AD NUMBER

AD884869

LIMITATION CHANGES

TO:

Approved for public release; distribution is unlimited.

FROM:

Distribution authorized to U.S. Gov't. agencies only; Test and Evaluation; 24 JUN 1971. Other requests shall be referred to U.S. Army Medical Research and Development Command, Washigton, DC 20314.

AUTHORITY

USAMRDC ltr, 19 Jun 1976

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 1969 – 30 June 1970

VOLUME II

WALTER REED ARMY INSTITUTE OF RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, D.C. 20012

Unclassified

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)	2a. REPORT SECURITY CLASSIFICATION
Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington, D. C. 20012	Unclassified 2b. GROUP NA

3. REPORT TITLE

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES, INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Annual Progress Report - 1 July 1969 to 30 June 1970

5. AUTHOR(S) (First name, middle initial, last name)

Listed at beginning of each work unit report

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
July 1970		NA

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
NA b. PROJECT NO. NA c. d.	NA 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report) NA

10. DISTRIBUTION STATEMENT

Qualified transmittal of this document outside the agencies of the U.S. Government must have prior approval of the Commanding General, U. S. Army Medical Research and Development Command, or the Director, Walter Reed Army Institute of Research

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
NA	U. S. Army Medical Research and Development Command Washington, D. C. 20314

13. ABSTRACT

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DD FORM 1 NOV 68 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

Unclassified

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Biological Sciences Medical Sciences Biochemistry Communicable Diseases Immunology Internal Medicine Nuclear Medicine Physiology Psychiatry Surgery Veterinary Medicine						

☆ U. S. GOVERNMENT PRINTING OFFICE : 1971 O - 421-232 (Vol. II)

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

ANNUAL PROGRESS REPORT

1 July 1969–30 July 1970

VOLUME II

WALTER REED ARMY INSTITUTE OF RESEARCH

WALTER REED ARMY MEDICAL CENTER

WASHINGTON, D.C. 20012

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 postion unless so designated by other authorized documents.

AD

RCS MEDDH-288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES, INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

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

Annual Progress Report
1 July 1969 - 30 June 1970

Volume II

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

~~Each transmittal of this document outside the agencies of the U. S. Government must have prior approval of the~~ Commanding General, U. S. Army Medical Research and Development Command, Washington, D. C. 20314, or the Director, Walter Reed Army Institute of Research.

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.

SUMMARY

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

FOREWORD

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

TABLE OF CONTENTS

VOLUME II

PROJECT 3A062110A806
MILITARY PREVENTIVE MEDICINE

Task 00
Military Preventive Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OA 6457	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A806	00	030
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) GLOBAL HEALTH DATA (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

005100 Documentation + J,007800 Hygiene and Sanitation, 007000 Geography

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 11	CONT	DA	C. In-House

17. CONTRACT/GRANT: NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 70	1	25
FISCAL YEAR CURRENT 71	1	25

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, Colonel W. H., MC

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Inst. of Research

ADDRESS: Div of Biometrics & Med Info Proc
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Fred, Ann C., M.D.

TELEPHONE: 202-576-2086

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

21. GENERAL USE

Foreign intelligence not considered

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Global Health Data; (U) Epidemiology; (U) Preventive Medicine; (U) Infectious Diseases; (U) Sanitation; (U) Ecology; (U) Climate; (U) Geography

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

(23) Health Data Publications on selected foreign countries are researched, written, published and issued. (U) Unclassified health and disease data on all foreign countries accrued, analyzed, stored and made available to approved requesters. (U) Consultation and assistance rendered to Medical Officers and to Global Medical Courses.

(24) (U) Preventive medicine oriented Health Data Publications on individual foreign countries are unclassified reports on the infectious and communicable diseases and health and sanitary conditions in the country.

(25) (U) 69 07 - 70 06. Pending decision on organization and staff, no new Health Data Publications have been issued. The staff of two (1 professional and 1 administrative) continue to research for data and write draft Health Data Publications; to accrue data and make it available to Medical and Research requesters. Health Data Publications on Jordan, Syria, Upper Volta, Uganda, North Korea, Cambodia and Ethiopia are ready for finalization and issuance when organization and staffing pattern has been made. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 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 3A062110A806 MILITARY PREVENTIVE MEDICINE

Task 00, Military Preventive Medicine

Work Unit 030, Global Health Data

Investigators:

Principal, Colonel Hinton J. Baker, MC
Associate, Ann C. Fred, M.D.

Description:

Health Data Reports are prepared for the use of Army Medical Service Officers and contain unclassified information regarding the health and sanitary conditions likely to be encountered in foreign countries to which they are deployed. They describe the geography, climate, religion, animals and plants of medical importance, water supplies, methods of waste and sewage disposal, diseases present, medical facilities, etc., of each country reported on.

Progress:

At the end of FY 1970, Health Data Reports had been completed and published on 46 countries. Updating, with complete revision of text and maps, is in the completion stage on Ethiopia. Rewrites of Syria and Jordan are continuing. A revised Cambodia is in progress and the complete text has been submitted on Mauritania and Dahomey.

PROJECT 3A062110A811
MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00
Tropical and Subtropical Military Medical Research

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6465	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO /CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	044
b. CONTRIBUTING				
[redacted]	CDOG 1412A (2)			

11. TITLE (Procede with Security Classification Code)

(U) Virus Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 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

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	5	295
FISCAL YEAR CURRENT 71	5	295

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)

NAME: Altstatt, LTC L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Smith, LTC T. J.

NAME: Johnsen, MAJ D. O. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Virus Ecology; (U) Arbovirus; (U) Rabies Virus; (U) Acquired Immunity; (U) Natural Immunity; (U) Infectious Diseases

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) To define the ecology of viruses of military importance in Southeast Asia thus providing a rational basis for decisions which involve association with or control of that or a similar viral ecosystem.

24. (U) Component parts of the natural viral ecosystem (e.g. vectors, hosts, reservoirs) and variables which affect these component parts (e.g. rainfall, topography, immunity) are identified and quantified through the disciplines of clinical medicine, medical entomology, epidemiology, veterinary medicine and virology.

25. (U) 69 07 - 70 06 Diagnostic increases in antibody titer in encephalitis patients from Vietnam and the isolation of JEV from the brain in one fatal case indicate that transmission of the virus is occurring in south Vietnam. Titer rises plus the isolation of the virus from an epidemic in Chiengmai indicate that JEV was also involved in that summer epidemic. Antibody titer changes in sera from patients in Vietnam suggest that mycoplasma is involved in 40% of the lower respiratory infections sampled. No new strains of influenza were encountered during this reporting period. For technical reports see SEATO Annual Progress Report, 1 Apr 69 - 31 Mar 70.

* Available to contractors upon originator's approval.

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

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 044, Virus diseases of man and animals

Investigators.

Principal: Thomas J. Smith, LTC, MC

Associate: Salvadore Arellano, SP5; Keith Arnold, MAJ, MC*; Anan Boonkanoke; Attaya Boonyakanist; Aree Boriharnvanakett, B.S.; Chumpan Chavachati, B.S.; Ming Choochong; Supatra Chulachombok, R.N.; Howard B. Emory, M.D.; Hilary Evans, MAJ, MC; Samuel Fulton, SFC; Douglas J. Gould, Ph.D.; Richard A. Grossman, MAJ, MC; Pairatana Gunakasem, M.D.; Robert L. Hickman, MAJ, MC; Somsak Imlarp; Dennis O. Johnsen, MAJ, VC; Chalobon Kachanaprakorn, M.S.; Sopana Kanchana; Nonglak Khananuraksa, B.S.; Kwanyuen Lawhaswasdi, D.V.M.; Raveewan Leelasatayakul, B.S.; Somboon Maneechai; Samarn Maneewongse; George S. Manning, CPT, MSC; Pethai Mansuwan, M.D.**; Amara Markvong; Joe T. Marshall, Ph.D.; Kol Mongkolpanya; Dephanom Muangman, M.D., D.P.H.; Sumitda Narupiti, B.S.; William A. Neill, SP5, E5; Anan Nisalak, M.D.; Vandee Nongngork; Chumnong Noigamol; Howard E. Noyes, Ph.D.; Naowayubol Nuthumkang, B.S.; Lloyd Olson, LTC, MC; Vichit Phunkitchar; Boonsri [illegible]vichit, M.D.***; Phuangthong Puengkaew, R.N.; Larp Puntusiri; Rattana Rattanawongse, M.D.; Pranee Sithisarn, B.S.; Phanu Sittisomwongse, D.D.S.; James P. Slowey, SFC, E7; Paul C. Smith, MAJ, VC; Laddawal Sookcharoen; Rapin Snitbhan, M.D.; Panor Srisongkram, B. S.; Harold E. Stark, Ph. D.; Thanomchit Suwapant, M. D.;***; Michael F. Sullivan, CPT, MSC; Jiraporn Supavadee, B. S.; Moragot Tanticharoen, B.S.; Prayot Tanticharoenyos, D. V. M.; Prapai Thong-Ngarm, B. S.; Roypim Tiptanatoranin, B. S.; Pranom Tuntrakool, B. S.; Suchinda Udomsakdi, M. D.;

---

* USA Med Rsch Team (WRAIR) Team

** Director, Children's Hospital, Bangkok

*** Staff physician, Children's Hospital

Suwana Vithanomsat, R. N.; Milton Willhight, SFC;
James E. Williams, CPT, MC; Ronald G. Wilson,
M. D.; William Wooding, MAJ, MC

Ecology of Japanese Encephalitis Virus

Principal Investigators: Ananda Nisalak, M.D.
Dennis O. Johnsen, MAJ, VC
Douglas J. Gould, Ph. D.
James E. Williams, CPT, MSC
Joe T. Marshall, Ph. D.
Richard A. Grossman, MAJ, MC
Suchinda Udomsakdi, M. D.
Thomas J. Smith, LTC, MC

Project Coordinator: Thomas J. Smith, LTC, MC

OBJECTIVE: To investigate the ecology of Japanese encephalitis virus in Thailand, with particular reference to aspects contributory to infection in humans. Specific objectives include the following:

1. to assess the extent of human infection, and its seasonal variation;

2. to observe clinical manifestations of human infection;

3. to determine which species of mosquitoes are transmitting human infections;

4. to investigate animal reservoirs of JE virus;

5. to ascertain environmental factors bearing on the incidence of human infection;

6. to attempt laboratory measurement of virus virulence or host defenses which bear on the nature of host-parasite relationship;

7. to study the inter-relationships of multiple co-existing group B arboviruses in a discrete area; whether competition, antagonism or synergism obtains between these agents, their mosquito vectors and the immune systems of their human hosts.

DESCRIPTION: Japanese encephalitis (JE) virus has been known to be endemic in Thailand for a number of years. Reports of outbreaks of clinical encephalitis have been received from the north and north-central plains area since at least 1962. In that year the existence of known JE virus mosquito vectors was documented, and subsequently group B arbovirus antibody was detected in sera of potential or known animal reservoirs of this agent. In 1964 an extensive outbreak of encephalitis at Pitsanuloke was serologically confirmed as JE, and the virus was isolated from a human case of encephalitis at Chiengmai.

Extensive ecologic studies of JE virus have been carried out by SMRL at Bang Phra, Southeastern Thailand. JE virus was isolated repeatedly from two species of mosquitoes, Culex gelidus and tritaeniorhynchus. Evidence was also accumulated suggesting that several species of domestic and wild vertebrates were involved in JE virus transmission cycles. Serologic evidence, however, indicated only low levels of JE virus infection in a nearby population of school children.

During the period June-September, 1969, an epidemic of encephalitis was reported in Chiengmai province that ultimately affected hundreds of children. Subsequent information indicated that the epidemic actually occurred throughout a wide area of the north and central plains area of Thailand. In the Chiengmai area alone 232 cases of encephalitis were reported, of whom 68 died. Paired sera were available from 55 children admitted to the McCormick and University Hospitals. Thirty-two of these showed evidence of recent infection with a group B arbovirus, and 15 monotypic HI antibody response to JE virus. JE virus was isolated from brain tissue of a patient dying of encephalitis. Initial serologic surveys of large domestic animals (pig, dog, buffalo and cattle) in the area indicate a high incidence of past infection with group B arbovirus. Preliminary mosquito collections revealed the abundant existence of the potential vectors C. gelidus, C. tritaeniorhynchus, and the C. vishnui complex.

In contrast to other areas of the world where JE virus ecology has been studied (e.g. Japan and Taiwan), however, other members of the group

B arbovirus complex are endemic in northern Thailand. The clinical existence of Thai hemorrhagic fever has been known for a number of years in this area and follows the same epidemiologic patterns as seen elsewhere in Thailand. Serological results of patients have been identical to those seen in dengue hemorrhagic fever and indicate the hyperendemicity of dengue virus in the area. Because infection(s) with dengue virus may result in broadly-reacting group B arbovirus antibody, which in at least some instances will protect the host to challenge with heterologous virus, the coexistence of JE and dengue viruses provides an opportunity to study this interaction in human populations.

In preparation of the anticipated increase in JE virus transmission to occur during the 1970 rainy season, study populations have been delineated. A comprehensive program to define mosquito populations in the area has been initiated. Light traps, bait-trap, and biting collections in several representative habitats have been collected. Pools of culicine mosquitoes have been tested for JE virus by mouse inoculation, and subsequently isolation systems will compare both mouse and LLC-MK_2 cell systems. Collections of Aedes will also be tested for dengue virus. Culicine larval populations have been surveyed by quantitative sampling methods. Simultaneous insecticide susceptibility tests have been established on colonized strains of vector species.

Serologic surveys are being carried out on potential reservoir hosts, based on data found both previously by SMRL at Bang Phra and in other countries. Antibody-free swine have been established in study areas as sentinel hosts. Studies in wild animals emphasize collection and analysis of suspect species in order to determine whether any are naturally infected to the same frequency as found previously in other areas of Thailand and whether the incidence of such infection relates to infection in humans.

Other aspects of this study are being reported separately in the section on Mosquitoes.

PROGRESS: Four villages in scattered locations of the Chiengmai Valley have been selected as study sites. Each has been completely censused and characterized, and approximately 20% sample of the population selected as demographically representative of the larger population. Initial bleeding was carried out in early November, the beginning of the dry season. In addition children from a school in the urban area of Chiengmai were bled.

Table 1 presents the age prevalence distribution for each area. Because of the cross-reactivity, interpretation is provisional. It seems clear, though, that most of the antibody prevalence in villages (A) and (B) is due to JE virus, while results in (C) and (D) probably represent both JE and dengue antibody. The city school (E) more clearly represents dengue prevalence. This is suggested not only by the prevalence difference between the two viruses but also by the level of geometric mean titers (Table II) for each area. Only in the city school are the dengue titers greater than the JE titers. Villages A and B are farther away and more rural and isolated from Chiengmai city than are villages C and D. Although only 6-8 year olds were sampled in Chiengmai city the picture looks like that of Bangkok for dengue prevalence and it is probable that virtually 100% of the population has been infected by group B virus(es) by early adulthood.

Table III shows the overall village prevalences and their 95% confidence limits for JE. Only (B) and (C) do not overlap but it appears that these villages appear similar in overall prevalence. The age prevalence distributions are likewise fairly similar (Figure 1) except for $\geq$ 20 year population of village (C).

