluenza SV5 vaccine for the prevention of respiratory disease in military dogs.

Over the past 12 years epizootics of respiratory disease have occurred at the DOD procurement and training centers at Lackland AFB (LAFB), Texas, and Ft. Benning, Ga.^{6,7} (WRAIR Annual Report 1975). During the disease outbreaks, approximately one-fourth of the dogs developed signs of respiratory disease and the great majority of the remaining dogs had inapparent infections. The epizootics seriously disrupted the training and deployment of military dogs. Parainfluenza SV5 virus

had been recovered consistently from affected dogs and the virus was demonstrated to be highly communicable. In December 1974, a vaccine containing attenuated parainfluenza SV5 was licensed for the immunization of dogs. The following May in conjunction with the DOD military dog center at LAFB a prospective study was initiated to evaluate the new vaccine in recruit military dogs. The present report summarizes the initial findings.

The vaccine evaluation study involved approximately 250 dogs; half received the standard bivalent canine distemper (CD)-infectious canine hepatitis (ICH) vaccine and the remaining half, the new CD-ICH-SV5 vaccine. Each dog was given 2 vaccinations at 30 day intervals by the subcutaneous route. The dogs were bled before vaccination on arrival at LAFB and at intervals thereafter as indicated below. Serums were forwarded to WRAIR for SV5 serum neutralization tests. The vaccinated dogs were examined for signs of disease and any reactions to vaccination.

Untoward vaccine reactions were not reported in nearly 100 SV5 vaccinated dogs. During the study period only a few new dogs have exhibited minor transient signs of respiratory disease and there have been no indications of secondary cases.

Laboratory studies to measure the SV5 antibody response have been carried out on approximately 30 vaccinated dogs. In addition, tests were conducted to detect SV5 infections in a similar number of dogs receiving the standard CD-ICH vaccine. With 2 possible exceptions, evidence of infections was not detected in dogs given the CD-ICH vaccine. One dog arrived with a low SV5 antibody titer (1:16) and had a rise in titer and a second dog developed a low neutralizing antibody titer after the second dose of standard vaccine. These findings indicated that SV5 virus infections of vaccine or natural origin were rare events if they occurred at all during this study period.

The antibody response of seronegative dogs given SV5 vaccine is summarized in Tables 1 and 2. In the initial study, the development of SV5 antibody in 8 dogs was examined (Table 1). Low levels of SV5 neutralizing antibody were first detected at 14 days after the initial dose. At 30 days, the titers were similar or slightly lower. Following the second vaccination, a 4-fold increase in antibody titer occurred and 7 of the 8 dogs were serotest positive. In a larger group of dogs, a similar antibody response was observed (Table 2). More than 60% (23 of 37 dogs) developed low levels of neutralizing antibody and 90% of the dogs had antibody after 2 doses. However, the levels of antibody were approximately 1/8th of that obtained after natural infection.

The level of vaccine induced antibody required for protection is unknown. It would be prudent, however, to explore the means of inducing higher levels of antibody. Data provided by the manufacturer indicates that the intramuscular route induces 2- to 4-fold higher antibody titers. Accordingly, studies are in progress comparing the response of military dogs to SV5 vaccine given by the subcutaneous and intramuscular routes.

In summary, a new SV5 vaccine is undergoing safety and potency tests to prevent respiratory disease in recruit military dogs. Following 2 doses of vaccine administered subcutaneously, 90% of the dogs develop low serum neutralizing antibody titers. The SV5 vaccine virus did not spread to unvaccinated dogs and untoward reactions were not reported. During the observation period, only a few dogs showed serological evidence of SV5 infection. These findings support the conclusion that wild strains of SV5 were not being transmitted. Consequently, it was impossible to evaluate the ability of the vaccine to protect dogs against field challenge with SV5 virus. Further studies are in progress, to increase the antibody titers produced by vaccination.

3. Detection and definition of canine intestinal carbohydrases using a standardized method.

Previous investigations of German shepherd puppies with chronic diarrhea showed a relationship between high starch diets and (1) chronic diarrhea, (2) frank starch in the feces, (3) numerous trichomonads and giardia in the feces, (4) malabsorption of xylose, and (5) mucosal injury of the small intestine. However, when constituent analysis for fat, protein, starch, and hemicellulose were conducted on the diarrhea stools from adult G. shepherds and from stools from adult inbred hounds, both breeds showed surprisingly comparable digestive efficiency (WRAIR Annual Report 1975). In conjunction with these studies, investigations were started for detection and definition of canine intestinal carbohydrase activity.

A survey of the literature on dog nutrition had brought to our attention significant differences in the reported levels of canine carbohydrate digesting enzymes. These differences could have been due to a host of variables, such as assays conducted on mixed or different breeds, and variations in feeding, environment, specimen collection and enzyme assay as well as different interpretations of test results^{8,9,10,11,12}. In fact, the inconsistencies encountered in our earliest attempts to repeat the studies of other authors, pointed out that a comparison of methods and their standardization was needed. This report describes the qualitative and quantitative carbohydrase contents of the jejunum and pancreas in purebred beagle dogs, as determined by a standardized methodology. The enzymes

investigated were α -amylase E.C.3.2.1.1, maltase and isomaltase E.C.3.2.1.20, sucrase E.C.3.2.1.26, lactase E.C.3.2.1.23, amyloglucosidase E.C.3.2.1.3 and cellobiase E.C.3.2.1.4.

Jejunal samples were obtained from 18 purebred, healthy inbred hounds whose ages ranged from 17 to 33 weeks. They were fed a daily diet of approximately 230 Gm. of Respond 2000 plus 116 Gm. of Kenl Ration, but fasted overnight prior to sampling. Specimens taken from the mid-jejunum region were chilled on wet ice and sliced longitudinally. Feces were removed by gently washing the mucosa with 0.85% NaCl - 0.02 CaCl₂. These mucosa samples were stored at -60 C. Pancreatic samples were stored at -60 C. Mucosa carefully scraped from thawed sections of jejunum with a glass slide was weighed and suspended in 4 volumes of cold 0.85% NaCl. Suspensions were sonicated in an ice bath using a sonicator set at 90 watts for either one or four minutes and stored at -60 C. Pancreatic samples were thawed, weighed and suspended in 20 vol of cold 0.85% NaCl + 0.02M CaCl₂. After mincing with scissors, the tissue was sonicated for 2 minutes and frozen at -60 C. Some of the sonicated mucosal homogenates were enzymatically solublized according to the method of Dahlquist¹³. After centrifugation, the clear supernatant extracts were removed and frozen at -60 C. All samples were diluted 1:100 v/v in 0.85% NaCl and assayed for protein by the method of Lowry et al¹⁴. Disaccharidase activities were measured by using a commercially prepared tris-glucose oxidase kit. The procedure was the same as that used by Dahlquist¹⁵. All the substrates, maltose, isomaltose, sucrose, lactose and cellobiose were prepared according to the method of Dahlquist except for the soluble starch. Contaminating glucose and maltose were removed from the soluble starch substrate by modifying the method used by Friedemann et al¹⁶. In this modification one gram of soluble starch was precipitated for 1 hr in 100 ml of 70% isopropanol, filtered and reprecipitated 4 more times. The slurry was then stirred in 100% isopropanol to remove the water from the starch, filtered and dessicated to dryness. Due to the variations in the size of the molecules of starch, the number of glucose residues per starch molecule is inestimable and consequently problematical. Therefore, in calculating amyloglucosidase activity, starch degradation was interpreted as a disaccharide yielding one glucose molecule per enzymatic degradation. The α -amylase activity was determined by measuring the liberation of reducing sugars¹⁷. Units of activity are expressed as nanomoles of maltose produced per mg protein per min. Amyloglucosidase activity was assayed after heating samples at 55 C for 15 min to inactivate the heat labile α -amylase¹⁸. Isomaltase activity was demonstrated using chromatographically homogeneous isomaltose as the substrate. Heating at 54 C was used to inactivate isomaltase¹⁹. Units of disaccharidase activity were expressed as nanomoles of disaccharide hydrolyzed per mg protein per min. The enzymes of solublized mucosal samples were separated

by polyacrylamide gel electrophoresis (PAGE: 20,21). The PAGE preparation was composed of 15 drops of glycerol, 4 drops of 0.1% aqueous bromophenol blue and 1.3 ml enzyme mixture. The enzyme mixture was adjusted so that 300 μ l of the PAGE preparation contained 400 μ g protein. The 300 μ l PAGE preparation was layered on the separation gel within glass tubes and electrophoresed. Both the discontinuous tris-HCl²¹ and the continuous imidazole buffer systems²¹ were each used in similar procedures for separating the enzymes. All gels were electrophoresed at 2ma per tube at 4 C for approximately 4 hours. Gels were sliced at 2 mm intervals and each segment was assayed for disaccharidase activity²⁰. Duplicate gels were stained for protein with Coomassie blue dye.