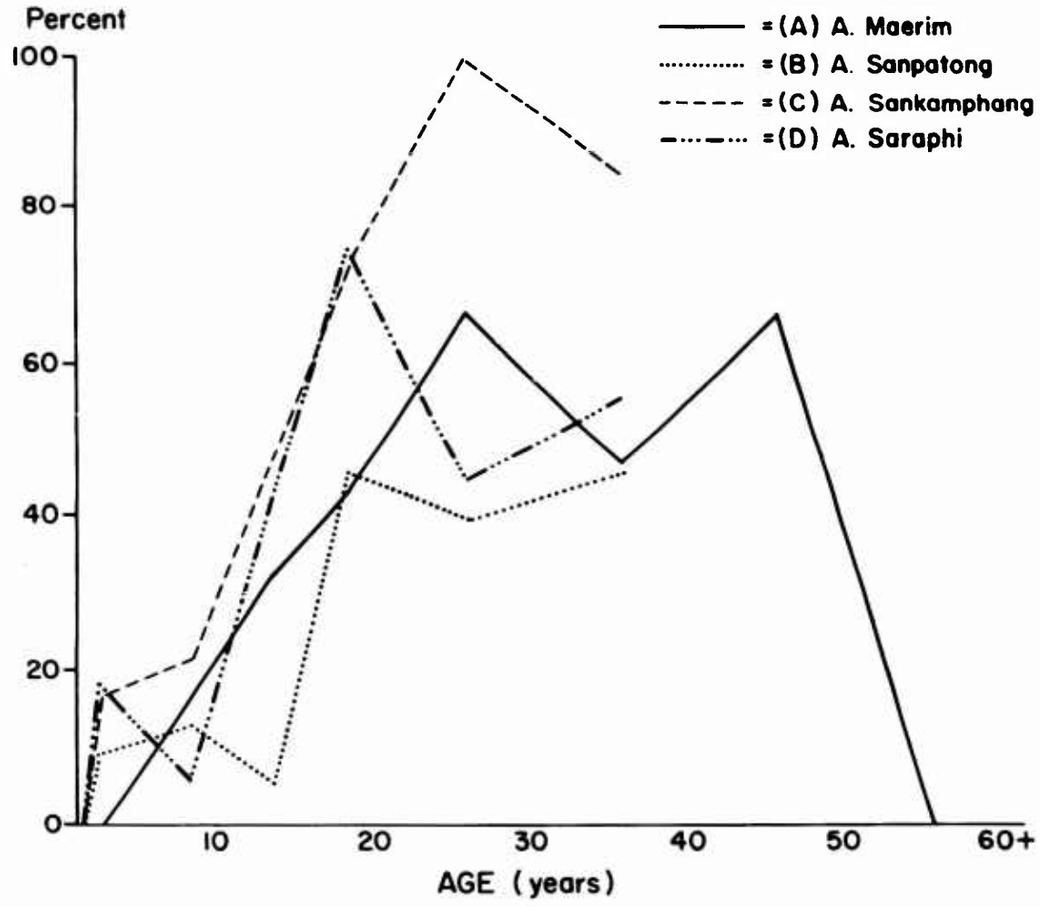
Chikungunya activity has evidently been absent in villages (A), (B), and (C), while present in village (D) and Chiengmai city. This is interesting and may deserve further entomologic and sero-epidemiologic investigation.

The similar prevalence of JE in males and females (Table IV) is surprising in view of the marked male preponderance of overt hospitalized encephalitis cases. There are several possible explanations, of course, and this certainly deserves thorough investigation in the coming season. It does suggest equal male-female exposure (and for each age group) to infection. From Figure 1 note the curve for village (A) in Amphur Maerim. Assuming this represents true JE prevalence the picture is as expected from the known epidemiology. Exposure to infected mosquitoes (presumed to be Culex) is most likely out in the nearby fields that are farmed by almost every family. Only the elderly and very young usually do not go to the fields and the low prevalence, $<$ 5 and $<$ 50 years, is shown.

The picture in Figure 1 is also compatible with that seen in a population exposed endemically with epidemics superimposed (assuming that HI positivity either remains $>$ 1 year or periodic exposures keep boosting HI levels). This can be analyzed by fitting a catalytic model to the prevalence data. This was done for Area (C) and appears reasonable (Table V).

FIGURE I

Percentage Age Distributions of Prevalence of JE Antibody (HI)
Chiengmai Villages — Nov. 1969



Thus, the estimated force of infection of 74 effective contacts per 1000 population per year for village (C) is quite high. Again this may be a combined Dengue-JE picture in village (C), which would be of interest epidemiologically and virologically.

The findings of marked differences in A. aegypti indices during the same month (November) as this survey and in the same villages suggested that there would also be differences in Dengue prevalence (Table VI). Indeed there is virtually perfect correlation, highly significant despite having only 5 data pairs to compare. Table VII presents data on incidence of group B arbovirus HI antibody as found in sera of domestic animals in the Chiengmai area. It is apparent that all species of domestic animals show a very high rate of exposure to group B arbovirus, and most probably this is JE virus. Preliminary neutralization test data confirms this but is not yet complete. Further sera collections of these animals are being made, as well as a wide variety of wild animal vertebrate species, in an effort to define which may be involved in the maintenance of group B arboviruses in nature.

It is recognized that to date unresolved difficulties in interpretation of serological data limit the ability to differentiate previous infection with JE and dengue viruses. Experiments are in progress to try to improve laboratory methods in this regard.

Continuing mosquito collections are being carried out. During the period October 1969 to March 1970, 1042 pools of Culex species have been tested for virus isolation. No viruses were isolated.

Table 1. Age Prevalence of JE, DENGUE and CHIKUNGUNYA HI Antibodies Chiengmai (Nov 1969)

Age	(A) Maerim		(B) Sanpatong		(C) Sankamphang		(D) Saraphi			(E) Chiengmai City			
	JE	D	JE	D	JE	D	JE	D	CHIK*	AGE	JE	D	CHIK
1-4	0	14.3	9.1	0	16.7	16.7	18.2	9.1	9.1				
5-9	16.7	16.7	13.3	13.3	21.7	34.8	5.9	35.3	5.9	6	75.0	87.5	25.0
10-14	31.6	10.5	5.5	5.5	47.0	64.7	36.4	50.0	4.5	7	64.0	81.3	12.0
15-19	42.8	28.6	45.4	18.2	72.7	63.6	75.0	75.0	12.5	8	55.5	85.2	0
20-29	66.7	33.3	40.0	30.0	100.0	100.0	44.0	66.7	11.1				
30-39	47.0	5.9	45.4	9.1	84.2	78.9	55.0	60.0	15.0				
40+	33.3	16.7	-	-	-	-	-	-	-				
Total	36.6	15.8	23.7	11.8	53.1	58.0	36.8	48.3	9.2	-	62.8	82.7	10.0
Sample Size	82		96		81		87			110			

+Titers ≥ 1:40
*CHIK completely negative in the villages (A), (B), and (C).

TABLE II

Geometric Mean Titers of those with HI Antibody*
Present-Chiengmai (Nov 1969)

	(A)	(B)	(C)	(D)	(E)
JE	56.6	105	185	132	117
Dengue**	44.5	74.1	142	127	129

* Titers ≥ 1:40
** Highest D1-D4 Titer used

TABLE III

Overall Prevalence (%) of JE Antibody (HI)*
and 95% Confidence Limits Chiengmai (Nov 1969)

Area	Prevalence(%)	Sp**	95% Confidence Limits
(A) Maerim	36.6	.0442	27.4 - 45.8
(B) Sanpatong	23.7	.0501	13.2 - 34.2
(C) Sankamphang	53.1	.0386	45.0 - 61.2
(D) Saraphi	36.8	.0595	26.3 - 47.3

*Titers ≥ 1:40
** S_p = Standard error of sample % positive

TABLE IV

Sex Prevalence of JE, DENGUE and CHIKUNGUNYA HI Antibodies Chiengmai (Nov 1969)

Area	No. Tests		PERCENTAGE POSITIVE*					
			JE		DENGUE		CHIK	
	Male	Female	Male	Female	Male	Female	Male	Female
(A) Maerim	36	46	41.7	32.6	16.7	15.2	0	0
(B) Sanpatong	36	40	22.2	25.0	11.1	12.5	0	0
(C) Sankamphang	49	32	55.1	50.0	51.0	68.7	0	0
(D) Saraphi	43	44	34.9	38.6	51.2	45.4	7.0	11.4
Total (A)-(D)	164	162	33.5	35.8	34.8	33.3	-	-
(E) Chiengmai City	57	53	61.4	64.1	82.4	83.0	10.5	9.4
TOTAL	221	215	-	-	-	-	-	-

*Titers ≥ 1:40

TABLE V

Fitting a Simple Catalytic Model to Area (C)-JE (HI) Prevalence Data

Age	t*	y	w	A	0.074t	$e^{-0.074t}$	y
1-4	2.0	.167	4	.668	.148	.8624	.1376
5-9	6.5	.217	5	1.085	.481	.6132	.3868
10-14	11.5	.470	5	2.350	.851	.4270	.5730
15-19	16.5	.727	5	3.635	1.219	.2955	.7045
20-29	24.0	1,000	10	10.000	1.776	.1693	.8307
30-39	34.0	.842	10	8.420	2.516	.0808	.9192
-	-	-	A = 26.158		-	-	-

*t = Age-1 year. Thus the t scale has been shifted to begin at age 1 instead of 0, the value of t on the scale is therefore always 1 year less than the population age at that point, and the total space covered by the data, from ages 1-40, is 39 years wide.

$'A = 26.158 \times \frac{100}{39} = 67.1$

$r' = .029$

$r = \frac{.029}{.39} = .074$ or estimated force of infection produces 74 effective contacts per 1000 pop. per year.

The fraction-positive pop. proceeds at rather less than the average rate for the years until about age 15 when the reverse is largely the case. This can be rationalized as the effect of extra exposure in the rice fields by the young and middle-aged population that does not attend school. The basic hypothesis here, of course, is that JE in this area is an endemic-epidemic disease and the curve tends to uphold this.

TABLE VI

Correlation of Aedes aegypti House Index and Prevalence of Dengue Antibody (HI) Chiengmai (Nov 1969)

Area	X = A. aegypti Index	Y = Dengue HI Prevalence
(A)	.019	.155
(B)	.001*	.117
(C)	.300	.580
(D)	.156	.461
(F)	.570	.827
Σ_2	1.046	2.140
Σ_2	0.4396	1.2706
$(\Sigma)/N$	0.2188	0.9159
C...	0.2208	0.3547
$\Sigma XY = .7204$ $\Sigma X \Sigma Y/N = .4477$ $C_{X,Y} = .2727$		

$$r = \frac{.2727}{\sqrt{(.2208)(.3547)}}$$

$r = 0.974$

$p < .01$

* Actual index was zero (.000). Correction used for analysis purposes.

TABLE VII

Incidence of HI Antibody in Sera of Domestic Animals in Chiengmai

	Per Cent with Antibody to :			
Animal	Chikungunya	Dengue-1	JE(Nak)*	JE(CM)**
Cattle	17	7	84	96
Pig	29	27	73	81
Dog	10	7	86	86
Buffalo	11	2	80	95

* Nakayama prototype strain
** Chiengmai strain

Ecology of Japanese Encephalitis Vectors

Principal Investigators : Douglas J. Gould, Ph.D.
Richard A. Grossman, MAJ, MC
Dennis O. Johnsen, MAJ, VC
Thomas J. Smith, LTC, MC
Michael F. Sullivan, CPT, MSC

Assistant Investigators : Attaya Boonyakanist
Samarn Maneewongse
Kol Mongkolpanya
Chumnong Noigamol
Larp Puntusiri

OBJECTIVES : To determine the identity of mosquitoes involved in the transmission of JE virus in the Chiengmai area and to measure the seasonal density, host preferences, insecticide susceptibility status and flight dispersal characteristics of suspected vector species.

DESCRIPTION : Between June and September 1969 an epidemic of encephalitis due to JE virus occurred in Chiengmai and other northern provinces of Thailand. In collaboration with the Epidemiology and Virology Departments of SMRL an initial survey of the affected districts in Chiengmai province was made in July, and approximately 11,000 culicine mosquitoes were collected for virus isolation attempts. In October 1969 routine collections of adult and larval mosquitoes were begun in seven districts of Chiengmai province. In order to measure mosquito population densities and to provide material for virus isolation attempts, 10 New Jersey type and 16 CDC (battery-powered) type light traps are operated weekly at sites in Mae Rim, San Sai, Doi Saket, Saraphi, Muang, Sankampaeng and Sanpatong districts. Bi-monthly larval surveys of culicine mosquitoes are made to determine population densities, types and location of breeding sites in the study sites.

PROGRESS : Between October 1969 and April 1970, 31,431 mosquitoes, consisting primarily of Culex fuscocephala, C. gelidus, C. tritaeniorhynchus, C. vishnui complex, Aedes linea-

topennis, A. mediolineatus and A. vexans, were collected for virus isolation attempts. Results of isolation attempts are given in the report of Virology Department. As measured by light trap collections of female mosquitoes, populations of the two suspected vector species, C. gelidus and C. tritaeniorhynchus, declined in December and remained at a low level until March. The numbers of C. tritaeniorhynchus began to rise in March, but there was little change in the C. gelidus population (Fig. 1).

During November 1969 and March 1970 houses in the study sites in Saraphi, Sankampaeng, Sanpatong, Mae Rim and Muang districts were surveyed for the presence of Aedes aegypti. The results of these surveys indicated that the A. aegypti population densities varied greatly between study sites; villages in Sanpatong district had no A. aegypti while in the Muang district (Chiengmai city) at least 50% of the houses were infested (Table 1). The A. aegypti indices represent the proportion of houses visited in which larvae of that species were found. These indices showed a strong positive correlation with the data for dengue prevalence in these areas (See report of Epidemiology Department).

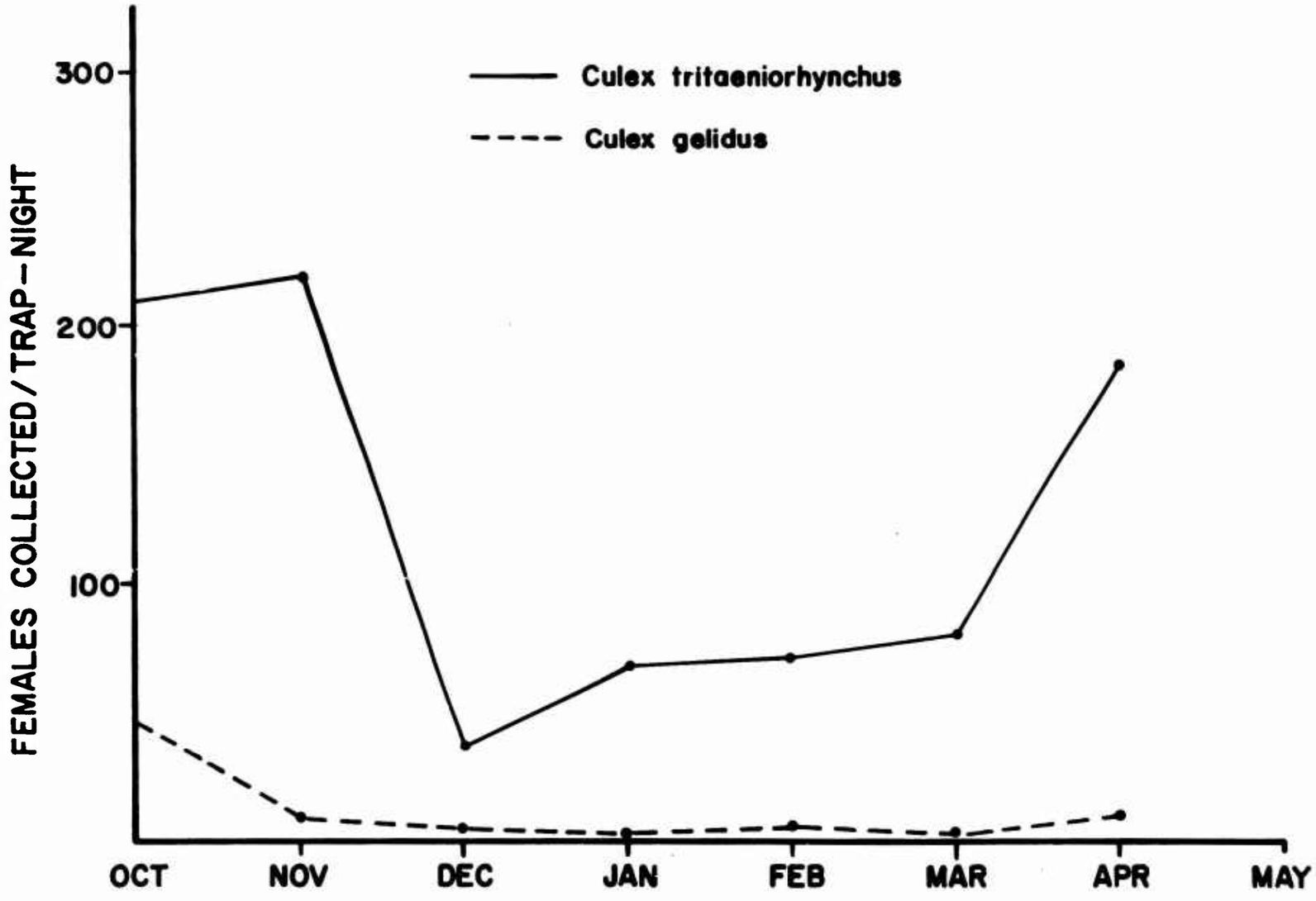
The stomach contents of blood engorged mosquitoes collected in light-traps in the Chiengmai area were tested by the agar-gel diffusion test to determine sources of their blood meals. The majority of those tested had fed on cattle and/or buffalo, while smaller numbers had pig, horse, chicken and humans (in that order) blood in their guts (Table 2).