Results

Hill²² has shown that the digestion of starch by the canine is initiated by the pancreatic α -amylases secreted into the lumen of the small intestine. The resulting starch fragments and disaccharides are then degraded further by enzymes associated with the cells and membranes of the brush borders of the small intestine. Table 3 describes these processes including isomaltase activity not previously reported by other authors.

Table 4 presents a comparison of disaccharidase activities found after different extraction treatments of inbred hounds intestinal mucosa. The mucosal homogenates were sonicated for 1 min, 4 min, and 4 min followed by enzyme solubilization. Homogenates sonicated for 4 min without solubilization showed the highest disaccharidase activity for maltase, sucrase, and amyloglucosidase. Only the lactase activity decreased after this prolonged sonication. The samples which were sonicated for 4 min and then solubilized with papain all lost activity.

An excess of the 4 minute-sonicated samples afforded an opportunity to measure the cellobiase and isomaltase activities of these samples. It can be seen that inbred hounds do have some ability to degrade β -linkages of disaccharides derived from cellulose (Table 4). Isomaltase activity was demonstrated as a separate isozyme in 8 of the 9 dogs studied (Table 5). Only one dog had no isomaltase activity and in this dog and 2 others the total maltase activity disappeared upon heating at 54 C for 1 hr.

The α amylase activity of the pancreas was 22.621 \pm 5.08 units and the total amylase activity (α amylase+amyloglucosidase) of the intestinal mucosa was 0.031 \pm 0.02 units (Table 4).

When papain solubilized homogenates from 2 dogs were applied to 7.5% polyacrylamide gels, electrophoresed and stained for protein, four major protein bands were detected, one fast moving and three slower

moving bands. In the discontinuous tris-HCl buffer system, maltase and amyloglucosidase were found in the sliced gels. In Figure 1A two samples are compared. Sample Ts shows two peaks of maltase activity and Ys shows only one peak. Figure 1B depicts amyloglucosidase activity in both samples with sample Ts possessing a small second peak of doubtful significance. In the continuous imidazole buffer system maltase, amyloglucosidase and sucrase were demonstrated in the sliced gels. Again the Ts sample displayed two peaks of maltase activity and the Ys sample only one peak. The amyloglucosidase assayed gels revealed only one peak in each of the samples. A third set of gels showed sucrase activity trailing over a broad range of slices. Lactase activity was not found in either buffer system and will be described later.

In these experiments it was seen that the method of treatment of intestinal tissue greatly affected the detection and quantification of disaccharidase activity in the microvilli of the mucosal epithelium. For example, the samples which were sonicated for only one minute had amyloglucosidase activities which were barely detectable. However, upon prolonged sonification a nearly 25-fold increase in activity was observed. The maltase activity increased more than threefold, and the sucrase activity improved slightly. Only lactase showed a reduction in activity after sonification for 4 minutes. This may be due to its sensitivity to heat and oxidation²³. In contrast, the solubilization treatment proposed by Dahlquist and Teleneus¹³ had a deleterious effect on the activities of all the enzymes tested. These variations may explain the inconsistencies seen when comparing the results of other authors. Hill²², in his excellent study on malabsorption in dogs, used two methods for enzyme extraction. A Griffiths tube homogenizer was used for manual assay whereas, an ultrasonicator was used for assay in an autoanalyzer. The sonicated samples always yielded higher activities. He stated that this increased activity was probably due to untimed enzyme hydrolysis in the dialyzer of the autoanalyzer. However, early studies in our laboratory comparing methods of tissue disruption indicated that sonicated tissue yielded higher enzymatic activity. Thus, we believe that some of the increased activity found by Hill could be due to greater release of these membrane-bound enzymes. It should also be noted that previous studies presented carbohydrase values from mongrels or as averages from two or more purebreds. The present investigation has defined carbohydrase values for one purebred dog.

The multiplicity of canine maltases has not yet been defined. Auricchio has separated five different human maltases¹⁹. Three of these are heat inactivated at 54 C for 1 hr, maltases III and IV each of which degrades maltose and sucrose and maltase V which degrades maltose, palatinose and isomaltose. The presence of a canine

maltase type V has not been described in previous investigations. Therefore, to show isomaltase as a separate isozyme of maltase in canines, we measured maltase and isomaltase activities of 4 min-sonicated homogenates before and after heating. The results showed a heat sensitive isomaltase in 8 of the 9 dogs sampled (Table 5). Three of the samples including that with no isomaltase, had a total maltase activity which disappeared upon heating at 54 C. According to Auricchio's scheme, these 3 samples do not show the presence of the heat stable maltases I and II. On the basis of the nine dogs studied, we have shown that canines do possess several maltases: heat stable (54 C, 1 hr) maltase types I and II, heat labile maltase types III and IV and a heat labile isomaltase type V (Table 3).

The tris-glucose-oxidase method of Dahlquist used in these experiments employs o-dianisidine as a chromagen. Because of its suspected carcinogenic effects, this method was supplanted by the Trinder method²⁴ which utilizes 4-aminophenazone and phenol. The results obtained with the Trinder method were comparable to those of the Dahlquist method when the same protocol was used.

In this study, a unit of disaccharidase activity is defined as nanomoles of disaccharide hydrolyzed per mg protein per minute. A mg of protein was chosen as the criterion rather than a mg wet weight of tissue because it is a more reproducible method of measurement. Hill²² came to this same conclusion after using the wet weight method of measurement.

α -amylase has been widely investigated using different techniques which employ various units of activity and it is therefore difficult to compare the results of different investigators^{12,25,26}. The Dahlquist method of reducing sugars was chosen for these studies because its unit of amylase activity is defined according to the recommendations for clinical and biochemical enzyme units made by the Joint Sub-Commission on Clinical Enzymes Units of the International Union of Biochemistry and the International Union of Pure and Applied Chemistry¹⁷.

In the polyacrylamide gel electrophoresis experiments neither sucrase nor lactase activity was detected in the tris-HCl buffer system. This was probably due to the inhibitory nature of the tris buffer on disaccharidases²⁰. For this reason, the imidazole buffer was used instead. Sucrase activity was detected and a 2- to 3-fold increase in activity was found for maltase and amyloglucosidase. However, the resolution of enzyme activity in the imidazole system was not as discrete as the discontinuous tris-HCl system. Our inability to detect lactase activity in either buffer may have been due to a loss of activity during the solubilizing of the samples, some inhibitory effect of the PAGE system, or a combination of both, as lactase is

inactivated by heat and by oxidation²³.

The reproduction of the two peak pattern of maltase activity in different electrophoretic buffer systems indicated that one of the peaks in the Ts sample is a valid and separable enzymatic activity and it may represent the presence of isomaltase which was subsequently demonstrated in the heat inactivation studies. The absence of this maltase-isomaltase enzyme in one dog's mucosa indicated that it was either a non-induced, inducible enzyme or a variable genetic characteristic of this breed.

Our data indicate that thermal, chemical, or mechanical treatment of intestinal mucosa prior to or during assay contributes to quantitative variations when measuring levels of digestive carbohydrases. This becomes a major consideration when digestive disorders are suspected to be due to enzymatic deficiencies. A statistical analysis of variance [$\sigma^2_{\text{Total}} = \frac{\sigma^2_{\text{Between}}}{N} + \frac{\sigma^2_{\text{within}}}{N \cdot n}$] of the results obtained using the procedures outlined in this report revealed that the variation within sample is extremely low. The variations reflected in the standard deviations in Table 4 are primarily contributed by the variation among the dogs. Thus even among a group of purebred dogs, the intestinal enzyme levels span a range of specific activity. Therefore, it is important to reduce variations stemming from method of treatment and assay to a minimum. The procedure presented in this report is an effort to refine the method of detection in order to further define the composition and concentration of canine intestinal carbohydrases.

In summary, intestinal mucosa and pancreas from inbred hounds were assayed for carbohydrase activity using several methods of tissue treatment. The enzymes found and studied were α -amylase, sucrase, lactase, amyloglucosidase, cellobiase, maltase and isomaltase. Experiments using polyacrylamide gel columns and heat inactivation showed the presence of an isoenzyme of maltase which degrades isomaltose. This activity has not been previously demonstrated in canines. An optimal standard procedure is presented for the preparation and assay of canine digestive enzymes. A statistical analysis of variance of the results showed that the variance was primarily associated with differences among dogs and not by variance within the procedure. When the different extraction procedures were used, results indicated that the level of enzymes detected differed with the method of treatment.