Table 1. Results of survey of houses in Chiengmai study sites for presence of Aedes aegypti-1969-70.

Site	November 1969		March 1970	
	No. Houses inspected	Aedes aegypti index	No. Houses inspected	Aedes aegypti index
Amphur Mae Rim	52	.02	82	.00
Amphur Muang	128	.68	144	.58
Amphur Sankam-paeng	92	.29	72	.39
Amphur Sanpatong	103	.00	93	.00
Amphur Saraphi	64	.16	78	.12

Table 2. Blood meal sources of mosquitoes identified by agar-diffusion technique - Chiengmai, October-November 1969.

Species	Host				
	Human	Buffalo-Cow	Pig	Horse	Chicken
Culex fuscocephala	0	169	8	3	0
Culex gelidus	0	69	3	0	0
Culex tritaeniorhynchus	0	190	2	0	1
Culex vishnui complex	3	222	5	3	9


Culex tritaeniorhynchus
Culex gelidus
FEMALES COLLECTED / TRAP – NIGHT
300
200
100
OCT
NOV
DEC
JAN
FEB
MAR
APR
MAY

Combined Investigation of Dengue Hemorrhagic Fever in an Urban Population

Project Coordinator:	Thomas J. Smith, LTC, MC
Principal Investigators:	Ananda Nisalak, M.D. Boonsri Phuvichit*, M.D. Curtis Bourgeois, Jr., LTC, MC Douglas Gould, Ph. D. Pethai Mansuwan, **M.D. Sophana Kanchanapilant,* M.D. Richard Grossman, MAJ, MC Thomas Smith, LTC, MC Suchinda Udomsakdi, M.D.
Associate Investigators:	Hilary Evans, MAJ, MC Phanu Sittisomwongse, D.D.S. Salvadore Arellano, SP5 Samuel Fulton, SFC
Assistant Investigators:	Aree Boriharnvanakett, B.S. Chumpan Chavachati, B.S. Nonglak Khananuraksa, B.S. Panor Srisongkram, B.S. Phuangthong Puengkaew, R.N. Supatra Chulachumbok, R.N. Suvana Vithanomsat, R.N.

OBJECTIVE: To study clinical and biochemical abnormalities associated with dengue hemorrhagic fever, and investigate more efficient and specific virological methods of determining the etiology. In addition, to investigate ecological conditions which potentially contribute to favorable virus transmission by mosquito vectors.

DESCRIPTION: Previous investigations of dengue hemorrhagic fever have provided an epidemiological description of the disease as it occurs in a

---

* Staff Physician, Children's Hospital
** Director, Children's Hospital

relatively isolated (insular) population. In this situation the fact of ecologic isolation apparently restricted annual epidemic disease to a single serotype (Annual Reports 1968, 1969). In a large urban population such as Bangkok, however, a multiplicity of ecologic situations with a large human population would seem to potentially offer totally different conditions.

The coordinated investigation by a number of different disciplines of all children admitted with dengue hemorrhagic fever over the course of a year offered the opportunity to study such urban dengue hemorrhagic fever. During the year an attempt was made to include all children admitted to Children's Hospital, Bangkok. Upon admission a physician recorded a complete history and all abnormal physical findings. Specimens were collected for hematology, clinical chemistry, serology and virus isolation. Complete blood counts, urinalyses, platelet count, bleeding time and prothrombin and partial thromboplastin times were measured. The battery of chemistry determinations included BUN, sodium, chloride, CO_2, glucose, SGOT, SGPT, LDH and isozymes, bilirubin, total protein and protein electrophoresis.

Home environment, family history, epidemiologic information and mosquito populations were assessed on the day of admission by a home visit. This team consisted of public health nurses and entomology technicians. Background information concerning the present illness, past medical history, family history and a general description of pertinent features of the home environment was recorded, and interview and sera collected from family members. Indoor daytime collections of Aedes aegypti mosquitoes resting or bitting and by pyrethrum spray knock-down were done, identified, and then frozen for virus isolation attempts. Artificial water containers in and around homes were enumerated and the number containing larvae recorded, and a representative sample collection recorded for identification. Mosquito evaluations included both the patient's home and the 10-20 immediately surrounding homes, in as many instances as possible.

A specific virus etiology was sought in each case. Acute sera were cultured for virus, and acute and convalescent sera from the patient and members of his family were tested serologically by HI, CF, and in some cases, neutralizing antibody.

Available evidence, obtained in this laboratory during the past several years, indicates that the lesion of dengue hemorrhagic fever is the

result of host hypersensitivity or immunity to dengue virus antigen. Immunologic data indicate that children with severe hemorrhagic fever have had previous experience with members of the dengue virus complex. This experience is reflected in the early appearance of high-titered, broadly-reactive group B antibody, and the rapid clearing of dengue virus from the circulation. The latter has been interpreted to mean that the virus is bound by antibody, and that "immune-complexes" of antigen, antibody and complement reacting on the surface of endothelial cells may result in cellular injury. As part of the present study direct evidence for the presence of cytophilic antibody in hemorrhagic fever was sought utilizing specimens obtained from hospitalized children. In addition the persistence of dengue virus otherwise obscured by antidengue antibody was sought in cells of the reticuloendothelial and lymphoid systems.

PROGRESS: During the study period April 1969 to March 1970, 273 patients were admitted to the study. This represented 68% of the 406 total cases clinically diagnosed at Children's Hospital during this period, most of those not studied having been private patients. Of these 273 patients, 210 were confirmed in the laboratory as dengue infections (see below). The monthly admission rates are shown in Table 1, along with the age distribution of cases. For comparison, the number of cases admitted to 16 other hospitals in Bangkok-Thonburi during 1968-1969 is also shown.

Of the 273 total cases 118 were male and 155 female, 31 of the cases were fatal, or 11.4%. Of the fatal cases, mean age was 6.5 years and median age 5.0 years, with the patients dying an average 3.7 days after hospitalization.

Cases admitted were diagnosed serologically according to previously described criteria (see Annual Report 1968). A summary of serological results are shown in Tables II and III. Of cases with dengue shock syndrome all 32 showed a secondary type serological response, whereas 4 of hemorrhagic fever cases showed a primary, and 165 a secondary, response.

A summary of clinical features is shown in Table IV with special emphasis on hemorrhagic and hematological features. Table V shows results of fibrinogen and lactic dehydrogenase (LDH) isoenzyme determinations. The homes of 189 patients were visited as soon after admission as possible by public health nurses for family histories

and blood sample collection from family members, and 147 homes were visited by an entomological field team. As shown in Table VI, 14% of family members on whom paired sera were available had evidence of concomitant dengue virus infection.

Virus isolations were attempted from four sources:

1. mosquitoes collected in and around the patient's house;
2. from acute phase sera of the patient;
3. autopsy specimens in fatal cases;
4. bone marrow aspirates during the acute phase.

A summary of virus isolates is presented in Table VII. Dengue 2 virus was most commonly isolated with 23 isolates, followed by 11 strains of dengue 3, 3 Chikungunya and one each dengue 1, Sindbis and unknown. At the time virus was present in the bone marrow it was also recovered from the blood of two patients.

Additional mosquito collection data is presented in the SEATO Medical Project on Mosquitoes report.

Table 1. Monthly admission rates and age of patients.

Month	No. Patients		Age
April 1969	17	6	∠1 yr
May	28	7	1
June	36	15	2
July	43	26	3
Aug	36	22	4
Sept	20	26	5
Oct	16	29	6
Nov	10	13	7
Dec	4	15	8
		13	9
		15	10
		23	>11

16 other Bangkok-Thonburi hospitals :

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
1968 (1894)	62	32	38	44	68	104	209	214	357	272	242	252
1969 (3,342)	278	174	156	170	241	310	497	516	500	358	141	48

Table II. HI Serology Summary (N = 273 Cases)

Dengue Status	No. Cases	% of Grand Total	Dengue Isolates	CHIKUNGUNYA	
				No. Sero-Positive	CHIK Isolate
Primary Response	4	1.6	I	-	-
Secondary Response	197	81.4	-	5	-
a) 2^{o} rise or fall	157	64.9	III(2)	4	-
b) Fixed Positive	40	16.5	-	1	-
Dengue Unclassifiable	9	3.7	III	-	-
Total Dengue Positive	210	86.8	4	5	-
Total Dengue Negative	32	13.2	1	5	3
a) True Negative	17	7.0	-	3	2
b) Fixed Negative	15	6.2	II	2	1
GRAND TOTAL	242*	100.0	5	10	3

* Of the 31 cases with inadequate specimens, 30 were rapidly fatal cases.

Notes :

(1) Of Dengue positive 1.9% had 1^{o} response
93.8% had 2^{o} response

(2) Proportion of case with CHIK diagnosis = 5/242 = 4.1%

(3) Proportion with CHIK and Dengue diagnosis = 5/242 = 2.1%

(4) Proportion of non-fatal study cases with serologic diagnosis of Dengue and/or CHIK = 215/242 = 88.8%
Thus the % nonfatal cases undiagnosed = 11.2%

Table III HI Serologic DX by Age

Age (Yrs)	Tested		1°	2°	%	Fixed(+)		Dengue Unclass	Total Dengue Positives		True Neg	Fix Neg	Tot Neg	CHIK
	No.	% of Total	No.	No.		No.	%	No.	No.	%	No.	No.	No.	No.
<1	7	2.9	1	-	-	-	-	5	6	2.8	-	1	1	-
1-	26	10.7	2	17	10.8	2	5.0	-	21	10.0	3	2	5	1
3-	49	20.2	-	37	23.6	10	25.0	1	48	22.8	1	-	1	-
5-	58	24.0	-	42	26.8	11	27.5	2	55	26.2	1	2	3	2
7-	35	14.5	-	23	14.6	5	12.5	-	28	13.3	4	3	7	1
9-	35	14.5	1	20	12.7	6	15.0	1	28	13.3	5	2	7	2
11-14	32	13.2	-	18	11.5	6	15.0	-	24	11.4	3	5	8	4
Total	242	100.0	4	157	100.0	40	100.0	9	210	99.8	17	15	32	10
Mean Age		6.80	-	6.70		7.2		-	6.6		-	-	8.2	9.4
Median Age		6.6	1-	6.21		6.5		<1	6.3		8-	8-	8.8	10
% Male		43.0	75.0	40:1		40.0		66.7	41.9% M:F	or 1:2.4				

TABLE IV. Clinical and Laboratory Data, THF

	Dengue Serologic Diagnosis						Fatal Cases N=31	
	Shock N= 32		Non-Shock N=178		Total N=210			
	No.	%	No.	%	No.	%	No.	%
Shock Cases	-	-	-	-	-	-	6/26	23.1
(+) Tourniquet Test	16/29	55.2	106/161	65.8	122/190	64.2	10/21	47.6
Rec. Plasma/(+)T.T.	15/16	93.8	61/106	57.5	76/122	62.3	9/10	90.0
Received Plasma	31	96.9	103	57.9	134	63.8	21	67.7
Adm. Temp. > 37.5°C	5/32	15.6	47/178	26.4	52/210	24.8	11/27	40.7
Petechiae	11/32	34.4	92/178	51.7	103/210	49.0	10/21	47.6
Liver	25/32	78.1	139/178	78.1	164/210	78.1	19/21	90.5
Lymphadenopathy	27/32	84.4	142/178	79.8	169/210	80.5	16/21	76.2
Pharyngitis	25/32	78.1	126/178	70.8	151/210	71.9	9/21	42.8
Conjunctivitis	4/32	12.5	18/178	10.1	22/210	10.5	0/21	0
History of Bleeding	6/32	18.7	77/178	43.2	83/210	39.5	12/21	57.1
Hemoconc. Index ≥ 0.2	24/25	96.0	82/119	68.9	106/144	73.6	4/7	57.1

TABLE IV (Continued)

	Dengue Serologic Diagnosis						Fatal Cases N=31	
	Shock N=32		Non-Shock N=178		Total N=210			
	No.	%	No.	%	No.	%	No.	%
Platelet Ct. ≤ 60,000	18/30	60.0	66/146	45.2	84/176	47.7	7/17	41.2
Hct ≥ 45%	25/31	80.6	74/169	43.8	99/200	49.5	13/21	61.9
SGOT > 125 SF Unit	8/22	36.4	42/138	30.4	50/160	31.2	9/12	75.0
TSP ≤ 5.5 G%	14/26	53.8	49/150	32.7	63/176	35.8	6/14	42.8
WBC < 4,500	2/24	8.3	14/149	9.4	16/173	9.2	1/13	7.7
WBC ≥ 10,000	7/24	29.2	65/149	43.6	72/173	41.6	7/13	53.8
Hemoglobin G% (Average)	13.21	n=21	11.91	n=113	12.11	n=134	12.36	n=5
BUN > 20 Mg%	10/26	38.5	46/154	29.9	56/180	31.1	12/15	80.0
Na < 135	15/30	50.0	60/89	67.4	75/119	63.0	4/7	57.1
Co_2 ≤ 15	11/19	57.9	37/113	32.7	48/132	36.4	5/8	62.5

TABLE V. LDH and Fribinogen Data

Test		Dengue Serologic Diagnosis			Fatal Cases n=31	CHIK Diagnosis n=10	No. Sero-Dx (non-fatal) n=32	U.S. Normal
		Shock n=32	Non-Shock n=178	Total n=210				
Fibrinogen	No. Test	8	34	42	4	-	9	400.700
	Mean	150.0	246.1	227.8	262.5	-	480.1	
	Median	125	225	216.5	250	-	375	
Total LDH (Units)	No. Test	15	100	115	12	9	15	< 250
	Mean	303.3	337.1	332.7	592.2	286.1	305.0	
	Median	288.5	328	322.8	275	300	270	
LDH Components No.		15	97	112	12	10	15	
%	Test							
LDH 1	Median	7.8	7.2	7.3	20.0	4.2	4.0	< 5%
LDH 2	Median	12.5	12.4	12.4	12.5	9.2	9.2	< 8%
LDH 3	Median	24.6	25.4	25.3	20.0	24.5	21.7	< 25%
LDH 4	Median	31.2	29.7	29.9	24.4	33.8	35.0	< 45%
LDH 5	Median	24.3	24.8	24.7	23.1	29.5	29.0	< 30%

TABLE VI. Family Sera from Dengue-Diagnosed Cases

Age (Yrs)	True Neg.	Fixed Neg.	Total Neg.	Primary 1° Response	Secondary 2° Response			Dengue Unclassified	Total Dengue (+)	Total
					↑	↓	Fix(+)			
0-4	5	3	8	2	3	-	1	-	6	14
5-9	8	9	17	-	3	1	3	1	8	25
10-14	4	3	7	-	2	-	-	-	2	9
15-19	1	7	8	-	-	-	-	-	-	8
20-29	1	35	36	-	2	1	1	2	6	42
30-39	1	70	71	-	2	2	-	1	5	76
40-49	1	14	15	-	-	-	-	1	1	16
50-	2	7	9	-	-	-	-	-	-	9
TOTALS	23	148	171	2	12	4	5	5	28	199

Notes : (1) Average No. paired sera per family = $\frac{199}{164}$ = 1.2

(2) 0/23 paired sera from 19 non-dengue-diagnosed cases were Dengue (+)

(3) 164/210 (78.1%) Cases had one or more family sera drawn.

TABLE VII. Virus Isolates

Source	Virus	No. Strains
A. aegypti	dengue 2	13
	dengue 3	8
C. quinquefasciatus	dengue 2	1
	Sindbis	1
	unknown*	1
Acute sera	dengue 1	1
	dengue 2	3
	dengue 3	3
	Chinkungunya	3
Lymph node	dengue 2	3
Bone marrow	dengue 2	3

*not neutralized with dengue 1-4, JE, Chik, Sindbis, Batai or Wesselsbron antisera.

Combined Investigation of Central Nervous System Infection in Bangkok Children.

Principal Investigators: Curtis Bourgeois, Jr., LTC, MC
Douglas Gould, Ph.D.
Lloyd Olson, MAJ, MC
Pethai Mansuwan,* M.D.
Rapin Snitbhan, M.D.
Ratana Rattanawongse, M.D.
Richard Grossman, MAJ, MC
Suchinda Udomsakdi, M.D.
Thanomchit Suwapant, M.D.**
Thomas Smith, LTC, MC

Associate Investigators: Milton Willhight, SFC
Phanu Sittisomwongse, D.D.S.
Salvadore Arellano, SP/5
Samuel Fulton, SFC
Sumitda Narupiti, B.Sc.