Table 1: The development of parainfluenza SV5 neutralizing antibody in seronegative dogs given an attenuated CDV-ICH-SV5 vaccine (preliminary study)*

Vaccination schedule		Day post vaccination examined	No. of dogs with antibody titer								Geom. mean. titer	No. sero positive**	
Dose	Day		0	2	4	8	16	32	64	128		No. tested	(%)
1	0	7	8	-	-	-	-	-	-	-	0	0/8	(0)
		14	2	3	1	2					2.6	6/8	(75)
		30	4	1	2	1					2.0	4/8	(50)
2	30	45	1	-	3	1	1	1	1		8.0	7/8	(88)
		70	1	2	1	2	-	2	-		5.7	7/8	(88)

*Tissuvax D-H-P (Pittman-Moore Inc., Washington Crossing, NJ 08560) by the Subcutaneous route.

**Titer 1:2 or greater.

Table 2: The parainfluenza SV5 neutralizing antibody response of seronegative dogs given attenuated CDV-ICH-SV5 vaccine by the subcutaneous route

Vaccination schedule		Day post vaccination examined	No. of dogs with antibody titer								Geom. mean. titer	No. sero positive	
Dose	Day		0	2	4	8	16	32	64	128		No. tested	(%)
1	0	30	14	10	9	3	1				2.2	23/37	(62)
2	30	45	4	1	9	9	5	5	1	1	7.8	31/35	(89)
		70	3	8	9	4	3	3			4.5	27/30	(90)

Table 3: Location and Sequence of Canine Enzymatic Carbohydrate Digestion*

Intraluminal	Brush border	Maltase type
Starch		
Amylose $\xrightarrow{\alpha\text{-Amylase}}$	Maltose, Maltotriose $\xrightarrow{\text{Maltase}}$	Glucose I \rightarrow V
Amylopectin $\xrightarrow{\alpha\text{-Amylase}}$	Maltose $\xrightarrow{\text{Maltase}}$	Glucose I \rightarrow V
	Isomaltose $\xrightarrow{\text{Isomaltase}}$	Glucose V
	Oligosaccharises $\xrightarrow{\text{Amyloglucosidase}}$	Glucose I
	Lactose $\xrightarrow{\text{Lactase}}$	Glucose, Galactose
	Sucrose $\xrightarrow{\text{Sucrase}}$	Glucose, Fructose III & IV

*This table is in part derived from references 19 and 22.

Table 4: Carbohydrases of Inbred Hounds*

Treatment of samples:	Specific activity of enzymes found in intestines				Amylases units/mg. protein/min.†
	Disaccharidases units/mg. protein/min.**				
	Maltase	Sucrase	Lactase	Amyloglucosidase	Cellobiase
Homogenate sonicated 1 min.	0.069±0.02‡	0.049±0.02	0.045±0.02	0.002±0.00	ND
Homogenate sonicated 4 min.	0.238±0.13	0.057±0.02	0.026±0.01	0.049±0.03	0.006±0.00
Solubilized Extract sonicated 4 min.	0.145±0.14	0.019±0.02	0.011±0.01	0.015±0.01	ND
Pancreatic α-Amylase activity units/mg. protein/min.†					
Homogenate sonicated 2 min.	22.621±5.08				

*Results derived from 10-12 samples each. Tests performed in triplicate.

**Nanomole disaccharide hydrolyzed per mg. protein per minute.

†Nanomole Maltose produced per mg. protein per minute.

‡ \bar{x} ± Standard Deviation.

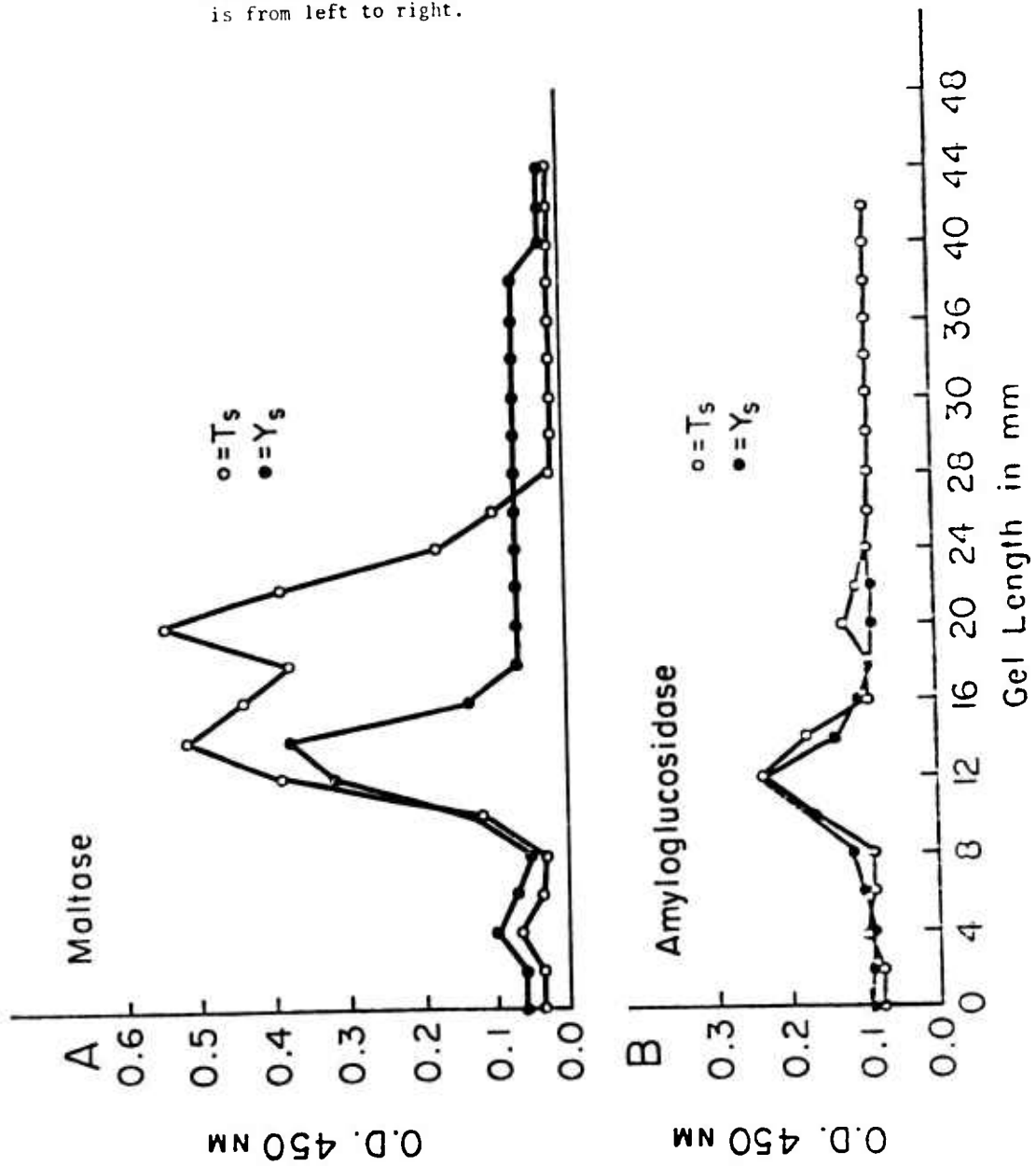
ND - Not determined.

Table 5: Maltase and Isomaltase Activities in heated and unheated mucosal homogenates sonicated for 4 min.

Enzyme: Maltase		Isomaltase		
Substrate: maltose		isomaltose		
	Unheated	Heated 54 C/hr	Unheated	Heated 54 C/hr
Sample J	0.3999*	0.2163	0.0589	0.0000
T	0.3801	0.2095	0.0670	0.0000
G	0.3245	0.1517	0.0599	0.0000
A	0.3000	0.0800	0.0482	0.0000
H	0.2504	0.0000	0.0000	0.0000
E	0.2205	0.0835	0.0423	0.0000
F	0.1835	0.0565	0.0221	0.0000
I	0.1554	0.0000	0.0220	0.0000
K	0.1502	0.0000	0.0204	0.0000

*units - nanomole disaccharide hydrolyzed per mg. protein per minute.

Figure 1: Separation of disaccharidases by polyacrylamide gel electrophoresis. Discontinuous electrophoreses using tris-HCl buffer system. Direction of electrophoresis is from left to right.



Project 3A762760A837 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 056 Diseases of Military Animals

Literature Cited.

References:

1. Nyindo, M. B. A., M. Ristic, D. L. Huxsoll, and A. R. Smith. 1971. Tropical canine pancytopenia: In vitro cultivation of the causative agent - Ehrlichia canis. Amer. J. Vet. Res. 32: 1651-1658.
2. Storz, J. 1971. Chlamydia and chlamydia-induced diseases. Charles C. Thomas, Springfield, IL.
3. Gordon, F. B., H. R. Dressler, A. L. Quan, W. T. McQuilkin, and J. I. Thomas. 1972. Effect of ionizing irradiation on susceptibility of McCoy cell cultures to Chlamydia trachomatis. Appl. Microbiol. 23:123-129.
4. Osterman, J. V. 1975. Personal communication.
5. Gimenez, D. F. 1964. Staining rickettsiae in yolk-sac cultures. Stain Technol. 39:135-140.
6. Binn, L. N., E. C. Lazar, M. Rogul, V. M. Shepler, L. J. Swango, T. Claypoole, D. W. Hubbard, S. G. Asbill, and A. D. Alexander. 1968. Upper respiratory disease in military dogs: Bacterial, mycoplasma, and viral studies. Amer. J. Vet. Res. 29: 1809-1815.
7. Crandell, R. A., W. B. Brunslow, and V. E. Davison. 1968. Isolation of a parainfluenza virus from sentry dogs with upper respiratory disease. Amer. J. Vet. Res. 29:2141-2147.
8. Hill, F. W. G., and D. F. Kelly. 1974. Naturally Occurring Intestinal Malabsorption in the Dog. Digest. Dis. 19:649-665.
9. Hore, P., and M. Messer. 1968. Studies of Disaccharidase Activities of the Intestine of the Domestic Cat and Other Carnivorous Mammals. Comp. Biochem. and Physiol. 24:717-725.
10. Toofanian, F., D. E. Kidder, and F. W. G. Hill. 1974. The Amylases of Calf Small Intestine. Brit. Vet. J. 130:XIX-XXI.

11. Welsh, J. D., and A. Walker. 1965. Intestinal Disaccharidase and Alkaline Phosphatase Activity in the Dog. *Proc. Soc. Exp. Biol. and Med.* 120:525-527.
12. Ziene, L., W. C. Vogel, and W. D. Kelly. 1963. Species Difference in Pancreatic Lipolytic and Amylolytic Enzymes. *J. App. Physiol.* 18:77-82.
13. Dahlquist, A., and U. Telenius. 1969. Column Chromatography of Human Small Intestinal Maltase, Isomaltase and Invertase Activities. *Biochem. J.* 111:139-146.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* 193:265-275.
15. Dahlquist, A. 1964. Method of Assay of Intestinal Disaccharidases. *Anal. Biochem.* 7:18-25.
16. Friedemann, T. E., N. F. Witt, and B. W. Neighbors. 1967. Determination of Starch and Soluble Carbohydrates. I. Development of a Method for Grains, Stock Feeds, Cereal Foods, Fruits and Vegetables. *J. AOAC* 50:944.
17. Dahlquist, A. 1962. A Method for the Determination of Amylase in the Intestinal Content. *Scand. J. Clin. and Lab. Invest.* 14:145-151.
18. Kidder, D. E., F. W. G. Hill, and J. A. Stevens. 1972. Automatic Measurement of Some Mucosal Carbohydrases. *Clin. Chim. Acta*, 37:491-501.
19. Auricchio, S., G. Semenza, and A. Rubino. 1965. Multiplicity of Human Intestinal Disaccharidases. II. Characterization of the Individual Maltases. *Biochim. Biophys. Acta* 96:498-507.
20. Alpers, D. H. 1969. Separation and Isolation of Rat and Human Intestinal β -Galactosidases. *J. Biol. Chem.* 244:1238-1246.
21. Ornstein, L. 1961. *Disc Electrophoresis*, Canal Industrial Corp., Bethesda, MD.
22. Hill, F. W. G., Ph.D. Thesis. 1971. Univ. of Bristol, Dept Vet Med, Bristol, England.
23. Birkenmeier, E., and D. H. Alpers. 1974. Enzymatic Properties of Rat Lactase-Phlorizin Hydrolase. *Biochim. Biophys. Acta* 350:100-112.

24. Trinder, P. 1969. Determination of Glucose in Blood Using Glucose Oxidase with an Alternative Oxygen Acceptor. *Ann. Clin. Biochem.* 6:24-26.

25. McGeachin, R. L., J. R. Gleason, and M. R. Adams. 1958. Amylase Distribution in Extrapaneatic, Extrasalivary Tissues. *Arch. Biochem. and Biophys.* 75:403-411.

26. Rajasingham, R., J. L. Bell, and D. N. Baron. 1971. A comparative study of the isoenzymes of mammalian α -amylase. *Enz.* 12:180-186.

Publications:

1. Buhles, W. C., Jr., D. L. Huxsoll, and P. K. Hildebrandt. 1975. Tropical canine pancytopenia: Role of aplastic anemia in the pathogenesis of severe disease. *J. Comp. Pathol.* 85:511-521.

2. Buhles, W. C., D. L. Huxsoll, G. Ruch, R. H. Kenyon, and B. L. Elisberg. 1975. Evaluation of primary blood monocyte and bone marrow cell culture for the isolation of Rickettsia rickettsii. *Infect. Immun.* 12:1457-1463.

3. Davidson, D. E., Jr., G. S. Dill, Jr., M. Tingpalapong, S. Premabutra, P. L. Nguen, E. H. Stephenson, and M. Ristic. 1975. Canine ehrlichiosis (tropical canine pancytopenia) in Thailand. *Southeast Asia J. Trop. Med. Pub. Hlth.* 6:541-543.

4. DeShazo, R. D., J. R. Boyce, J. V. Osterman, and E. H. Stephenson. 1976. Early diagnosis of Rocky Mountain spotted fever using the primary monocyte culture technique. *J. Amer. Med. Ass.* 235:1353-1355.

5. Groves, M. G., G. L. Dennis, H. L. Amyx, and D. L. Huxsoll. 1975. Transmission of Ehrlichia canis by ticks (Rhipicephalus sanguineus). *Amer. J. Vet. Res.* 36:937-940.

6. Lewis, G., D. Renquist, I. Hemelt, D. Huxsoll, M. Ristic, and E. H. Stephenson. 1975. Experimental inoculation of Macaca mulatta with Ehrlichia canis-infected canine monocyte cell culture. *Proc. 56th Ann. Meet. Conf. Res. Workers Anml. Dis.* p.15.

7. Smith, R. D., D. M. Sells, E. H. Stephenson, M. Ristic, and D. L. Huxsoll. 1976. Development of Ehrlichia canis, causative agent of canine ehrlichiosis, in the tick Rhipicephalus sanguineus and its differentiation from a symbiotic rickettsia. *Amer. J. Vet. Res.* 37:119-126.

8. Stephenson, E. H., and J. Storz. 1975. Protein profiles of dense-centered forms of five chlamydial strains of animal origin. *Amer. J. Vet. Res.* 36:881-887.

9. Storz, J., E. J. Carroll, E. H. Stephenson, L. Ball, and A. K. Eugster. 1976. Urogenital infection and seminal excretion after inoculation of bulls and rams with chlamydiae. *Amer. J. Vet. Res.* 37:517-520.

Diseases of Military Animals Section II

Investigators:

Principal: LTC T. J. Keefe, VC

Associate: L. N. Binn, Ph.D.; R. E. Sims, B.S.; CPT j. R. Boyce, VC; MAJ R. J. Beattie, VC; MAJ C. A. Montgomery, VC; R. H. Marchwicki, B.S., CPT J. P. Alford, VC; MAJ M. J. Reardon, VC; SP4 R. Barriere; PFC J. A. Lee; and PFC A. E. Kanthack

Description:

To investigate diseases and/or conditions affecting laboratory animals at the WRAIR to enhance production efficiency and health care management, and to provide research animals free of known or potential pathogens. A major effort is directed toward defining the etiology of respiratory and enteric pathogens, developing rapid, specific and economical diagnostic techniques, and developing rapid analytical surveillance methods for early detection of known or potential pathogens.

During the reporting period, research activities have included investigations on: (1) respiratory disease in random source laboratory dogs, (2) antibiotic-induced lethal diarrhea in hamsters, and (3) the role of enterotoxigenic E. coli in diarrhea of monkeys and hamsters.