Project Coordinator: Thomas J. Smith, LTC, MC

OBJECTIVE: To determine the relative incidence of encephalitis occurring in Bangkok children, the spectrum of clinical disease, and etiological agents associated with the disease.

DESCRIPTION: At the present time essentially no information is available on the causes of CNS infections in the urban population of Bangkok. A number of arboviruses are known to be present, but their behavior with respect to this form of disease is unknown. For example, instances of dengue hemorrhagic fever with CNS symptoms are not unusual, and the wide variety of ecological situations present in the sprawling metropolitan area of Bangkok potentially offers suitable microhabitats for a full range of mosquito vectors. At the same time the population is exposed to a very high infectious force by enteroviruses known to be present as a result of the watery environment heavily contaminated by lack of developed sewage and refuse disposal systems. Finally, the syndrome of encephalopathy and fatty degeneration of the viscera is known to occur to an unknown extent in Bangkok, even though in northeastern Thailand this syndrome follows an almost exclusively rural distribution.

---

* Director, Children's Hospital, Bangkok
** Staff Physician, Children's Hospital, Bangkok.

To evaluate these and other aspects of encephalitis, all children admitted to Children's Hospital with this admission diagnosis or aseptic meningitis or poliomyelitis were studied. History and physical examination data were recorded on a standard form, blood obtained for hematology, chemistries and acute sera, and specimens obtained for attempted virus isolation. A home visit was made the same day for family history, assessment of the environment and mosquito collection. In fatal cases, where possible, an autopsy was performed to obtain an anatomical diagnosis and tissue for virus isolation attempts. In survivors, convalescent sera were obtained for serological studies.

PROGRESS: During the period April 1969 to March 1970, 64 patients were admitted with a presumptive diagnosis of non-bacterial infections of the central nervous system. Twenty-four (38%) of these were fatal, but only eleven were autopsied. A paucity of clinical data, lack of information on hospital course, and limited number of clinical investigations also contribute to difficulties in establishing a definitive diagnosis. Fifty-eight were admitted with suspected encephalitis with only 6 admitted with aseptic meningitis and 2 with poliomyelitis. Other syndromes subsequently established (clinically) but initially diagnosed as encephalitis included febrile convulsions, infantile beri-beri, shiggellosis, tuberculous meningitis, purulent meningitis and hepatic coma. Only 34 patients were from Bangkok-Thonburi, the remainder from outlying provinces as distant as Pitsanuloke and Mahasarakarm.

From analysis of admission history and physical examination, ten patients met the criteria as established for EFDV syndrome, outlined elsewhere in this report. Seven were from the metropolitan area. All ten died within two days of admission.

Only one arbovirus was isolated from mosquitoes collected in the homes of 21 encephalitis patients; a dengue 2 was isolated from Aedes aegypti. Of paired sera available on 20 patients two were positive for recent group B arbovirus infection. Neither patient was from the metropolitan area; one was from Chachoengsao and the other from Samutprakarn and both sera were broadly reactive and not specific for JE virus. Paired sera were available from 17 family members of the patients in this series. Only one, the mother of a patient from Bangkok, showed evidence of recent infection with a group B arbovirus. Thus, evidence of arbovirus participation in CNS infections of Bangkok children is virtually negative.

CSF and throat and rectal swabs were received from all patients, as well as 63 specimens of tissue from the eleven autopsied cases. Twenty-four isolates were made from 17 patients, and are shown in Table VIII. An enterovirus etiology is thus suggested on at least nine of the patients.

In addition, sera collected from 19 other patients showed an increase in poliovirus antibody in five patients: two to polio type 1, and 3 to polio type 2.

TABLE VIII. Virus isolates from patients with CNS infections.

Patient	Specimen	Isolate
21	RS	Unidentified enterovirus
27	RS	echo 17
45	CSF, brain, RS	unident. enterovirus
58	TS,RS	myxovirus
64	TS	CMV
65	RS	echo 22
73	TS	CMV
83	TS	unident. enterovirus
	RS	Coxsackie B3
104	RS, TS	unidentified
164	RS	echo 6
170	TS	unidentified
185	TS, RS, CSF	Coxsackie B4
227	RS	incomplete
299	RS	"
301	TS	"
181	TS	"
313	RS	"

RS = rectal swab; TS = throat swab

Bangkok Dengue Vectors

Principal Investigators : Douglas J. Gould, Ph.D.
Richard A. Grossman, MAJ, MC
Thomas J. Smith, LTC, MC

Associate Investigators : Salvador Arellano, SP 5
Samuel E. Fulton, SFC

Assistant Investigators : Attaya Boonyakanist
Vichit Phunkitchar
Chamnong Noigamol

INTRODUCTION : The study described below was part of a collaborative investigation into the epidemiology of dengue hemorrhagic fever in the Bangkok-Thonburi metropolitan area carried out together with the Departments of Epidemiology and Virology. Pertinent epidemiological and virologic aspects of this study are to be found in the reports of the above departments.

OBJECTIVES : To determine the relative density of dengue vector populations in the vicinity of houses of dengue hemorrhagic fever patients admitted to Children's Hospital and to collect mosquitoes from these sites for virus isolation attempts.

DESCRIPTION : Collections of adult mosquitoes for virus isolation attempts were made in the houses of dengue hemorrhagic fever patients admitted to Bangkok Children's Hospitals and in at least nine houses adjoining or near the patients' houses. Indoor daylight collections of mosquitoes, were made by human biting collections and/or the pyrethrin-spray knockdown technique in these houses. Mosquitoes collected by these methods were identified, frozen in pools of suitable size and forwarded to the Virology Department for virus isolation attempts. All artificial containers inside and around all houses visited were examined for mosquito larvae and the number of infested containers and species of mosquito recorded. The sources of blood-meals taken by engorged mosquitoes in the pyrethrin-knockdown collections were determined by the agar-gel diffusion test.

PROGRESS : Between 11 April 1969 and 25 March 1970, 3952 pools containing a total of 6504 A. aegypti and 3927 pools containing a total of 84,339 C. quinquefasciatus, collected from houses in the Bangkok-Thonburi area, were submitted to the Virology Department for virus isolation attempts. These mosquitoes were collected from 3147 houses; 307 of these were houses of dengue hemorrhagic fever cases and the balance were houses adjoining or located nearby the case houses. Dengue viruses were isolated from 20 of the A. aegypti pools; 14 of these were dengue type 2 and the other were dengue type 3. One pool of C. quinquefasciatus yielded a strain of Sindbis virus. Only 5 of the above isolates came from case houses, while the balance of positive pools came from nearby houses, which were located at distances of from 1 to 30 meters from case houses. All of the isolations came from mosquitoes collected between May and November 1969, and the majority of isolations (16/20) were made from mosquitoes collected during the rainy season (June-October). Agar gel diffusion tests on engorged mosquitoes from pyrethrin-knockdown collection indicated that all 110 A. aegypti tested had fed on humans; 56 per cent of engorged C. quinquefasciatus had fed upon humans, 31 per cent on dogs and 6.5 per cent on chickens. Analysis of larval and adult mosquito population data accumulated during this study is presently underway.

Laboratory Study of Arboviruses

Principal Investigators: Ananda Nisalak, M.D.
Dephanom Muangman, M.D., D.P.H.
Pairatana Gunakasem, M.D.
Suchinda Udomsakdi, M.D.
Thomas J. Smith, LTC, MC

Associate Investigators: Phanu Sittisomwongse, DDS
Wattana Wattanavicharn, M.Sc.

Assistant Investigators: Anan Boonkanoke
Aree Boriharnvanakett, B.S.
Chumpan Chavachati, B.S.
Jiraporn Supavadee, B.S.
Laddawal Sookcharoen, B.S.
Ming Choohong
Moragot Tanticharoen, B.S.
Panor Srisongkra[illegible], B.S.
Pranom Tuntrakoo, B.S.
Suwanna Vithanomsat, B.S.

OBJECTIVE: To investigate basic biological properties of the arboviruses, with particular reference to dengue viruses.

DESCRIPTION: Many strains of dengue 2 virus isolated from mosquito pools in 1967 epidemic of hemorrhagic fever at Koh Samui Island showed a mixture of small and large plaque size viruses on LLC-MK_2 cell culture. Pure cultures of small and large plaque viruses were prepared from strains BKM 551 in stock culture by methods as previously described. For the purpose of biological studies in various tests these two viruses were designated as SP-BKM 551 and LP-BKM 551 viruses. The passage level of MK_2 seed of SP-BKM 551 and LP-BKM 551 viruses used in these studies are the 13th and 14th.

PROGRESS: From the results of previous reports, SP-BKM 551 and LP-BKM 551 viruses were proved to be dengue 2 viruses but SP-BKM 551 was neutralized by monkey antiserum prepared from dengue 2 (NG-C) virus at a significantly less degree than LP-BKM 551. The other evidence that SP-BKM 551 and LP-BKM 551 are dengue 2 virus strains is that dengue 2 (NG-C) are neutralized to the same degree by monkey antisera immunized with SP-BKM 551 and LP-BKM 551 viruses.

One-day old mice inoculated with 5-530 PFU of SP-BKM 551 virus showed no sign or symptom but developed resistance to intracerebral challenge dose

of 200-10,000 mouse LD_{50} of dengue 2 (NG-C) virus 21 days after inoculation. Mice inoculated with 3-300 PFU of LP-BKM 551 virus showed sickness and death 8-10 days after inoculation.

In addition to the above studies small plaque size virus can be demonstrated in brains of infected mice after inoculation with SP-BKM 551 virus.

To exclude the contamination of dengue or other viruses in the seed of SP-BKM 551 virus, many attempts have been tried to illustrate SP-BKM 551 virus seed is a pure population.

1. After several passages in MK_2 cells there is stability of plaque size from passage 8 to passage 24.

2. LP-BKM 551 virus was introduced into SP-BKM 551 virus in different PFU ratios and the pools were inoculated intracerebrally into one-day old mice. The results shown in Table 1 illustrate when 0-30 PFU of LP-BKM 551 mixed with 240000 PFU of SP-BKM 551 deaths in mice occurred. None of the mice receiving 240000 PFU of SP-BKM 551 virus showed sickness or deaths.

3. From the results previously reported, three blind-passages of 1:10 dilution of 10% mouse brain suspension revealed no sickness or death in inoculated mice.

4. SP-BKM 551, LP-BKM 551 and BKM 551 (parent strain of SP and LP virus before cloning) were passed 4 times in one-day old mice. HA antigens were prepared by the sucrose acetone method from infected mouse brain for each of the three viruses and were tested against mouse immune ascetic fluids prepared against each of the four dengue serotype.

HI results are shown in Table 2, and indicate that all are identical to each other and to New Guinea C prototype.

When infected mouse brain suspensions of SP-BKM 551 virus, mouse passage 4, was tested with specific dengue monkeys antisera type 1, 2, 3 and 4 by plaque reduction neutralization test, neutralization ratios were 15/640, 640/640, 10/8 and 15/400, respectively.

5. SP-BKM 551 virus from mouse passage 4 was used to immunize monkeys. Viruses were recovered from viremic monkeys and reinoculated into another monkey. Virus was also recovered from the second monkey. Sera at two-month bleeding were tested for type-specific dengue antibodies. The results in Table 3 indicate SP-BKM 551 from 4th mouse passage, first monkey passage and second monkey passage showed the

same neutralization ratio when tested against dengue specific monkey antisera.

The first and second passage monkey isolates showed the same specific antibody response against dengue 2 virus as shown in Table 4.

Shifting of small plaque size to large plaque size.

Purification of small and large plaque virus for a pure clone was done with passage 8 of mixed-plaque BKM 551 virus. When the small plaque and large plaque were passed in MK_2 cells up to passage 24, the two viruses showed stability in plaque size, but there is evidence of shifting of small plaque size to large plaque size when the hosts were changed from MK_2 cells.

1. MK_2 seed of SP-BKM 551 virus was passed subcutaneously and intravenously into a monkey and then the viruses recovered from viremic blood were passed into another monkey. After the first monkey passage, the virus recovered from viremic blood was large plaque variant. The virus recovered from viremic blood of the second monkey shows the same large plaque size as that of the first monkey.

2. SP-BKM 551 virus was passed intracerebrally in one-day old mice with the appearance of large plaque size virus on mouse passage 4.

Changing of mouse virulence after monkey passage of SP-BKM 551.

There is an increase in mouse virulence after monkey passage of SP-BKM 551 prepared from MK_2 cells. Two consecutive inoculations of monkeys by subcutaneous and intravenous route were done as described. In Table 5 the virus recovered from first viremic monkey showed mouse adaptation on second mouse passage, but at first only 1 of total 16 died on first mouse passage. When the first monkey virus was passed into another monkey, the virus recovered from viremic blood of the second monkey caused death in all inoculated mice at first mouse passage.

The studies of the challenge virus resistance in mice after IC inoculation of SP-BKM 551 (MK_2-14).

Table 6 illustrates the results of challenge 21 days after IC inoculations of dilution of small plaque virus in newborn mice. Such mice resist IC challenge not only to dengue 2 New Guinea C, but also to chikungunya virus, a Group A arbovirus. While the protection endpoint was missed in this experiment, it can be seen that protection against challenge virus corresponds fairly closely to the estimated intracerebral dose as expressed

in terms of PFU, measured by a simultaneous titration of the inoculum in MK_2 cells. Serum antibody was measured in some of these mice at time of challenge, and the results are shown in Table 7.

While low levels of antibody could be detected against dengue 2 virus in mice, which received the largest inoculum of small plaque virus (4×10^5 PFU), no chikungunya antibody could be detected, nor could dengue antibody be measured in any of the mice which received higher dilutions of small plaque virus. Thus, it would seem that the protection of mice on day 21 is not the result of antibody against challenge virus. Virus content of brain tissue was measured during the same experiment on day 7, 14 and 21 following inoculation of small plaque virus, and the results are shown in Table 8.

At each interval, small plaque virus could be detected in brain tissue of mice which received doses ranging from 400,000 to 4 PFU, providing evidence for multiplication of the virus. It is clear from this table that titrations of brain tissue do not show a ten fold reduction in virus content corresponding to ten fold dilutions of the tissue, and this has been observed in several experiments. In fact, in some experiments very little or no virus can be detected in undiluted samples of brain tissue, suggesting that this virus demonstrates the phenomenon of autointerference.

It has been shown that no SP virus is detectable in the mouse brain after inoculation with a high dose (400,000, 40,000 and 4,000 PFU) but SP viruses persist in mouse brain inoculated with lower dose up to day 21 of inoculation.

The inoculated mice in every group inoculated with various doses from 4-400,000 PFU developed resistance to challenge virus so that simple persistence of SP virus in mouse brain is probably not the basis of protection.

Interferon studies in mouse brain inoculated with SP-BKM 551 virus.

Interferon-like substance was prepared from mouse brain harvested at interval after inoculation of SP-BKM 551 (MK_2-14), LP-BKM 551 (MK_2 -14) and dengue 2 (NG-C) intracerebrally into one-day old mice. Twenty percent brain suspensions were centrifuged at 40,000 rpm for 4 hrs. The supernates were dialyzed in Hank's BSS at pH 2.0 for hr hrs. and dialyzed back at pH 7.4 for 12 hrs. The preparations were found to be free of infectivity by plaque methods in MK_2 cell cultures. L cells were used in assay of mouse interferon.

To test interferon activity, 2.0 ml dilutions of each preparation were left

for 18 hrs on cell sheets. After one washing with Hank's BSS, challenge viruses (Sindbis (cgLt 599) for L cells and Chikungunya (Ross) for MK_2 cells) were inoculated onto cell sheets in the dose of 30-100 PFU. Other plaque techniques followed the methods of dengue plaque on LLC-MK_2 cells.

The results illustrated in Table 9 reveal interferon action is probably not a mediator of mouse challenge virus resistance after SP-BKM 551 (MK_2-14) inoculation. The peaks of virus titer, challenge virus resistance and interferon titer appeared on day 14. There was decrease in virus titer and interferon to undetectable levels by day 35.

The mouse challenge virus resistance on day 35 without detectable virus and interferon in brains suggests other mechanisms as another role in mouse immunity developed after SP-BKM 551 (MK_2-14) inoculation. In Table 5 LP-BKM 551 (MK_2-14) and dengue 2 (NG-C) showed interferon titers of 1:2560 and 1:10240. SP-BKM 551 (MK_2-14), which caused no symptom in mice, produced interferon to a titer of only 1:80.

The interferon preparations of SP and dengue 2 (NG-C) showed no effect on MK_2 cells. Other physical properties of interferon of SP virus from mouse brain are being studied.