1. Respiratory disease in random source laboratory dogs.

Severe and often fatal respiratory disease continues as the most important problem in the conditioning of random source dogs (RSD) for laboratory use¹. Experience at the WRAIR more than a decade ago indicated that up to 70% of these dogs developed respiratory disease and 20% of the affected dogs died^{2,3}. Clinical observations, pathologic examination, and virus studies have provided clear evidence of the importance of canine distemper (CD) virus infections in the majority of severe cases of respiratory disease. However, respiratory disease of varying severity occurred in dogs immune to CD virus. The severe disease occurred notwithstanding prompt immunization of newly purchased dogs with attenuated CD virus and infectious canine hepatitis (ICH) virus vaccines. Moreover, intensive antimicrobial treatment of sick dogs was largely unsuccessful. Laboratory virus studies of these RSD showed the presence of 6 viruses and the common

occurrence of multiple virus infections^{2,3}. In addition to CD virus, infections with ICH virus, Toronto A26/61 adenovirus, parainfluenza SV5, canine herpesvirus and reovirus type 1 were detected. The respiratory disease problem encountered by this institute a decade ago was resolved when colony bred hounds were purchased. At the colony, the hounds were given CD-ICH vaccines and many dogs were naturally infected with SV5 and canine herpesvirus. Due to changing contractual requirements, in December 1975, WRAIR again began to buy unconditioned RSD. As anticipated, during the conditioning of these dogs, a high incidence of severe respiratory disease has occurred. A prospective study is in progress to define the current etiology and epizootiology. The objectives of this investigation are the reduction of the number and severity of respiratory disease cases and better clinical management of the remaining affected dogs. This report summarizes the clinical observations and laboratory studies carried out to date.

Groups of RSD were purchased from a licensed vendor (Biomedics Associates, Inc., Friedensburg, PA 17933). On arrival, the dogs were weighed, examined for signs of disease, heartworms, and unsuitable dogs rejected. Many shipments contained dogs exhibiting overt signs of respiratory disease. The newly procured dogs were conditioned in building 511 as customary. Each dog was identified by number and given CD-ICH vaccine within 48 hours after arrival. Blood specimens for virus serology, and CBC were collected on the day of arrival from each dog. At the onset of signs of respiratory disease, nose, throat, and rectal swab specimens were obtained from dogs for bacterial, mycoplasma, and virus isolation tests. A blood specimen for serology and CBC was also obtained at this time and a convalescent blood specimen 21 days later. In addition, post-mortem tissue specimens for histopathologic, microbiologic, virus, and mycoplasma studies were collected from dogs with fatal infections. The procedure for microbiological and virus studies have been described elsewhere^{2,4} in detail. Primary dog kidney (PDK) and the Walter Reed canine cell (WRCC) were used for isolation of virus.

During the conditioning of each of six groups of RSD severe respiratory disease occurred with one or more fatal infections (Table 1). More than half (86 of 152) of the new dogs developed respiratory disease and 40% (34 of 86) of the sick dogs died. Signs of upper respiratory disease (URD) were detected as early as 2 days after arrival with most cases beginning 10-14 days after arrival. The clinical nature of disease could be divided into 2 types depending on the clinical signs, duration of illness, and outcome. Initially all affected dogs exhibited signs of URD, i.e., conjunctivitis, nasal discharge and elevated body temperature. Following treatment

for 5 days with tylosin, sulfonamide, antihistamine, vitamin C, and expectorants, 40% (36) dogs recovered. These dogs were considered to have had uncomplicated URD. The remaining 50 dogs developed a second clinical course consistent with lower respiratory disease and a diagnosis of distemper, i.e., cough, pneumonia, diarrhea, dehydration and depression. Approximately one-third (16) of these severely ill dogs made a slow recovery without sequelae. The remaining 34 dogs died or developed signs of central nervous system disease and were euthanatized (Table 1). Histopathogenic examination of 17 of 34 fatal cases confirmed the clinical diagnosis of CD, in that pathognomonic virus inclusions were evident in 14 dogs, and the remaining 3 dogs were reported as CD suspect cases. The remaining fatal cases were not available for histopathologic examination.

Studies were carried out on the CD and ICH antibody status of the dog on arrival. Forty-three percent (67 of 157) of the dogs had significant CD virus neutralizing antibody and 52% (81 of 159) had ICH virus neutralizing antibody (Table 2). Fifty-six of the 67 dogs (84%) with CDV antibody also had ICH virus neutralizing antibody. The relationship of these antibody test results to subsequent respiratory disease during the conditioning period is summarized in Table 3. As anticipated, dogs with CD virus and ICH virus antibodies had the lowest incidence (23%) of respiratory disease and dogs without these antibodies had the highest incidence (83%). Although the number of dogs with only CD virus antibody was small, these dogs also had a similar low incidence of disease (27%) as dogs with both types of antibody. The dogs with only ICH virus antibody has a significantly higher rate of disease (48%) than dogs with CD virus antibody. Consistent with the previously described relationship between severe, lower respiratory diseases and histopathologically confirmed CD, dogs arriving with CD antibodies experienced relatively mild URD. Dogs arriving without CD antibodies developed the severe, lower respiratory disease of CD. The findings emphasize the importance of prior immunity to CD in preventing severe respiratory disease during conditioning. However, it is important to note the occurrence of respiratory disease in approximately one-fourth the dogs with CD virus antibody.

Virus isolation studies were done on the first 3 groups of dogs. Table 4 summarizes the results of isolation attempts from nose, throat, and rectal swab specimens in PDK and WRCC cells. The hemadsorbing viruses and adenoviruses were recovered most frequently. Only a few canine herpesviruses and minute viruses of canines (MVC) were isolated. Adenoviruses and herpesviruses were recovered equally well in PDK and WRCC cell cultures. However, the hemadsorbing viruses were isolated more frequently in PDK cell than WRCC cultures. The hemadsorbing viruses were recovered from upper respiratory specimens

but not from rectal specimens. In contrast, adenoviruses were recovered from all three sources, frequently from the rectum and infrequently from nasal secretions. Different isolates from each dog were identified by serum neutralization tests and the results summarized in Table 5. Viruses were recovered from 29 of 39 dogs tested. Parainfluenza SV5 and Toronto A26/61 were recovered from each group of dogs. Individual dogs in each group shed the MVC. Canine herpesvirus was recovered only from dogs in the last group tested. In 6 dogs, 2 viruses were simultaneously recovered; 5 had SV5 and Toronto A26/61 viruses and 1 had Toronto A26/61 and canine herpesviruses. The findings provide evidence of multiple virus infection during the quarantine of each group of dogs. In this regard, one of these multiple virus recoveries, SV5 and Toronto A26/61 virus, was from a dog rejected on arrival with signs of URD. The latter findings clearly indicate that these viruses are introduced into our facilities. Viruses were recovered from the post-mortem tissues of 2 of 3 dogs. SV5 was recovered from the tracheae, lung, and pulmonary lymph node of dog 66123. This dog shed SV5 from the nose and throat 2 weeks before death and was shedding Toronto A26/61 virus and the MVC prior to that. The histopathological studies on this dog indicated it died with concurrent CD. In addition, an agent unidentified to date has been recovered in PDK cells from the lungs of another dog (65908). Although the dog has pathognomic CD, the isolate was not neutralized by CD virus antiserum. Further studies on its identification are in progress.

Serological tests are in progress to measure the transmission of selected canine viruses among the sick dogs. The use of CD and ICH vaccines precluded studying the natural transmission of CD, ICH, and Toronto A26/61 viruses. Results of SV5 and canine virus neutralization tests on each group of dogs are summarized in Table 6. SV5 infections occurred in each group of dogs and 83% of the sick dogs had a rise in titer. In contrast, canine herpesvirus infections were detected in only one group of 21 dogs and only 4 (19%) had a rise in titer. Each of these 4 dogs had rises also to SV5. The serological studies were consistent with the virus isolation results.

Bacterial culture of nose and throat specimens resulted in the isolation of commensal organisms, e.g., staphylococcus, coliform, and Neisseria sp. However, Bordetella bronchiseptica and beta hemolytic streptococci were recovered from the lungs of individual dogs with fatal respiratory disease. These 2 dogs also had CD. Mycoplasma sp. were recovered on appropriate media from throat specimens of all 13 sick dogs assayed. The lung suspension from the dog cited above with B. bronchiseptica also contained mycoplasma. Hyperimmune specific antiserum against the 6 prevalent canine mycoplasma species have been prepared in rabbits for identification of isolates. In

addition, mycoplasma have been recovered from subcultures of canine cell cultures originally inoculated with nasal or throat specimens from 3 sick dogs. The latter 3 isolates were detected by focal areas of hemadsorption. The hemadsorption was not present in cell cultures containing chlortetracycline. The recovery of non-viral hemadsorbing agents in cell cultures complicates the recovery and identification of parainfluenza SV5 from dogs. The significance of mycoplasma in canine respiratory disease has not been resolved.