Studies of the production of interferon and virus titer in mouse brain at interval of time after high and low doses of SP-BKM 551 on successive passages are in progress. The studies on mouse lethality of SP-BKM 551 are one of the main interests of this investigation because of its apparent determinant of virulence.

Table 1. Results of Mouse Mortality from Inoculation of Pools of SP-BKM 551 and LP-BKM 551, LP-BKM 551 and SP-BKM 551 Viruses.

Dose of Pool of LP-BKM 551 and SP-BKM 551 (PFU)				Dose of LP-BKM 551 (PFU)			SP-BKM 551 (PFU)		
LP	SP	Mortality		LP	Mortality		SP		
3000	240000	8/8	8/8*	3000	8/8	3/8	240000	0/8	0/8
300	240000	8/8	8/8	300	8/8	8/8	240000	0/8	0/8
30	240000	7/8	8/8	30	6/8	6/8			
3	240000	2/8	2/8	3	6/8	3/8			
0	240000	1/8	0/8	0	2/8	2/8			

* Mortality ratio represents Deaths/Total inoculated

Table 2. Hemagglutination-inhibition by D1-4 Immune Ascetic Fluids of D-2 (NG-C), BKM 551, LP-BKM 551 and SP-BKM 551 antigens.

Prototype Immune Ascetic Fluid	Reciprocal HI Titer of Ascitic Fluid Against Indicated Antigen			
	D-2 (NG-C)	BKM 551	LP-BKM 551	SP-BKM 551
D-1	80	320	80	160
D-2	1280	1280	1280	2560
D-3	160	160	160	160
D-4	80	320	80	320

Table 3. Plaque Reduction Neutralization of Virus Recovered from 2 Monkey-passages of SP-BKM 551 at Fourth Mouse-passage.

Virus from Monkey Passage	Antisera			
	Dengue 1	Dengue 2	Dengue 3	Dengue 4
Passage 1	<10	140	<10	<10
Passage 2	10	640	<10	<10
Homologous Titer	640	640	80	400

Table 4. Antibody Response of Monkeys Inoculated with Mouse Passage 4 of SP-BKM 551 Virus.

Monkey Passage Level	Reciprocal 50% Plaque Reduction Against Indicated Virus			
	Dengue 1	Dengue 2	Dengue 3	Dengue 4
Passage 1	80	>640	10	60
Passage 2	30	550	>10	45
Homologous titer	640	640	80	400

Table 5. Mouse Pathogenicity of SP-BKM 551 After Two Passages in Monkeys.

Monkey Passage	Mouse Passage (Intracerebral)					
	First		Second		Third	
	Mortality	Virus Titer (PFU/0.3 gm.) of Mouse Brain	Mortality	Virus Titer (PFU/0.3 gm.) of Mouse Brain	Mortality	Virus Titer (PFU/0.3 gm.) of Mouse Brain
(800) Before monkey passage	0/8*	$4.0 \times 10^{2.7}$	0/8	$2.3 \times 10^{5.7}$	0/8	$5 \times 10^{5.7}$
(36) First passage	0/8 1/8	$1.1 \times 10^{5.3}$	8/8 8/8	$1.9 \times 10^{5.2}$	8/8 8/8	$4.6 \times 10^{7.3}$
(60) Second Passage	8/8 8/8	$1.3 \times 10^{5.3}$	8/8 8/8	$2.0 \times 10^{7.3}$	8/8 8/8	$2.0 \times 10^{7.3}$

* Mortality ratio represents deaths/total inoculated

( ) Indicates PFU dose of mouse intracerebral inoculation

Table 6. Challenge Virus Resistance in Mice 22 Days after IC Inoculation of BKM 551-SP (MK2-14).

Inoculum Dilution of (BKM 551-SP)	Estimated IC Dose (PFU/0.3 Ml)	Mortality		
		21 Days after BKM 551-SP Inoc.	11 Days after Challenge with >10,000 Mouse LD of	
			D-2 (NG-C)	Chikungunya
Undiluted	400,000	0/24*	0/8	0/8
-1	40,000	0/24	0/8	0/8
-2	4,000	0/24	0/8	0/8
-3	400	0/24	0/8	0/8
-4	40	0/24	0/8	0/8
-5	4	0/24	2/8	0/8

* Number dead/Number inoculated

Table 7. Antibody Response 21 Days After Inoculation of BKM 551-SP (MK2-14) in Newborn Mice.

Inoculum (Dilution of BKM 551-SP)	Estimated IC Dose (PFU/0.03 Ml)	Reciprocal 50% Plaque Reduction Titer Against Indicated Virus	
		D-2 (NG-C)	Chikungunya
Undil	400,000	40	<10
-1	40,000	20	<10
-2	4,000	<10	<10
-3	400	<10	<10
-4	40	<10	<10
-5	4	<10	<10

Table 8. Growth of BKM 551-SP (MK2-14) in CNS of Newborn Mice.

Inoculum Dilution of (BKM 551-SP)	Estimated IC Dose (PFU/0.03 Ml)	Virus Content (PFU/0.3 Ml)* of Brain Tissue on Indicated Day of Infection											
		Day 7				Day 14				Day 21			
		Undil.	-1	-2	-3	Undil.	-1	-2	-3	Undil.	-1	-2	-3
Undil.	400,000	TNTC	74	42	9	69	43	67	33	0	0	0	0
-1	40,000	70	36	65	6	50	36	53	18	0	0	0	0
-2	4,000	100	17	18	2	17	5	2	1	0	0	0	0
-3	400	88	38	13	3	67	52	72	29	25	22	8	5
-4	40	25	14	3	0	113	56	19	9	25	14	5	2
-5	4	28	15	3	0	120	33	31	5	TNTC	TNTC	60	16

* Expressed as Mean Count of Triplicate Bottle Cultures

Table 9. Correlation of Virus Titer, Challenge Virus Resistance and Interferon Titer in One-day Old Mice Inoculated Intracerebrally with SP-BKM 551 (MK_2-14).

Day of Inoculation	Virus Titer PFU/0.3 gm Brain	Day After Challenge with 10,000 Mouse LD_{50} of Chikungunya Virus	Interferon in e.o. ml of 10% Mouse Brain
1	0	8/8*	<10**
2	$10^{3.0}$	8/8	<10
3	$2.2\times10^{3.0}$	8/8	<10
4	$2\times10^{3.0}$	8/8	<10
5	$1.7\times10^{4.0}$	7/8	<10
6	$1.4\times10^{4.0}$	3/8	<10
7	$3.2\times10^{4.0}$	2/8	20
14	$1.0\times10^{6.7}$	0/8	80
21	$1.0\times10^{5.7}$	0/8	20
28	$0.6\times10^{1.0}$	0/8	0
35	0	0/8	0

* Mortality ratio = Deaths/Total inoculated

** Reciprocal 50% plaque reduction of interferon dilutions to CgLt 599

Table 10. Effect of Interferon Preparation from SP-BKM 551, LP-BKM 551 and Dengue 2 (NG-C) to L Cells and MK_2 Cells.

Viruses	Reciprocal 50% Plaque Reduction of Interferon Dilution	
	L Cells	MK_2 Cells
SP-BKM 551 (MK_2-14)	80	<10
LP-BKM 551 (MK_2-14)	2560	-
Dengue 2 (NG-C)	10240	<1:20

* Challenge virus after interferon P_X = CgLt 599

** Challenge virus after interferon P_X = Chikungunya (Ross)

Longitudinal Surveillance of Respiratory Viruses in Bangkok

Principal Investigator: Lloyd C. Olson, MAJ, MC

Assistant Investigators: Pranee Sithisarn, B.S.
Prapai Thong-Ngarm, B.S.

OBJECTIVE: To determine over an extended period of time which viruses are associated with respiratory illnesses in children in Bangkok.

DESCRIPTION: During the period January 1968-April 1969, 476 children with respiratory illnesses were studied for nasopharyngeal microorganisms. Paired sera were collected during acute and convalescent phase of illness and tested for a variety of antibodies to known respiratory viruses. A general summary of viruses isolated and significance of the high frequency of cytomegalovirus was reported in the Annual Report of April 1969.

PROGRESS: Virus isolation and identification and serological testing has been completed on all patients. Four hundred and three (403) of the 476 patients studied suffered from clinically uncomplicated febrile upper respiratory illnesses. Thirty-nine also showed exanthems (13 clinically consistent with rubella, 8 with rubeola and 18 "non-specific"), 19 had associated diarrhea, 8 bronchitis, 2 pneumonitis, 3 herpangina, and one, pertussis.

Viruses isolated and serological results are shown in Table 1. Not considering cytomegalovirus, a potential etiologic agent was identified in 38% of the cases. The most common infectious agents identified were the parainfluenza viruses (15.5%) followed by approximately 5% each with adenoviruses, respiratory syncytial and influenza A2 viruses.

By calculating the age - specific incidence of antibody, some appreciation of the transmissibility of various respiratory agents in the local population can be gained. Table 2 outlines such data for six agents. In general these data agree with that found in temperate zone countries.

Analysis of clinical data showed no correlation of peripheral white blood cell counts with nasopharyngeal flora (bacterial and/or viral), nor was there any correlation between recovery of a given virus and the presence of a certain species of bacteria.

SUMMARY: Parainfluenza viruses were found to be the respiratory viruses most commonly associated with febrile upper respiratory tract infection in Bangkok children, accounting for approximately 15% of infections. Adenoviruses, respiratory, syncytial and rhinoviruses each account for 5% of infections. Age-specific antibody incidence suggest most respiratory

viruses circulate through local populations in a manner similar to that found in temperate zone countries.

Table 1. Summary of virus isolation and serological results by month of study.

		Viruses Isolated								Additional Positives by Serology				
Month	No.	Cmv	Aden	Pflu	Rhino	Rub	Cox.B	Flu	Misc*	Ad	RS	HS	Pfl	Flu
Jan	27	6		2		4	2			Sera				
Feb	52	10	2			2	5			Not				
Mar	57	9	7	4					2	Available				
Apr	22	6	1	2					1					
May	29	3		6			1		2	2	2		2	
June	49	10	3	15					1	1	5		2	1
July	45	11	2	5	5		1			1	8	1	7	
Aug	23	1		1	1			1			9	1		7
Sep	35	3		1				8					1	9
Oct	14	1						7						
Nov	23	3		6				1						3
Dec	28	2		1					1	1		1		1
Jan	31			7	2		2			1			2	
Feb	25	1		2						1			1	
Mar	17	2		6									1	
	476	68	15	58	8	6	11	17	7	7	24	3	16	21

* Includes two herpes simplex, and one each polio 2, echo 18, RS, reovirus 1, and rubeola

Table does not include ten instances of dual infection, and 4 serologically proven measles. Adenoviruses isolated: type 1 (1); type 2 (3); type 3 (6); type 5 (4) and type 11 (1). Parainfluenza viruses: type 1 (22); type 2 (11); and type 3 (25).

Table 2. Age-specific incidence of antibody

Age	No.	% with antibody to: H.S.	R.S.	Adeno	Pflu-1	Pflu-2	Pflu-3
<1	59	4	17	31	34	3	29
1	78	23	21	49	37	10	23
2	55	20	42	61	49	31	49
3	38	26	47	57	41	34	60
4	35	40	58	70	63	46	74
5	17	52	39	64	65	30	82
6-10	43	84	30	61	75	35	73

The Etiology of Lower Respiratory Tract Infections in American Troops.

Coordinator: Thomas J. Smith, LTC, MC

Principal Investigators: Keith Arnold, MAJ, MC*
Lloyd Olson, MAJ, MC
Rapin Snitbhan, M.D.
Thomas Smith, LTC MC

Assistant Investigators: Aree Boriharnwannakett, B.S.
Laddawal Sookcharoen, B.Sc.
Phanor Srisongkram, B.Sc.
Sumitda Narupiti, B.Sc.

OBJECTIVE: To determine the etiology of non-bacterial pneumonitis occuring in American troops stationed in the Republic of Vietnam.

DESCRIPTION: During the middle period of 1969, a number of patients were seen at the 3rd Field Hospital, Saigon, with an unusual form of pneumonia. The syndrome was unusual in the following respects.

1. The patients frequently presented as a non-specific FUO with normal chest X-rays and several days elapsed before the clinical and X-ray features of pneumonitis made their appearance.
2. Many of the pneumonias were severe, associated with hypoxemia, pleuritic involvement and effusion with a prolonged (10-14 days) course. Complete recovery, however, was the rule, whether the patient was treated or not with antibiotics.
3. Sputum cultures in the majority of patients were negative.

As a result all patients admitted with, or developing pneumonitis not secondary to other conditions, were enrolled in a prospective study. Clinical radiographic and bacteriologic findings were the responsibility of personnel at the 3rd Field Hospital and 9th Med. Lab. Indirect immunofluorescence for scrub and murine typhus and melioidosis and cold agglutinin antibody titers were determined at the 9th Med. Lab. Sputum cultures for virus isolation and acute and convalescent sera were submitted to the Virus Dept., SMRL.

PROGRESS: Paired sera were received from 56 patients, and sputa from 50 relations are being analyzed by the WRAIR Research Team. Thirty seven sputa have been cultured for viruses, and only one was positive, an influenza A_2 Hong Kong variant being isolated. All sera have been tested for, and were negative to, CF antibody to respiratory syncytial and adenoviruses. One serum, in addition to the above, was positive for an increase in HI antibody to A_2/Hong Kong/68, and one to influenza B/Taiwan/62.

Twenty (20) sera pairs have been tested to parainfluenza types 1-3 and were negative.

Of unusual interest is that of the 56 sera pairs tested, 22 showed significant increases of CF antibody to mycoplasma pneumoniae. Since convalescent sera were collected 14 days after admission it might be expected that an even larger number suffered from mycoplasma pneumonia.

SUMMARY: A serological survey of patients admitted to one hospital in Saigon, South Vietnam, with non-bacterial pneumonia revealed 39% were probably due to mycoplasma pneumoniae.

---

* USA Med Rsch Team (WRAIR) Vietnam

Experimental infection of the Gibbon with influenza virus.

Coordinator: Thomas J. Smith, LTC, MC
Chief, Virus Dept.

Principal Investigators: Chalobon Karnjanaprakorn, M.S.
Dennis Johnsen, MAJ, VC
Lloyd Olson, MAJ, MC
Prayot Tanticharoenyos, D.V.M.
Rapin Snitbhan, M.D.
William Wooding, MAJ, VC

Assistant Investigator: Sumitda Narupiti, B.Sc.

OBJECTIVE: To determine the susceptibility of the gibbon to experimental infection with influenza A_2 viruses as a means of studying antigenic similarities and specificity of humoral and secretory antibodies formed in response to infection.

DESCRIPTION: In 1968 a major A_2 influenza virus variant appeared in the Far East. Although the agent was subsequently shown to be related, albeit distantly, to earlier A_2 strains, biologically it was distinct.

Thus, the host previously infected with earlier strains of A_2 virus, either naturally or via immunization, was not protected.

It is well known that immunity to infection with influenza virus does not correlate with the presence or absence of humoral antibody. It can thus be assumed that susceptibility probably depends on the presence of secretory antibody in the secretions of that organ constituting the portal of entry. This has been true of measles, parainfluenza polio-viruses.

The gibbon has been found to be strikingly similar to man insofar as his susceptibility and response to infection by a wide variety of human pathogens. Thus the response of the gibbon to intranasally and intravenously administered strains of A_2 influenza virus was studied.

PROGRESS: As a preliminary experiment, two gibbons were inoculated intranasally with A_2/Jap 305/57 virus. Neither possessed HI or neutralizing antibody to this agent at the time they were inoculated. Neither gibbon showed any signs of illness after inoculation, even though one gibbon shed virus for one week thereafter, and both developed significant levels of neutralizing antibody by three weeks post-inoculation. Since this suggested the virus was not unusually virulent in the host, twenty additional gibbons were inoculated according to the following scheme: (five gibbons per group)

Group A: A_2/Jap 305/57 intranasally
B: A_2/Jap 305/57 intravenously
C: A_2/Hong Kong/68 intranasally
D: A_2/Hong Kong/68 intravenously

None of these gibbons possessed serum neutralizing antibody to either agent prior to inoculation. Following inoculation animals were followed for 28 days. Every fourth day serum was collected from each animal, and tracheal washings collected. No animal showed any signs of clinical illness. Tables 3-6 show results of antibody titers to homologous virus in each animal. All sera were negative to heterologous A_2 virus when tested for neutralizing (hemadsorption-inhibition) antibody at a 1:10 dilution.