In summary, severe respiratory disease epizootics have occurred in random source dogs during the conditioning period. More than half of the introduced dogs developed respiratory diseases, and 40% of the affected dogs died. Clinical observations and laboratory studies have provided clear evidence the CD infections were responsible for most of the severe lower respiratory disease and associated mortality. SV5 and Toronto A26/61 adenovirus infections also occurred during the conditioning of each group of dogs. The latter viruses contributed to the severity of the CD infections and were responsible for most of the URD in CD-immune dogs. Both SV5 and Toronto A26/61 were isolated from sick dogs rejected on arrival, indicating these viruses were consistently being introduced into our facilities with new dogs. Canine herpesvirus and MVC were recovered from a few dogs. Although B. bronchiseptica and B. hemolytic streptococci were isolated from the lungs of individual dogs with CD, these and other pathogenic bacteria were not present in the respiratory secretions of other sick dogs. Further studies are needed to identify and evaluate the pathogenic role of the mycoplasmas which were recovered from each of 16 sick dogs examined. A virus, unidentified to date, was recovered from the lungs of one dog which died with CD. Evidence was presented for the transmission of 5 viruses, CD, Toronto A26/61, SV5, canine herpesvirus, and MVC, and the common occurrence of multiple infections. Results from this study clearly indicate that purchase of dogs immune to CD should reduce the morbidity of respiratory disease by 50% and the fatal cases by 75%. Studies are in progress to develop a rapid and inexpensive assay for detection of CD antibody.

2. Antibiotic-induced lethal diarrhea in hamsters.

Outbreaks of watery diarrhea are the principal causes of morbidity and mortality in hamster colonies⁵. Typically, young hamsters which have undergone a stress such as weaning or shipping are most affected. The enzootic nature of the diarrhea, its predilection for the young, and the sporadic occurrence of the diarrhea following stress, suggests an indigenous etiology. One of the most popular theories incriminates E. coli as the causal agent⁶. We, as well as others⁷, have observed that the intestinal contents of affected hamsters frequently contain large numbers of E. coli, and rarely other organisms not usually considered as pathogens, such as Proteus sp. and Pseudomonas sp.

Penicillin and other antibiotics inoculated parenterally into hamsters causes diarrhea within several days and usually results in death^{8,9}. The normal aerobic bacterial inhabitants of the intestinal tract are found in disproportionate numbers compared with untreated hamsters. The bacteria most often detected in large numbers were members of the Enterobacteriaceae, usually *E. coli*, *Klebsiella* sp., and *Enterobacter* sp. Antibiotic induced, lethal diarrhea appears to mimic the naturally occurring disease clinically, and with regard to the type and number^{8,10} of various Enterobacteriaceae commonly found in affected intestines^{8,10}. The present studies were designed to clarify the etiologic role of Enterobacteriaceae in the lethal diarrhea of hamsters induced by penicillin.

Pooled accumulations of weekly fecal pellets as well as minced whole intestine from dying hamsters were cultured by incubating the samples in 250 ml of Trypticase soy broth (TSB) at 37°C overnight. The broth cultures were then subcultured onto MacConkey's, eosine methylene blue (EMB) and sheep's blood agars. These plates were then incubated at 37°C for 24 hr and observed for several days at room temperature before discarding as negative. Colonies appearing on MacConkey's and/or EMB agar were selected for identification. Colonies appearing on sheep's blood agar were Gram stained. Quantitative culture of small intestine was performed by making serial 100-fold dilutions of contents in TSB. Dilutions were incubated at 37°C for 18-24 hr and then streaked on MacConkey's agar and further incubated 18-24 hr at 37°C. Colonies from the highest dilution showing growth were picked for identification using standard techniques. Hamsters were defined as free of Enterobacteriaceae when no facultative, anaerobic, oxidase negative, Gram negative rods were isolated on the above-mentioned media.

Hamsters with no detectable Enterobacteriaceae, as described above were (1) maintained in a laminar flow hood, (2) handled aseptically, (3) reared in sterilized cages with sterilized bedding, and (4) fed sterilized food and water. Twenty-one hamsters lacking Enterobacteriaceae were used for this experiment when 21 days old. Fecal accumulations from these animals, when cultured on sheep's blood agar, usually showed a variety of Gram + organisms, e.g., enterococci. These hamsters were born of four mothers also devoid of Enterobacteriaceae. Accumulated feces from mothers and babies of each litter were cultured weekly as previously described. By these culture techniques, these hamsters showed no evidence of Enterobacteriaceae, in spite of coprophagy. An equal number of 21-day old hamsters received from a commercial source were caged without attempts to restrict environmental contamination. Both groups of hamsters were inoculated intraperitoneally with 100,000 units of potassium penicillin G in 0.5 ml of saline. This dose and route of inoculation, on previous trials, was associated with 90%-100% mortality within one week post-inoculation (pi).

In both groups of hamsters, diarrhea was usually evident within 24 hr pi and persisted in varying degrees until death. The mortality in both groups was similar; 11 of 21 (52%) hamsters without intestinal Enterobacteriaceae died, and 7 of 12 (58%) conventional hamsters died. Survivors showed a self-limiting diarrhea which was absent by the fourth day pi. As expected, disproportionate numbers ($\geq 10^6$ ml) of Klebsiella sp. and E. coli were isolated from the small intestines of three dead hamsters from commercial origin. Nine additional commercial hamsters, found partially decomposed or eaten, also harbored Enterobacteriaceae. Intestinal contents cultured from the dead hamsters which were devoid of Enterobacteriaceae before challenge yielded growth only on sheep's blood agar.

In summary, these experiments showed that with strict sanitary precautions, hamsters can be raised and maintained healthy without harboring Enterobacteriaceae. However, contrary to what was expected and what has been implied by others, the absence of these organisms from the intestinal tract had no sparing affect on antibiotic-induced diarrhea and death.

3. The role of enterotoxigenic E. coli in the diarrhea of monkeys and hamsters.

Despite the advent of antibiotics, the feeding of more nutritious diets, and the use of improved animal housing facilities, diarrheal disease continues to be one of the principal causes of morbidity and mortality in laboratory animals. This is particularly true in colonies of newly acquired laboratory monkeys and weanling hamsters.

Numerous outbreaks of diarrhea in laboratory monkeys have been described in the literature. The vast majority of these have been attributed to infections with Shigella spp., usually Sh. flexneri^{11,12,13,14}. The second most commonly incriminated agents are the salmonellae^{11,12,14}. Our laboratory examined 184 diarrheal stools from monkeys during the 2-year period 1 July 1973 to 30 June 1975. Shigella spp. were isolated from 23% and Salmonella spp. from 1% of the specimens. The most striking finding, however, was the absence of either agent in 75% of the cases. Although it is recognized that many of these undiagnosed cases may represent nutritional, parasitic, or viral diarrheas, we have nevertheless observed that in a high percentage of these cases the stools contained large concentrations of E. coli. Weanling age hamsters, particularly when stressed, develop a highly lethal diarrhea. We, as well as others⁶, have observed that the small intestines of affected hamsters frequently contain very high numbers of E. coli ($\geq 10^6$ /ml). Although E. coli is not currently recognized as a cause of diarrhea in either of these two species, the ubiquitous and pathogenic nature of this organism in other species requires that it be considered as a possible cause of enteric disease.

Enterotoxin elaborating strains are also recognized as etiologic agents of diarrhea in humans, young swine, and calves^{15,16}. Serotyping cannot be used to evaluate enteropathogenicity of E. coli, since enterotoxin production is determined by a plasmid which can be passed to any serotype¹⁷. Systems for the assay of E. coli enterotoxin are based on animal intestinal loop models or indirect cyclic AMP measurements. Undoubtedly the tedious testing procedures required to evaluate a strain's pathogenicity partially accounts for the absence of reports of enterotoxigenic E. coli for laboratory animal species.

Fecal swabs from 131 monkeys experiencing diarrhea during early conditioning and before antibiotic treatment were streaked onto plates of MacConkey, salmonella-shigella, and brilliant green agars for detection of Salmonella sp. Shigella sp. and E. coli. Salmonella and Shigella bacteria were identified by standard methods. Lactose fermenting colonies from MacConkey's agar were further tested for indole production and their inability to grow on Simmon's citrate agar before being designated E. coli. Fecal swabs from 116 of 131 monkeys yielded 4, well-isolated colonies with the above-described biochemical reactions. These 464 strains were tested for enterotoxin production. Each colony was streaked onto trypticase soy agar (TSA) slants and stored at 25 C until processed for enterotoxin production. In addition, E. coli strains were similarly isolated from each of 25 juvenile hamsters which experienced severe diarrhea during naturally occurring epizootics subsequent to the stress of shipping.

Cultures were processed for production of heat labile enterotoxin by bacterial loop transfer from TSA slants into 0.5 ml of trypticase soy broth (TSB) within individual wells of the 24-well tissue culture plates (Falcon) and incubated at 37C/48 hr. Cultures were aerobically processed for production of heat stable enterotoxin by bacterial loop transfer from TSA slants into 0.5 ml of 1% peptone water and incubated overnight at 37 C. Then 0.01 ml was inoculated into 10 ml of TSB within a 50 ml erlenmeyer flask, shaken overnight at approximately 150 rpm at 37 C, clarified by centrifugation at 2500 rpm/20 min in a GSA rotor (Sorvall), and the supernate stored at -70 C until assayed. Cultures were anaerobically processed for production of heat stable enterotoxin by bacterial loop transfer from TSA slants into 3.0 ml of TSB within 12 mm X 56 mm screw cap vials and incubated with loosened caps anaerobically using a Gas-pak (BBL) at 37 C/48 hr. Cultures were clarified by centrifugation and the supernate stored frozen as previously described.

Heat-labile enterotoxin from aerobic growth of E. coli was detected by the morphologic changes it produces in monolayers of Y-1 mouse adrenal cells as described by Sack¹⁸. Cells were grown in the 24-well tissue culture plates and maintained in Ham F-10 medium enriched with 12.5% horse serum and 2.5% fetal calf serum and containing 100 units/ml potassium penicillin G and 25 ug/ml streptomycin sulfate. Each

well was seeded with approximately 40,000 cells in 0.6 ml of medium and used 3 days later when monolayers were between 50% and 75% confluent. Using a pasteur pipette, 2 drops of whole live culture were added to individual wells, adsorbed at 37 C/15 min, then removed by suction. Cells were then gently rinsed with Pucks saline and fresh medium was replaced. The cells were observed after 6 hr incubation at 37 C for typical rounding. Known positive and negative control strains of E. coli were used with each assay.

Heat stable enterotoxins, from aerobic or anaerobic growth of E. coli were detected in 2-day old, white, Swiss mice (WRAIR), as described by Dean¹⁹. At least 4 mice per strain were inoculated intra-gastrically with 0.1 ml of culture supernate which had been prepared and stored frozen no longer than 7 days previously. After 4 hours, the mice were euthanized under Co₂ and necropsied. Using an analytical balance (Mettlar type H), gut weights (pylorus to terminal rectum) and remaining body weights were obtained. The mean of the ratios of gut weight to remaining body weight were calculated for each strain. Indigo blue dye in each inoculum assured that at least 2 mice for each strain received the inoculum intra-gastrically.

Four strains of E. coli from each of 116 monkeys (464 strains) and each of 25 hamster strains were assayed in duplicate on Y-1 mouse adrenal cells for morphologic evidence of the presence of heat labile enterotoxin. By this assay, none of the above strains showed evidence of heat labile enterotoxin.

E. coli strains from 52 of 116 monkeys (208 strains) grown both aerobically and anerobically were assayed for heat stable enterotoxin in newborn mice. The overall mean ratio and standard deviation (SD) of gut weight to remaining body weight for the 208 strains grown aerobically and anerobically was 0.063 ± 0.007 and 0.065 ± 0.006 , respectively. These values are well within the range indicating the absence of stable enterotoxin as described by Dean¹⁹. By contrast, the mean ratio of the enterotoxigenic Calcutta strain 078:H12 when grown aerobically was 0.120, well beyond 3 SD (0.084). The mean ratio of two strains from one monkey (#346) when grown aerobically was also well beyond 3 SD (0.117) and comparable to the positive mean ratio produced by the Calcutta strain grown aerobically. Interestingly, mean ratios of the Calcutta strain (0.066) and the two strains from monkey #346 (0.064) were well within the normal range after anaerobic growth. Anaerobic growth conditions apparently enhanced enterotoxin production by these three strains, as described by Klipstein²⁰ with Klebsiella pneumoniae.

E. coli strains from the 25 hamsters with epizootic-related diarrhea were grown aerobically and assayed for heat stable enterotoxin in newborn mice. The overall mean ratio and SD of gut to remaining body

weight was 0.067 ± 0.005 . The range of individual mean ratios (0.061 to 0.078) were well within 3 SD (.082) and previously described "normal" values, indicating the absence of enterotoxin.

Salmonella sp. were not isolated from any of the 131 monkeys or 25 hamsters. Shigella sp. were recovered from 36 monkeys (27%), including monkey #346 from which enterotoxin producing E. coli were also isolated.

The absence of heat labile enterotoxin of E. coli from diarrhea specimens of monkeys when assayed by the Y-1 mouse adrenal cell culture system was not particularly surprising. Most enterotoxin producing strains of E. coli reported from non-human animals to date have produced the heat stable toxin only. The detection of enterotoxigenic E. coli from diarrhea specimens of monkeys is consistent with their presence in other species. However, only 4 of 52 (8%) monkey harbored enterotoxin producing E. coli during diarrhea episodes which occurred during conditioning. By contrast, 27% of these same monkeys harbored Shigella sp. These data suggest that the etiologic role of enterotoxigenic E. coli is minor in the diarrhea episodes which affect monkeys during conditioning. Although few hamsters were examined, the failure to detect enterotoxigenic E. coli is consistent with epidemiological and experimental data which incriminate indigenous, host-related factors rather than transmission of enteropathogenic organisms during juvenile diarrhea episodes.

In summary, four strains of E. coli from each of 116 monkeys (464 strains) affected with diarrhea during conditioning, and strains from each of 25 juvenile hamsters with epizootic-related diarrhea after shipping, failed to show any heat labile enterotoxin as detected and defined by cell culture assay. Additionally, the 25 hamster strains failed to show any heat stable enterotoxin, as detected and defined by intra-gastric inoculation of newborn mice. However, 4 of 52 monkeys (8%) harbored E. coli which produced heat stable enterotoxin. Interestingly, strains which produced enterotoxin after aerobic growth failed to do so when grown anaerobically, and vice-versa. Salmonella sp. was not isolated from hamsters or monkeys, but Shigella sp. was isolated from 27% of the monkeys.

New work, detailed in Section II above, will be separated for FY77 under title change "Health Care and Management of Laboratory Animals", Work Unit #057.

Table 1: Incidence of non-fatal and fatal respiratory disease in groups of random source laboratory dogs during conditioning.

Date group arrived	No. with signs/total (%)	No. died/No. with signs (%)
18 Dec 75	18/47 (38)	8/18 (44)
29 Dec 75	2/10 (20)	1/2 (50)
25 Feb 76	28/37 (76)	6/28 (21)
11 March 76	7/15 (47)	3/7 (43)
31 March 76	29/43 (67)	14/29 (48)
14 April 76	<u>2/12 (17)</u>	<u>2/2 (100)</u>
Totals	86/164 (52)	34/86 (40)

Table 2: Incidence of canine distemper virus (CDV) infectious canine hepatitis virus neutralizing antibodies in newly procured dogs.

Virus	No. dogs tested	Number seropositive	Percent seropositive
CDV	158	67*	43
ICHV	159	81**	52

*Based on the neutralization of 100-300 egg ID₅₀ of CDV by a 1:100 serum dilution.

**Based on the neutralization of 300 TCD₅₀ of ICHV by a 1:20 serum dilution.

Table 3: Relationship between canine distemper virus (CDV) and/or infectious canine hepatitis virus (ICHV) antibodies in newly procured dogs and subsequent non-fatal and fatal respiratory disease.

Antibodies present on arrival*	No. Dogs	No. with signs (%) (A)	No. with fatal disease (%) (B)	Total Sick (%) (A+B)
CDV + and ICHV +	56	10 (18)	3 (5)	13 (23)
CDV + and ICHV 0	11	3 (27)	0	3 (27)
CDV 0 and ICHV +	25	7 (28)	5 (20)	12 (48)
CDV 0 and ICHV 0	<u>66</u>	<u>30 (45)</u>	<u>25 (38)</u>	<u>55 (83)</u>
Totals	158	50 (32)	33 (21)	83 (53)

* + = seropositive

0 = seronegative

Table 4: Cytopathic and hemadsorbing viruses recovered from nose, throat, and rectal swab specimens of dogs with respiratory disease in Primary dog kidney (PDK) and the Walter Reed Canine Cell (WRCC) line.