An attempt was also made to characterize classes of serum anti-influenza immunoglobulin present in serially collected sera and tracheal washing by use of indirect immunofluorescence using anti-IGA, IGG and IGM antisera. Considerable technical difficulty prevented accurate determination, including antiserum cross-reactivity between monkey renal cells and virus grown in this system and degree of dilution of tracheal washing specimens.

Two to three weeks after inoculation of this experimental group, signs of upper respiratory tract infection began appearing in other members of the gibbon colony. During the subsequent five weeks, approximately 30% of the 120 members of the colony were clinically affected. Nasopharyngeal swabs were collected from twelve animals, and A_2/ Hong Kong/68 influenza virus were recovered from 5.

During the epidemic four animals died. Two of these were in the experimentally inoculated group who died 30 days after inoculation. Autopsy findings were virtually identical to those seen in primary human influenza pneumonitis. The other two animals died during acute respiratory diseases. Autopsy findings in these animals were similar to that seen in humans with influenza complicated by superimposed bacterial infection.

A serological survey suggested that A2/H.K./68 was widely disseminated throughout the colony during this epizootic. Approximately 80% of animals, where adequate pre-and post epizootic sera pairs were available, showed evidence of infection. Table 7 illustrates antibody titers found in the colony before and after the epizootic and does not include the 22 animals experimentally infected with the virus.

Table 3. Antibody responses after intranasal inoculation of A_2/Jap 305/57

Gibbon No.	Hemagglutination-inhibiting antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	21/2mos.	4 mos.
P-14	<20	<20	<20	<20	<20	<20	<20	<20	<20
S-12	<20	<20	<20	20	<20	<20	<20	<20	<20
S-18	<20	<20	<20	40	40	40	40	<20	<20
S-25	<20	<20	<20	<20	<20	<20	<20	<20	<20
S-36	<20	<20	<20	<20	<20	<20	<20	<20	<20
Gibbon No.	Neutralizing antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	21/2mos.	4 mos.
P-14	<10	<10	<10	<10	<10	<10	<10	<10	<10
S-12	<10	16	100	100	80	80	80	<10	<10
S-18	<10	<10	50	160	200	200	200	32	32
S-25	<10	<10	16	25	50	50	100	<10	<10
S-36	<10	<10	50	63	32	32	32	16	<10

[a]Expressed as reciprocal of serum dilution.

Table 4. Antibody responses after intravenous inoculation of A_2/Jap 305/57

Gibbon No.	Hemagglutination-inhibiting antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	2 1/2 mos.	4 mos.
S-70	<20	<20	<20	<20	<20	<20	<20	<20	<20
VM-5	<20	<20	<20	<20	<20	<20	20	<20	<20
VM-6	<20	<20	<20	<20	<20	<20	<20	<20	<20
VM-8	<20	<20	40	40	40	40	40	<20	<20
VM-9	<20	<20	20	20	<20	<20	<20	<20	<20

Gibbon No.	Neutralizing antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	2 1/2 mos.	4 mos.
S-70	<10	<10	<10	<10	<10	10	16	<10	<10
VM-5	<10	16	16	16	16	16	32	63	63
VM-6	<10	<10	16	16	32	32	32	16	<10
VM-8	<10	16	63	100	126	126	126	63	63
VM-9	<10	16	16	16	16	16	16	33	<10

[a]Expressed as reciprocal of serum dilution.

Table 5. Antibody responses after intranasal inoculation of A_2/KT1/68

Gibbon No.	Hemagglutination-inhibiting antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	2 1/2 mos.	4 mos.
P-9	< 20	80	320	320	320	320	640	320	80
S-5	< 20	20	80	160	160	320	320	80	80
S-20	< 20	< 20	160	160	320	320	320	160	40
S-21	< 20	80	160	320	640	640	640	160	40
S-39	< 20	40	160	640	640	640	640	80	40
Gibbon No.	Neutralizing antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	2 1/2 mos.	4 mos.
P-9	< 10	10	250	250	400	640	1000	250	80
S-5	< 10	< 10	32	63	80	100	100	63	63
S-20	< 10	< 10	160	250	500	500	500	63	40
S-21	< 10	< 10	80	250	500	500	500	250	80
S-39	< 10	< 10	200	250	250	250	400	80	63

[a]Expressed as reciprocal of serum dilution.

Table 6. Antibody responses after intravenous inoculation of A_2/KT1/68

Gibbon No.	Hemagglutination-inhibiting antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	2 1/2 mos.	4 mos.
S-65	<20	<20	20	80	80	80	80	80	20
S-80	<20	<20	80	320	320	320	320	*	
S-87	<20	<20	160	320	320	320	640	**	
B-18-S	<20	<20	<20	160	320	640	640	320	320
B-16-S	<20	20	80	640	640	640	1280	640	160
Gibbon No.	Neutralizing antibody titre[a] at indicated time								
	day 0-4	day 8	day 12	day 16	day 20	day 24	day 28	2 1/2 mos.	4 mos.
S-65	<10	<10	<10	40	40	50	50	40	20
S-80	<10	<10	20	126	126	126	320	*	
S-87	<10	<10	50	80	126	160	160	**	
B-18-S	<10	<10	<10	32	80	200	200	640	640
B-16-S	<10	<10	50	400	500	500	795	640	160

[a]Expressed as reciprocal of serum dilution.

* Died day 29 post-inoculation.

** Died day 33 post inoculation.

Table 7. Gibbon Colony HAI antibody to A_2/Hong Kong/68 influenza virus

Serum Titre	Number of Gibbons	
	Pre-epizootic	Post-epizootic
<1:20	18	14
1:40	6	10
1:80	13	17
1:160	none	26
1:320	"	22
1:640	"	1
1:1280	"	2

Ecology of Arboviruses in Thailand

Principal Investigators: Joe T. Marshall, Ph.D.
Ananda Nisalak, M.D.
Thomas J. Smith, LTC, MC
Harold E. Stark, Ph.D.
James E. Williams, CPT, MSC

Associate Investigators: Anan Boonkanoke
Pranom Tuntrakool, B.Sc.
Somsak Imlarp
Raveevun Leelasatayakul, B.Sc.
Roypim Tiptanatoranin, B.Sc.

OBJECTIVE: To investigate factors in ecology, particularly with respect to vertebrate populations, influencing and maintaining the transmission of arboviruses in nature.

DESCRIPTION: As outlined in previous Annual Reports, especially 1968.

PROGRESS: A serological survey of wild vertebrates at Bang Phra found considerable neutralizing antibody to JE in rodents, including Rattus rattus (40%) R. rajah (=surifer, 25%), Menetes berdmorei (33%), and Bandicota indica (47%). Neutralizing antibody to Wesselsbron (WESS) virus was detected in R. rattus (18%), M. berdmorei (5%), and B. indica (30%). Those data were obtained using the metabolic inhibition test. Additional rodent sera have been tested now using tube neutralization and plaque tests in efforts to confirm the survey results and to detect antibody in animals collected at other places in Thailand.

Blood samples were taken by heart puncture and spun down minutes thereafter. Sera were kept at -20°C or colder temperatures until inactivation at 56°C in a waterbath for 1/2 hr. Sera then were diluted 1:5 for use in tube neutralization tests. After challenging with virus, serum-virus mixtures were incubated 1/2 hr at 37°C, and two culture tubes of BHK-21 cell monolayers received 0.1 cc of inoculum for each specimen. Tubes were maintained at 37°C while observing for cytopathic effect (CPE). If less than 50% CPE occurred in both tubes for a given specimen after seven days, antibody was considered to be present in the animal's sera. Equivocals resulted if only one of two tubes showed evidence of neutralization. Positive and equivocal sera were retested by plaque tests if sufficient sera was available. The MK-2 plaque reduction test was used according to the procedure described previously.

No evidence for neutralizing antibody to JE was found in rodent sera tested by tube neutralization (Table 1). In most cases, the amount of virus used to challenge sera was 80-100 $TCID_{50}$. However, the virus dose used to test the 56 sera of Rattus rattus collected at Bang Phra was excessive, and 53 of the samples were retested by plaque test with sera diluted 1:10. Plaque reduction in 29 sera was within the error expected of controls and thus was not significant. Seven specimens showed plaque reduction which was significantly different from controls at $p<.01$. The percent plaque reduction was 72% in one and 74% in another. Reduction of plaques may have been due to specific neutralizing antibody but non-specific plaque inhibition could have been present at the 1:10 dilution of sera used in the test. In any case, JE antibody does not appear to be as prevalent as the Bang Phra survey indicated. Rodents have not been implicated as hosts of JE in Japan, Malaysia or India, and it is doubtful that they function as natural hosts in Thailand.

Indications of WESS antibody were found in Rattus rattus collected at Bang Phra and in the area of the Sakaerat research station in Nakorn Ratchasima Province. Also, a positive result was obtained for one R. surifer collected at Sakaerat on 18 July 1969. The positive and equivocal R. rattus from Sakaerat were distributed throughout the period June-December 1969. The percentage of Rattus rattus positives at Bang Phra was 18% which was the percentage found in the previous survey. Positives and equivocals in the Bang Phra series were retested by plaque test (Table 2). Plaque reduction was significant in most cases at the 1:5 dilution of sera. At 1:10 dilution of sera, however, most specimens were negative. Four were significantly different from controls at $p<.01$ and a fifth was different at $p<.001$. These specimens and the several solid positives in tube neutralization tests indicate that some neutralizing antibody to WESS virus may be present in rodents. Again, however, the involvement is not so great as the previous survey indicated. That rodents play a significant role in the ecology of WESS remains to be shown.

S-19-B Virus and Antibody Prevalence in the Wrinkle-lipped Bat (Tardarida plicata)

Shortly after the isolation of an unknown virus from dead bats collected in Saraburi Province (see elsewhere in this report), attempts were made: 1) to identify the virus and 2) to determine if antibody was present in living bats.

S-19-B was found to be ether sensitive and is probably an arbovirus. Therefore S-19-B was tested against specific sera for several arboviruses

known to occur in Thailand using the MK-2 plaque reduction technique (Table 3). No relationship to Chikungunya (CHIK), Sindbis (SIND), JE, Newcastle Disease (N.D.V.), Batai or Wesselsbron (WESS) was found. S-19-B formed big, clear plaques after three days of incubation at 37°C which were quite different from the small and hazy plaques produced by Dengue and Tembusu viruses given similar incubation. S-19-B plaques were intermediate in size between those of Chikungunya and Sindbis viruses. In terms of plaque morphology, S-19-B virus is related to group A. CF antigen has been prepared from guinea pigs with CF titers of 1:512 for use in further attempts to characterize S-19-B.

Tardarida plicata were mist-netted at evening exodus at a small cave on Khao Lam Phat, Kangkoi District, Saraburi Province. This cave is several hundred yards south and approximately 50 yards lower on the mountain than the large cave where S-19-B was originally isolated. The small cave may be a nursery cave. At the time of collection, several hundred-thousand T. plicata were resident there, whereas previous and later visits showed that T. plicata was not abundant. A second series of T. plicata was obtained from a large cave deep in the forest on Khao Phlong, near Amphur Phra Buddhabat, Saraburi Province. Blood was taken from the heart and diluted to give 1:3 sera dilution prior to centrifugation, which was accomplished soon after bleeding. In the laboratory, sera were incubated at 56°C for 1/2 hr and diluted to 1:5 for use in BHK-21 tube neutralization tests, which were performed as described in the previous section. Sera (1:5) were challenged with 10-25 $TCID_{50}$ of S-19-B virus prepared in BHK-21 cell culture. Positives and equivocals were retested, where possible, in a second tube neutralization test.

Sera of male and female bats neutralized 25 $TCID_{50}$ of S-19-B virus (Table 4). A greater proportion of females were positive than males in the Khao Lam Phat population, whereas the reverse was true of the Khao Phlong population. Thus, both sexes are susceptible and probably are infected at similar frequencies in nature. Approximately 30% of each population evidenced prior contact with S-19-B. If equivocals are included as showing evidence of antibody, rates increase to 35%. Thus, contact with S-19-B is common. Probably all T. plicata populations in the Saraburi region are involved with this virus. Further studies of antibody prevalence using the MK-2 plaque test are in progress. It remains to be shown whether or not S-19-B virus occurs elsewhere in Thailand or in other countries where T. plicata is found.

Table 1. BHK-21 tube neutralization tests of rodent sera

Location and Species	Period when animals collected	Virus dose in tests ($TCID_{50}$)		Number positive over number tested		Number equivocal over number tested	
		JE	WESS	JE	WESS	JE	WESS
Bang Phra, Cholburi Province: Rattus rattus	Nov. 1969	630	65	0/56	10/56	0/56	11/56
Siracha District, exclusive of Bang Phra Cholburi Province: Rattus rattus Rattus (Lenothrix) surifer Menetes berdmorei	Nov. 1969	80 and 100	100	 0/17 0/3 0/7	 0/6 - -	 0/17 0.3 0/7	 0/6 - -
Bangkok Rattus rattus Bandicota indica	Dec. 1969	100	25	 0/11 0/6	 0/2 0/1	 0/11 0/6	 0/2 0/1
Khao Nam Tok, Kangkoi District, Saraburi Province: Rattus rattus	Dec. 1969	100	100	 0/4	 0/1	 0/4	 0/1
Pak Thong Chai District, Nakorn Ratchasima Province: Rattus rattus Rattus (Lenothrix) surifer Rattus (Stenomys) sabanus Rattus (Maxomys) niviventer Menetes berdmorei Bandicota indica	June-Dec 1969	80 and 100	100	 0/46 0/29 0/13 0/7 0/5 0/5	 1/20 1/14 0/5 0/2 0/2 0/4	 0/46 0/29 0/13 0/7 0/5 0/5	 5/20 0/14 0/5 0/2 0/2 0/4

Table 2. Comparison of BHK-21 tube neutralization test and MK-2 plaque test results.

Tube Neutralization Test Result (Sera 1:5)	*R. rattus* Serum No.	% Plaque Reduction (Sera 1:5)	% Plaque Reduction (Sera 1:10)
Equivocal WESS	3464	80	59*
	3467	85	1
	3469	73	0
	3473	88	32
	3479	27	-
	3488	59	0
	3503	88	18
	3508	71	1
	3512	34	-
	3514	68	64*
Positive WESS	3471	56	100**
	3472	88	64*
	3476	73	0
	3483	76	0
	3491	83	0
	3501	80	15
	3505	83	49*

* = .01>p>.001 ** = .001>p

Table 3. S-19-B Virus versus specific sera in MK-2 plaque test.

Serum	Virus					
	S-19-B	Chik	Sind	JE	Betai	Wess
S-19-B (rabbit)	1.000*	<40	<10	<10	<10	<10
Chik (rabbit)	<10	640	≤10	<10	-	-
Sind (mouse)	<10	<10	10,240	-	-	-
JE (rabbit)	<10	<10	<10	640	<10	<10
N.D.V. (commercial)	<10	-	-	-	-	-
Batai (mouse)	<10	-	-	<10	>640	<10
Wess (mouse)	<10	-	-	<10	<10	10,240

* Reciprocal of serum dilution giving 50% plaque reduction.

Table 4. S-19-B antibody prevalence in two populations of T. plicata at Saraburi Province.

Location-Date-Sex	Number Tested	A % Positive	B %Equivocal	A.+B.
Khao Lom Phat (8 Dec 69)				
Males	34	26	6	
Females	26	35	3	
Both	60	30	5	35
Khao Phlong (10 Dec 69)				
Males	41	39	2	
Females	41	15	14	
Both	82	27	8	35

Vertebrate Ecology

Principal Investigator: Joe T. Marshall, Jr.

Associate Investigator: Amara Markvong, B.S.*

Assistant Investigator: Vandee Nongngork

OBJECTIVE: To provide correct identification and meaningful names for wild vertebrate animals from which blood samples are taken for studies on the ecology of Japanese encephalitis virus.

DESCRIPTION: Identifications usually present no problems and the ecologic studies can proceed without hindrance from lack of a name. Amphibians, reptiles, and most of the birds and mammals are keyed out with the aid of the excellent reference library maintained by SMRL. However, in the recent study of the ecology of Japanese encephalitis virus at Bang Phra, difficulties were encountered with leaf-warblers, bats, and the common rats and mice. Some of the warblers and all of the species of bats and rats and mice had significant amounts of antibody to the virus. Therefore, it was evident that straightening out their names would be of value not only to the Bang Phra study, but to all future studies of arboviruses in Thailand. (Specifically, the same animals occur at Chiengmai, the site of a current study).