Agent recovered*	Host Cells	No. of isolations made from		
		nose	throat	rectum
Hemadsorbing agent	PDK	18	14	0
	WRCC	3	3	0
Adenovirus	PDK	3	10	13
	WRCC	3	10	14
Herpesvirus	PDK	3	1	0
	WRCC	3	0	0
Minute virus of canines	PDK**	0	0	0
	WRCC	1	0	3

*Tentative grouping of isolates based on type of cytopathic effect or presence of hemadsorption.

**Minute virus of canines does not replicate in PDK.

Table 5: Viruses recovered from random source dogs with respiratory disease.

Date Arrived	No. dogs with virus/ No. examined (%)	No. dogs with virus			
		SV5	Toronto A26/61	Canine Herpes	Minute Virus of Canines
18 Dec 75	16/22 (73)	12*	8*	0	1
29 Dec 75	2**/2 (100)	1	1	0	1
25 Feb 76	11/13 (85)	6	5	3	1
Totals	29/37*** (78)	19	14	3	3

*Includes the recovery of SV5 and Toronto A26/61 from individual rejected dogs with overt signs of disease.

**Dog 66123 examined 3 times, Toronto A26/61 isolated from first specimen, SV5 from second and third.

***Two viruses recovered from 7 dogs. Six SV5-Toronto A26/61 combined isolations and 1 Toronto A26/61-canine herpesvirus.

Table 6: Parainfluenza SV5 and canine herpesvirus antibody studies of random source laboratory dogs with respiratory disease.

Date Arrived	No. dogs with increased neutralizing antibody titer/total tested (%)	
	SV5	canine herpesvirus
18 Dec 75	10/13 (77)	0/11 (0)
29 Dec 75	1/2 (50)	0/2 (0)
25 Feb 76	18/21 (86)	4*/21 (19)
11 March 76	1/2 (50)	0/2 (0)
31 March 76	17/25 (68)	0/25 (0)
Totals	47/63 (74)	4/61 (7)

*Each of these dogs also had a rise in titer to SV5.

Project 3A762760A837 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit Diseases of military animals

Literature Cited.

References:

1. Christie, R. J. 1975. Evaluation of a Conditioning Program for Random-Source Dogs. *Lab. Anim. Sci.*, 25:647.
2. Binn, L. N., G. A. Eddy, E. C. Lazar, J. Helms, and T. Murnane. 1967. Viruses Recovered from Laboratory Dogs with Respiratory Disease. *Proc. Soc. Exptl. Biol. & Med.*, 126:140-145.
3. Binn, L. N., E. C. Lazar, J. Helms, and R. E. Cross. 1970. Viral Antibody Patterns in Laboratory Dogs with Respiratory Disease. *Am. J. Vet. Res.*, 31:697-702.
4. Binn, L. N., E. C. Lazar, G. Eddy, and M. Kajima. 1970. Recovery and Characterization of a Minute Virus of Canines. *Inf. & Immun.*, 1:503-508.
5. Soave, O. A. 1963. Diagnosis and Control of Common Diseases of Hamsters, Rabbits, and Monkeys. *J. Am. Vet. Med. Assoc.* 142:285-290.
6. Thomlinson, J. R. 1975. "Wet Tail" in the Syrian hamster: a form of colibacillosis. *Vet. Rec.*, 96:42.
7. Sheffield, F. W., and E. Beveridge. 1962. Prophylaxis of "Wet Tail" in Hamsters. *Nature (London)*, 196:294-295.
8. Small, J. D. 1968. Fatal Enterocolitis in Hamsters given Lincomycin Hydrochloride. *Lab. Anim. Care*, 18:411-420.
9. Schneierson, S. Stanley, and E. Perlman. 1956. Toxicity of Penicillin for the Syrian Hamster. *Proc. Soc. Exp. Biol. Med.*, 91:229-230.
10. Frenkel, J. K. 1972. Infection and Immunity in Hamsters. *Prog. exp. Tumor Res.*, 16:341-344.
11. Arya, S. C., A. Verghese, D. S. Agarival, and S. C. Pal. 1973. Shigellosis in rhesus monkeys in quarantine. *Lab. Anim.*, 7:101-109.

12. Groves, M. G., J. Anandan, and C. E. Davis. 1969. Epizootic diarrhoea in a group of pig-tailed macaques (Macaca nemestrina). Kajian Veterinaire 2:23-28.

13. Lindsey, J. R., P. H. Hardy, Jr., H. J. Baker, and E. C. Melby, Jr. 1971. Observations on shigellosis and development of multiple resistant shigellas in Macaca mulatta. Lab. Anim. Care, 21:832-844.

14. Schneider, N. J., E. C. Prather, A. L. Leives, J. E. Scatterday, and A. V. Hardy. 1960. Enteric bacteriological studies in a large colony of primates. Ann. N.Y. Acad. Sci., 85:935-941.

15. Ogawa, H., A. Nakamura, and R. Sakazaki. 1968. Pathogenic properties of "enteropathogenic" Escherichia coli from diarrheal children and adults. Jap. J. Med. Sci. Biol., 21:333-349.

16. Smith, H. W., and S. Halls. 1967. Observations by the ligated intestinal segment and oral inoculation methods on Escherichia coli infections in pigs, calves, lambs, and rabbits. J. Pathol. Bacteriol., 93:499-529.

17. Gyles, C., N. So, and S. Falkow. 1974. The enterotoxin Plasmids of Escherichia coli. J. Infect. Dis., 130:40-49.

18. Sack, David A., and R. Bradley Sack. 1975. Test for enterotoxigenic Escherichia coli using Y-1 adrenal cells in miniculture. Inf. & Imm., 11:334-336.

19. Dean, A. G., Y. Ching, R. H. Williams, and L. B. Harden. 1972. Test for Escherichia coli enterotoxin using infant mice: Application in a study of diarrhea in children in Honolulu. J. Infect. Dis., 125:407-411.

20. Klipstein, F. A., and R. F. Ewert. 1976. Purification and properties of Klebsiella pneumoniae heat-stable enterotoxin. Inf. and Imm., 13:373-381.

Publications:

1. Binn, L. N., E. C. Lazar, K. P. Keenan, D. L. Huxsoll, R. H. Marchwicki, and A. J. Strano. 1975. Recovery and Characterization of a Coronavirus from Military Dogs with Diarrhea. Proc. 78th Ann. Meeting, U. S. Animal Hlth Assoc., 1974. pgs. 359-366.

2. Binn, L. N. 1975. Epizootiological Studies of Canine Virus Diseases. JAVMA 167:856 (Abstract).

3. Binn, L. N. 1975. Public Health Aspects of Canine Viral Diseases. JAVMA 167:858 (Abstract).

4. Keenan, K. P., H. R. Jarvis, R. H. Marchwicki, and L. N. Binn. 1976. Intestinal Infection of Neonatal Dogs with Canine Corona-virus 1-71: Studies by Virologic, Histologic, Histochemical, and Immunofluorescent Techniques. Am. J. Vet. Res. 37:247-256.

5. Takeuchi, A., L. N. Binn, H. R. Jarvis, K. P. Keenan, P. K. Hildebrandt, R. B. Valas, and F. F. Bland. 1976. Electron Microscope Study of experimental enteric infection in neonatal dogs with a canine coronavirus. Lab. Invest. 34:539-549.

DISTRIBUTION:

- 5 - U. S. Army Medical Research and Development Command, WASH DC 20314
- 12 - Defense Documentation Center, ATTN: DDC-TCA, Alexandria VA 22314
- 1 - Superintendent, Academy of Health Sciences, U. S. Army, ATTN: AHS-COM, Ft Sam Houston TX 78234
- 1 - Director of Biological and Medical Sciences Division, Office of Naval Research, 800 N. Quincy Street, Arlington VA 22217
- 1 - Commander, Naval Medical R&D Command, National Naval Medical Center, Bethesda, MD 20014
- 1 - Director of Professional Service Office of The Surgeon General, Department of the Air Force, Washing. DC 20314
- 1 - Director of Defense Research and Engineering, ATTN: Assistant Director (Environmental and Life Sciences), Washington DC 20301
- 1 - Commander, U. S. Army Training & Doctrine Command, Ft Monroe VA 23651
- 1 - Each USAMRDC Laboratory