Accordingly, an illustrated identification aid to the leaf-warblers was devised and published. Scientific specimens of the bats were prepared and sent to specialists who provided names in due course. But the nomenclature and species limits of the common rats and mice at Bang Phra were found to be confused in the current literature. It was necessary to undertake a systematic revision of them, and the Virology Department thus inherited a project that had earlier been set up in optimistic terms as a SMRL goal by the Department of Entomology (SMRL Annual Report, April 1964: 308-310). The method was collecting and examining scientific skins and skulls and extracting information on karyotype, ectoparasitic lice, and biochemical genetics (=enzyme polymorphism involving isozymes) from collaborators who were provided pertinent material by this laboratory.

PROGRESS: Delineation of the species of medically important rats and mice of Thailand has been complete, on the basis of skin and skull morphology. They have also been grouped into genera and subgenera consisting of closely related forms. Data from ecology, geographic overlap, biochemical genetics, ectoparasitic lice, chromosome number and chromosome morphology substantiate the new classification proposed. This

information has been widely disseminated in mimeograph form and two monographs are ready for publication, one on the rats, another on the mice. Table 1 summarizes the main points; the linear sequence reflects similarities in karyotype.

SUMMARY: Proper identification of animals and birds for studies of Japanese encephalitis ecology necessitated overhaul of the classification of medically important rats and mice of Thailand, using both classical and modern cytologic and biochemical methods. There are 33 species in Thailand. All but one, the Norway rat, are native to the country. No less than eleven species are specially adapted for living with man. The completion of this study now permits immediate field identification of reservoir hosts of human diseases in Thailand.

---

* Dept. of Biology, Kasetsart University.

Table 1. Species of Rats and Mice of Thailand

Groups of related species based on skull	scientific name	Chromosome number[1]	habitat	Distribution in Thailand	Mammary formula	Lice[7] Species of *Hoplopleura*	Species of *Polyplax*
Bandicota	Bandicota indica	46	rice field, marshy grass	all	3 + 3	malabarica	asiatica
	Bandicota bengalensis	44	grassland	central	3 + 3	malabarica	asiatica
"Berylmys"	Rattus bowersi	40	forest	all	2 + 2	diaphora	
	Rattus mackenziei		forest	North	3 + 2?		
	Rattus berdmorei	40	swampy forest and grassland	all except Central and Peninsula	3 + 2	kitti[3]	
"Stenomys"	Rattus mulleri	42	evergreen forest	Peninsula	2 + 2	dissicula	
"Rattus"	Rattus germaini	42	evergreen forest	Koh Samui	3 + 3		
	Rattus rattus	42[5]	all, incl. house	all	2 + 3[6]	pacifica	spinulosa
	Rattus sladeni	42	mountain evergreen forest	North	3 + 3		
	Rattus nitidus	42	mountain villages, in house	North	3 + 3		

Table 1. (Continued)

Groups of related species based on skull	scientific name	Chromosome Number[1]	Habitat	Distribution in Thailand	Mammary formula	Lice[7] Species of Hoplopleura	Species of Polyplax
"Rattus"	Rattus exiguus	42	rice field, truck garden	North, NE, SE	2 + 3		
	Rattus exulans	42	house	all	2 + 2	pacifica	spinulosa
	Rattus argentiventer	42	rice field	Peninsular, and Central	3 + 3	pacifica	
	Rattus norvegicus	42	buildings	Local[4]	3 + 3	pacifica	spinulosa
"Leopoldamys"	Rattus edwardsi	42	evergreen forest	Northeast	2 + 2		insulsa
	Rattus sabanus	42	evergreen forest	all	2 + 2	malaysiana	insula
"Lenothrix"	Rattus surifer	52	forest	all	2 + 2	(2)	
	Rattus rajah	36	evergreen forest	Peninsula	2 + 2		
	Rattus whiteheadi	36	evergreen forest	Peninsula	2 + 2		
"Maxomys"	Rattus niviventer	46	forest	all	2 + 2	(2)	pricei
	Rattus fulvescens	46	mountain forest	Peninsula and North	2 + 2		

Table 1. (Continued)

Groups of related species based on skull	scientific name	Chromosome Number[1]	Habitat	Distribution in Thailand	Mammary formula	Lice[7] Species of Hoplopleura	Species of Polyplax
"Maxomys"	Rattus cremoriventer	48	evergreen forest	Peninsula and East	2 + 2	sicata	
"Leggadilla" and "Coeomys"	Mus shortridgei	48	grass in deciduous forest	West, Northeast	3 + 2	new species	
	Mus pahari		mountain forest?	North	3 + 2		
"Mus"	Mus famulus	40	grass in pine forest	North	3 + 2		
	Mus nitidulus?		grass in deciduous	Northeast	3 + 2		
	Mus cervicolor	40	grass in deciduous forest	all except Peninsula	3 + 2	johnsonae	
	Mus caroli	40	rice field	all except Peninsula	3 + 2	johnsonae	
	Mus musculus castaneus	40	warehouse	Thonburi, Trang	3 + 2	captiosa	serrata

Table 1. (Continued)

Groups of related species based on skull scientific name	Chromosome Number[1]	Habitat	Distribution in Thailand	Mammary formula	Lice[7] Species of Hoplopleura	Lice[7] Species of Polyplax
Chiromyscus chiropus		forest (arboreal)	Northeast	2 + 2		
Vandeleuria oleracea 28	28	cane	North and Northeast	2 + 2?		
Chiropodomys gliroides		bamboo in forest	all	0 + 2		
Hapalomys longicaudatu		bamboo in forest	West and Peninsula	2 + 2		

Footnotes:

(1) Diploid number - data of Amara, Yong Hoi Sen, Selander, Duncan.
(2) Pectinata has been found.
(3) About the same as diaphora.
(4) In cities of Prachinburi, Bangkok, Bang Phra, Trad, Chumporn, and village on Samui island.
(5) Polymorphic 42-48 at Suriporn Hotel, Chiengmai, data, of Gropp.
(6) Post-axial pair twinned in North.
(7) Data of Emerson.

Ecological and Epidemiological Survey for Rabiesvirus in Cave Bat Populations.

Principal Investigators: William A. Neill, SP5 E-5
Dennis O. Johnsen, MAJ, VC
Robert L. Hickman, MAJ, VC
Paul C. Smith, MAJ, VC

Assistant Investigators: Kwanyuen Lawhaswasdi, D.V.M.
William L. Wooding, MAJ, VC
George S. Manning, CPT, MSC
Howard E. Noyes, Ph.D.
Mrs. Naowayubol Nutkumhang, B.Sc.

OBJECTIVE: The objectives of the study are to determine, whether rabies is present among cave bats, whether diseases and ecological factors may be responsible for the many deaths in bats that occur frequently, and the relationship of bat zoonoses to individuals that work in a large limestone cave in the Saraburi province district of Kangkoi.

DESCRIPTION: Dead bats are picked up from several well defined areas in the cave each month by a native who collects guano in the cave. A careful count is kept of the bats collected from each area, and they are then kept in dry ice for laboratory examination. Continuous temperature recordings are also made in each of these areas and as an indicator of the population fluctuations the rate of guano deposition is measured. The overall population of bats within the cave has been estimated by taking a series of rapid photographs of the evening exodus from the cave. A record of meteorological data in the vicinity of the cave has also been kept to determine its effect on the other observations. In addition to dead bats, live bats, wild rodents, various ectoparasites and human sera have been collected from the area for laboratory examination. Laboratory rats, mice, and rabbits were also placed in the cave as sentinel animals to determine the effect of the zoonotic diseases there on newly introduced, and perhaps susceptible, animals.

PROGRESS: The brains and salivary glands of the dead bats were inoculated intracranially into weanling mice for the purpose of screening for rabies virus. Many of these mice subsequently died showing signs of paralysis or convulsions. Examination of their brains showed no evidence of infection by rabies virus or bacterial contamination.

Following a second passage in suckling mice the isolates were placed in tissue culture using MK_2 cells where cytopathic effect was produced. One isolate, S-19-B, was characterized by several laboratory procedures, and has been used subsequently as an antigen for producing antisera in rabbits. Following this experience, all suspensions made from dead bat tissue which caused death in the first mouse passage were screened for rabies virus, passed a second time in mice and, if deaths occurred, placed on tissue culture. A total of 1014 bats were processed yielding 2028 tissue suspensions. From these suspensions 648 have been lethal for weanling mice. Following fluorescent antibody examination for rabies, 190 of the mouse isolates have been placed in tissue culture and 173 have produced cytopathic effects. As mentioned, antisera was prepared by inoculating rabbits with the viral isolate, S-19-B. Serum neutralization tests are currently being set up to determine if all the isolates made are serologically identical to S-19-B.

Material obtained from live bats, wild rodents, ectoparasites, and sentinel animals has been processed in a similar manner. One hundred and twenty three live bats were processed by passing brain and salivary gland material through both weanling and suckling mice. Four isolations have been made from mouse material but have not produced CPE when placed on tissue culture. Sixty-five wild rats, Rattus rattus, were collected and five isolates producing CPE on MK_2 cells have been obtained. Suspensions made from pools of collected ectoparasites, although they produced a few deaths in inoculated mice, have failed to produce any CPE in MK_2 cells. Five tissue culture isolates have been obtained from the various sentinel animals and of interest also is the fact that the serum of one of these rats had a 1:80 antibody titer in a neutralization test against the S-19-B isolate. Similarly, in neutralization tests run with the sera collected from fourteen people who worked in the cave, three have been shown to contain antibodies to the S-19-B isolate also.

Surveys of Domestic and Sylvatic Animals for
Rabies Virus Infections.

Principal Investigators: Robert L. Hickman, MAJ, VC
Kwanyuen Lawhaswasdi, D.V.M.
William A. Neill, SP5 E-5

Associate Investigators: Dennis O. Johnsen, Maj, VC
James P. Slowey, SFC E-7

OBJECTIVE: The purpose of this study is to define sylvatic and domestic reservoirs of rabies virus infections in Thailand.

DESCRIPTION: The Fluorescent Rabies Antibody test is used to examine all specimens and is followed up by mouse inoculation tests when necessary. Surveillance of three groups of mammals (bats, rodents, and dogs) is being maintained. The bat studies are confined to a single population of two species from a cave in Saraburi province (More fully described under Ecological and Epidemiological Survey for Rabies virus in Cave Bat Populations). The rodent studies are conducted on animals collected from several provinces in Thailand by the Department of Entomology. Dog specimens are collected and submitted by the Bangkok Department of Public Health and individual Air Force and Army Veterinarians assigned throughout Thailand. Because of the relatively few dog specimens submitted, the absence of pertinent data on individual specimen and the lack of any control over selection of specimens and feed back of information, most of these programs have been terminated as formal surveys. In their stead, two preliminary studies were initiated in an effort to generate more meaningful data. The first of these is a survey of dogs to be conducted by the Bangkok Department of Public Health in conjunction with the Department of Veterinary Medicine. The second is a survey of dogs to be conducted jointly by the Departments of Epidemiology and Veterinary Medicine.

PROGRESS: A total of 267 bats were examined without finding evidence of rabies virus. No rabies infections were diagnosed after examination of 210 rodents of a variety of species. Two of 20 "randomly selected" dogs from the Bangkok Municipal pound were found to be positive. Insufficient information is available to adequately identify all of the remaining dogs that were submitted as survey specimens so the results of these examinations are included in the routine diagnostic report (Department of Veterinary Medicine Support Activities). Progress to

date on the recently initiated dog surveys has been limited to obtaining clearance for undertaking the studies, selecting the geographical areas to be studied, and defining and collecting pre-survey data on the dog populations therein.

SUMMARY: Limited surveys of bats and rodents failed to substantiate previous findings of rabies virus intections in these two group of animals. Dogs continue to be the principal known reservoir of rabies virus in Thailand, and studies have been initiated to learn more about the epidemiology of rabies virus in this host.

Indirect Fluorescent Rabies Virus Antibody Test

Principal Investigators: Robert L. Hickman, MAJ, VC
Ronald G. Wilson, M.D.*
Howard B. Emory, M.D.*
Kwanyuen Lawhaswasdi, D.V.M.

Associate Investigators: William A. Neill, SP5, E-5

OBJECTIVE: The indirect fluorescent rabies virus antibody test is a valuable tool for rapidly determining the antibody response to immunization with rabies vaccine. In the past, several unsuccessful attempts have been made to correlate the titers of antibody as detected by this test and by the standard mouse neutralization test using individual sera from patients receiving post-exposure antirabies treatment. The objective of the current study is to repeat previous effort and expand on them by studying comparative titer responses in Peace Corps volunteers, all of whom receive pre-exposure antirabies immunizations. In addition to the comparative titer aspects of the study, this is an unparalleled opportunity to study the response to post-exposure treatment following pre-exposure antirabies immunization since 15-25% of the volunteers require such treatment during their tour in Thailand.

DESCRIPTION: The standard IFRA test is used to examine all human sera. Single serum samples from patients receiving post-exposure antirabies immunizations are coded and titrated by both the IFRA technique and the mouse neutralization test for rabies virus antibody. In the recently initiated study, serum is collected from each newly arrived Peace Corps volunteer, coded and tested for an IFRA titer. Additional sera are collected and tested throughout the pre-exposure course of immunizations. If and when a volunteer is exposed to rabies virus, a second series of sera specimens is collected for IFRA titer determination. Mouse neutralization tests are performed for comparative purposes using each of the sera specimens from randomly selected volunteers.

* Peace Corps Physician, c/o American Embassy, Bangkok, Thailand.

PROGRESS: During the reporting period a total of 324 sera were submitted and examined for antirabies titers by the IFRA technique. Of these, 54 were selected for IFRA - Mouse Neutralization comparisons. A quantitative relationship could not be demonstrated between the titers determined by the two methods. These specimens were, for the most part, collected from different individuals at different intervals following immunization. The current study provides sequential sera at prescribed intervals and permits a comparison of antibody appearance and development as well as quantity. The first group of 95 volunteers arrived in Thailand during the last month of the reporting period.

SUMMARY: Although the IFRA test continues to be a valuable tool for evaluating antibody response in man to rabies vaccination, efforts to compare results of this test with the results of the more widely accepted mouse neutralization test have failed. A new study was initiated using sequential sera specimens from Peace Corps volunteers in order to compare the time of appearance, the course of development, and the titer of the antibody detected by both the IFRA test and the mouse inoculation test. In addition, the antibody response to post-exposure treatment is being studied.

Feeding Habits and Preferences of Adult Mosquitoes-*Aedes albopictus*.

Principal Investigators : Michael F. Sullivan, CPT, MSC
Douglas J. Gould, Ph.D.

Assistant Investigator : Somboon Maneechai

OBJECTIVE : Both *Aedes aegypti* and *Aedes albopictus* are vectors of dengue viruses in Thailand, but much epidemiologic data suggests that the former species is the vector of primary importance. Previous studies on Koh Samui indicated that perhaps one explanation for this is that *A. aegypti* feeds primarily upon humans while *A. albopictus* is frequently diverted to a variety of other blood meal sources in addition to man. The object of this study was to determine which hosts are preferred by *A. albopictus*.

DESCRIPTION : Four series of simultaneous biting collections were made from humans and from four species of animals common to Thailand. In these studies one man (X) collected mosquitoes attracted to himself, while a second collected mosquitoes attracted to a buffalo (A), a pig (B), a dog (C), a chicken (D) or a man (E). Individuals in each of the above categories were designated numerically (e.g., first buffalo as A_1). A single trial consisted of a ten minute initial collection period in which X and one of the animals were placed in locations 15 to 30 meters apart; this was followed by a second ten minute period during which the positions of the two hosts were reversed. In the first series of trials X_1 was compared with A_1, A_2, B_1, B_2, C_1, C_2, D_1, D_2, E_1 and E_2, in the second series a second man (X_2) was compared with the same hosts as X_1, and in the third series X_3 was compared with A_3, A_4, B_3, B_4, C_3, C_4, D_3, D_4, E_3 and E_4. In the fourth series X_4 was compared with the same hosts as was X_3. The same person collected the mosquitoes attracted to hosts A through E in all of the trials. After each test the immediate collection site was not used again for a period of three hours. All collections were made in coconut plantations on the island of Koh Samui between 0700 and 1300 hours.

PROGRESS : The results of this study indicated that the host species used in the trials differ in attractiveness to A. albopictus. When the ratios of mosquito numbers collected from man and the other hosts were compared, the host-attractiveness was ranked as follows : 1) man, 2) pig, 3) and 4) buffalo and dog, and 5) chicken (Table 1). Total numbers of mosquitoes collected from the five host species were large enough to indicate all five are probably important natural hosts of A. albopictus.

Table 1. Results of host preference tests with Aedes albopictus on Koh Samui, 1969.

Hosts A-E	Series 1				2				3				4				Sum of Rank
	No.Mosq coll'd from		Ratio A-E/ X_1	Rank	No.Mosq coll'd from		Ratio A-E/ X_2	Rank	No.Mosq coll'd from		Ratio A-E/ X_3	Rank	No.Mosq coll'd from		Ratio A-E/ X_4	Rank	
	Hosts A-E	Man X_1			Hosts A-E	Man X_2			Hosts A-E	Man X_3			Hosts A-E	Man X_4			
Man(E)	50	33	1.52	1	49	45	1.09	1	23	13	1.77	1	9	9	1.00	1	4
Pig(B)	60	100	.60	2	61	62	.98	2	9	23	.39	2	8	28	.29	3	9
Dog(C)	16	38	.42	3	21	56	.38	3	5	23	.22	3	2	14	.14	5	14
Buffalo (A)	25	65	.38	4	24	68	.35	4	4	21	.19	4	10	14	.71	2	14
Chicken (D)	15	47	.31	5	5	45	.11	5	10	97	.10	5	8	32	.25	4	19

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 044, Virus diseases of man and animals

Literature Cited.

References:

1. Olson, L.C , Keutsinha, R., Mansuwan, P. and Snitbhan, R., Respiratory excretion of cytomegalovirus in Thai children. Jour. Ped. (In press).

2. Marshall, J. and Vandee Nongngork. Mammals of Samui Island, Thailand. Nat. Hist. Bull. Siam Society. (In press).

3. Marshall, J.T., Jr. Revision of the genus Mus (Rodentia, Muridae) in Southeast Asia. American Museum Novitates. (In press).

Publications:

1. Marshall, J.T., Jr. and Somsak Pantuwatana. Identification of leaf warblers in Thailand. Nat. Hist. Bull. Siam Society, 1969:23, pp. 1-8.

2. Somsak Pantuwatana, Somchai Imlarp and Marshall, J.T., Jr. Vertebrate ecology of Bang Phra. Nat. Hist. Bull. Siam. Society, 1969:23, pp. 133-183.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6466	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	045
b. CONTRIBUTING				
[redacted]	CDOG 1412A	2)		

11. TITLE (Precede with Security Classification Code)

(U) Bacterial and Mycotic Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 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

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 70	4	236
FISCAL YEAR — CURRENT 71	4	236

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Altstatt, LTC L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Noyes, H. E. Ph.D.

NAME: Johnsen, MAJ D. O. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Bacterial Diseases; (U) Mycotic Diseases; (U) Southeast Asia; (U) Drug Resistance; (U) Diarrhea; (U) Pneumonia; (U) Meningitis; (U) Septicemia

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To identify bacterial and mycotic diseases of military importance in Southeast Asia and to provide information to aid in the diagnosis, treatment, and control of the diseases.

24. (U) Disease occurrence is certified by clinical and laboratory methods. Where relevant, long term surveillance of a population for occurrence of particular bacterial or mycotic diseases is instituted. Variables affecting transmission and virulence are studied in vivo and in vitro.

25. (U) 69 07 - 70 06 Meningitis due to meningococcal disease was found to be rare in a study of meningitis in Thai children. Preliminary data indicate a poor correlation between the oral and pulmonary flora in lower respiratory tract disease in Thai children. A potential pathogen intermediate in type between pathogenic E. coli and shigella has been isolated from patients with diarrhea in Vietnam. The hypothesis of water borne infection by Pseudomonas pseudomallei was not substantiated by field observations. For technical reports see SEATO Annual Progress Report, 1 Apr 69 - 31 Mar 70.

*Available to contractors upon originator's approval

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

BLANK PAGE

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 045, Bacterial and mycotic diseases of man and animals

Investigators.
Principal: Howard E. Noyes, Ph.D.
Associate: Yachai Ampaipast; John C. Bell, SSG; Panyasri Benjadol, M.S.; Paibul Busapathamrong, MAJ, MC, RTA; Santiparb Chaiwongkiat; Yupin Charoenvit, M.S.; Henry H. Higaki, MSG; Yupa Kasemsanta, P.H.N.; Supanee Kethsuwan, M.D.; Ovath Khunphol, R.N.; Kanchana Leelasiri, B.S.; Pethai Mansuvan, M.D.; Lakshana Nagarajara, M.D.; Tatsanee Occeno, B.S.; Lloyd C. Olson, MAJ, MC; Pavanee Prayon Prayongratana, R.N.; Yongyuth Raengpradub, B.S.; Swish Rardjamroensook, M.D., M.P.H.; Chantana Ratanavaraha, B.S.; Prani Ratarasarn; Pravit Songsaengterm, M.D.; Chanphen Srimunta, B.S.; Ronald K. Stackhouse, SP5; Curtis A. Stewart, SP6; Veeravat Supradish, M.D.; Tasna Tamaarree, Dip. Med. Tech.; Prayoth Tanticharoenyos, D.V.M.; Pairoj Tanvichien, M.D.; Pitsawas Thongphakdi, M.D.; Markpol Tingpalapong, D.V.M.; Susri Tondhavudho, M.D.; Prapin Tugkanon, M.D.; Malinee Udhitanonda, B.S.; Pisit Uttamote, M.D.; Suthee Vallikul, M.D.; Ravi Vanichsarn, M.D.; Saridwongsa Wongsathuaythong, LTC, MC, RTA; William L. Wooding, MAJ, VC; Sunee Yuvachitti, R.N.

Mycotic Diseases

Coordinator: Howard E. Noyes, Ph.D., Chief, Department of Bacteriology & Mycology

Principal Investigators: Renoo Kotrajaras, M.D.*
Howard E. Noyes, Ph.D.

Associate Investigators: Yupin Charoenvit, M.S.
Malinee Udhitanonda, B.S.

OBJECTIVE: The objective of these studies is to gather information on the prevalence and distribution of mycoses in this area of the world. In addition to these survey activities, one study was carried out to determine the in vitro effects of griseofulvin on morphological changes of selected dermatophytes isolated in Thailand and another was done to identify the bacterial components of dermatologic infections.

DESCRIPTION: Survey studies were prompted by the major medical problems

*Dermatologist at Women's Hospital, Bangkok, Thailand

the dermatophytic fungi can present to the military in times of stress. Specimens were usually collected by a member of this department from patients with dermatologic problems. Cultures were prepared by cleaning the lesion with 70 percent ethanol and transferring material (hair, skin, nail) directly to 2 plates of Sabouraud - Cycloheximide - Chloramphenicol medium. The plates were sealed with paper tape to prevent contamination, and periodically examined during a 21 day incubation at 25°C. Blood agar plates were also inoculated and incubated at 37°C when clinical appearance of the lesion suggested bacterial infection.

Thirteen recent isolates of dermatophytes were exposed to concentrations of griseofulvin ranging from 0.1 to 30 mcg/ml in Sabouraud - Cycloheximide - Chloramphenicol agar or broth. Colonial and microscopic changes were observed at 5, 10 and 15 days after inoculation and incubation at 25°C.

In the study of the bacterial component of dermatologic infections, culture sites for bacteria and fungi included the site of the lesion, canal of the right ear, right nostril, beneath the index fingernail of the right hand, perineum and the fourth toeweb of the right foot. Procedures listed above were used for fungus cultures and a battery of culture media designed to favor different genera were used for bacterial cultures.

PROGRESS: During the period covered by this report 755 routine clinical specimens were received for mycological examinations. Included were 212 from Women's Hospital, and 36 from the U.S. Embassy Medical Unit and the 5th Field Hospital. Results in Table 1 show that the organisms most frequently isolated from Thai patients were Trichophyton rubrum and Candida albicans. Five of 36 dermatologic specimens from U.S. personnel and dependents were positive for C. albicans; two were positive for T. rubrum and 1 was positive for Trichophyton mentagrophytes (Table 2). In specimens from other than human sources there were 5 isolates of Microsporum canis from 6 gibbons and 5 isolates of Microsporum gypseum from 246 soil samples (Table 3).

The study of the intraction (s) of dermatophytes and griseofulvin was undertaken because of concern to clinicians about the likelihood that griseofulvin - resistant strains of dermatophytes would emerge and the possibility that resistant forms would have different characteristics which would complicate their identification. This study was concerned with sequential macroscopic and microscopic changes of recently isola ted dermatophytes in the presence of increasing concentrations of griseofulvin.

The presence of griseofulvin in media in which the dermatophyte cultures grew resulted in distinctive changes of growth rates of the organisms in both liquid and solid media. As expected the growth rates progressively decreased with increasing concentrations of the drugs. Minimum inhibition concentrations (MIC) in fluid media paralleled results

obtained with solid media but in most instances the MIC levels were lower in the former. It is assumed that this finding resulted from the organisms in the liquid media being in more intimate contact with the drug. Other morphological changes were those of texture, topography, and particularly, pigmentation which varied with different species. T. rubrum and T. mentagrophytes cultures developed increasing numbers of white tufts on the surface as the drug concentrations were increased. At 1.0 mcg/ml or more the colonies appeared as white and cottony; Trichophyton concentricum cultures changed from velvety to glabrous; M. canis changed from yellowish-brown with white cottony centers to light brown and glabrous throughout and the topography and pigmentation of Epidermophyton floccosum changed from flat, olivegreen colonies with white centers to pale olive or light brown. Only M. gypseum failed to show marked changes in texture. The inocula of all species were macerated at inhibitory concentrations of griseofulvin.

Although there were variations in the concentrations of griseofulvin eliciting morphological changes, the patterns of macroscopic and microscopic alterations wre similar for all dermatophytes studied. In absence of griseofulvin the mycelium were long, septate with straight, rarely - branched hyphal tips and contained homogenous cytoplasm. After exposure to increasing concentrations of griseofulvin the following sequential changes occurred in the mycelium of all isolates. The earliest changes were the appearance of lateral branches, curling of advancing hyphae and appearance of granular cytoplasm in mature areas of hyphae. At higher concentrations these were followed by increased branching, increased curling of the lateral branches at the ends of new mycelium and the presence of only granular cytoplasm.

At concentrations almost inhibiting new growth, branching and curling had progressed to the point of stunting and distortion and cytoplasm was granular and vacuolated. At inhibitory concentration of griseofulvin there was autolysis, vacuolated cytoplasm, and subsequent loss of mycelial contents. Griseofulvin incorporated in the culture medium resulted in fewer microconidia but their shapes and sizes were unaffected. Similar decreases of macroconidia were noted but morphologic changes were discernible even at low concentrations. These changes ranged from a slight bending at low concentrations to the appearance of distorted and ghost cells at inhibitory concentrations.

The similarity of the sequential changes in the presence of increasing griseofulvin concentrations suggests - but does not prove - similar mode of action of this drug for each of these organisms. The observation that the low concentrations first affected new growth and that the drug is mycostatic rather than mycocidal is in keeping with therapy experiences which show that griseofulvin appears to produce a favorable clinical effect by concentrating at the point of infection. There it inhibits the fungi and causes them to be shed with the normal outward growth and desquamation of the skin. Although the exact mode of action

of griseofulvin is not known, it appears to interfere with fungal mitosis, impairs synthesis of protein and nucleic acids and causes the breakdown of intracellular organelle membranes.

The interpretation of in vitro sensitivity determinations in terms of predictability of clinical effectiveness of griseofulvin is difficult. The usual criterion of relating microorganism sensitivities to blood levels does not pertain because these organisms are capable of in vitro growth at much higher concentrations of griseofulvin than is found in the blood after parenteral administration of the drug. It is concluded that therapeutic response to the drug depends on its concentration at the site of infection rather than in blood levels. A comparison of in vitro sensitivities of the strains used in this study to those reported in the literature suggests that even when one allows for differences of assay procedures, the dermatophytes isolated in Thailand were somewhat more resistant than those found elsewhere.

This study does not preclude the possibility that bizarre, mutant dermatophytes resulting from in vivo exposure to griseofulvin will not create diagnostic problems in the future. However the finding that morphologic changes of most of the griseofulvin induced morphologic changes of these dermatophytes were completely reversible on subsequent subculturing is encouraging from the standpoint of the clinical laboratory.

Reports of therapeutic failures with griseofulvin are appearing. The finding of griseofulvin - resistant dermatophytes in Thailand indicates that griseofulvin will become less effective as more fungi become resistant following exposure to the drug. To preclude unnecessary exposure to sensitive dermatophytes it is recommended diagnoses be confirmed by laboratory procedures prior to institution of griseofulvin therapy. Ideally there should be repeated cultures during therapy because the only criterion that can determine the required dosage levels and duration of treatment with griseofulvin and the final attainment of a biological cure are laboratory findings obtained by the medical mycologist.

Incomplete studies on the bacterial ecology of adult Thai females with dermatologic infections indicate that Staphylococcus epidermidis, Corynebacterium spp. Micrococcus spp. were found at most culture sites of patients infected with T. rubrum and of normal controls. Bacteria found less frequently at all culture sites included Staphylococcus aureus, Streptococcus fecalis, Pseudomonas spp. and alpha hemolytic streptococci. Conspicuous by its relative scarcity was Escherichia coli. This study will be completed by 30 June 1970.

SUMMARY: Studies on patients with dermatologic problems indicated that pathogenic fungi are present in Thailand and could represent a major problem to the military in times of stress. Pathogenic dermatophytes isolated most frequently were T. rubrum and C. albicans.

Macroscopic and microscopic changes resulting from exposure to graded concentrations of griseofulvin were observed for 31 recent isolates of dermatophytes. Most isolates were exposed to different concentrations of griseofulvin ranging from 0.1-30.0 mcg/ml in Sabouraud - Cycloheximide - Chloramphenicol Agar in petri dishes or Sabouraud - Cycloheximide - Chloramphenicol Broth in test tubes. Colonial and microscopic changes of cultures were observed at 5, 10 and 15 days at 25°C after inoculation. The results obtained indicated that griseofulvin created morphological changes of every isolate, especially on the microscopic structures of the mycelium. Discernible effects occurred at every concentration of the drug. The first change was the production of short, lateral hyphae from the primary mycelium followed, as the drug concentration increased, by curling, distorting and stunting of lateral branches and new mycelial growth. The cytoplasm of the mycelium was gradually changed from homogenous to granular to vacuolar and, at sub-inhibitory concentrations, the cytoplasm partially disappeared from the mycelium. Large, round chlamydospores germinated from the mature hyphae of most isolates at sub-inhibitory concentrations of griseofulvin. At inhibitory concentrations large round "ghost" cells or cells devoid of content occurred in most areas of the mycelium. The changes of shape and size of microconidia were little affected but they were reduced in number and they became poorly developed at high drug levels. Numbers of macroconidia decreased as the drug concentrations were increased and their shape became distorted in high concentrations. The macroscopic and microscopic changes of most isolates caused by griseofulvin appeared to be temporary. Most dermatophytes reverted to normal when sub-cultures of viable pleomorphic colonies were made on griseofulvin-free medium. The bacterial flora of adult Thai females with or without T. rubrum infections consisted predominantly of gram positive cocci and rods. Pseudomonas spp. was the gram negative organism found most frequently.

Table 1. Mycology Specimens from Patients at Women's Hospital, Bangkok, Thailand
1 April 1969 - 31 March 1970

Body areas	Total Specimen Examined	Negative for Fungus	Non-Pathogenic Fungi Isolated	Positive Cultures	
Body (Trunk, Face, Arms, Legs)	75	46	13	Trichophyton rubrum Epidermophyton floccosum Trichophyton mentagrophytes Candida albicans Pityrosporum orbicularae	9 1 3 2 1
Feet	54	24	26	Candida albicans Trichophyton rubrum	1 3
Hands	22	15	3	Trichophyton rubrum Candida albicans	1 3
Head	16	8	6	Trichophyton tonsurans Trichophyton rubrum	1 1
Nails	16	4	4	Candida albicans Trichophyton rubrum	7 1
Groin	14	1	1	Trichophyton rubrum Trichophyton mentagrophytes Epidermophyton floccosum Candida albicans Microsporum gypseum	2 4 1 4 1
Buttocks	8	3	1	Trichophyton rubrum	4
Vagina	3	-	3		-
Ear	2	2	-		-
Axilla	2	1	-	Trichophyton rubrum	1
Total	212	104	57		51

Table 2. Routine Mycology Specimens from American Nationals

1 April 1969 - 31 March 1970

Body areas	Total Specimens Examined	Negative for Fungus	Non-pathogenic Fungi Isolated	Positive cultures	
Body (Trunk, Face, Arms, Legs)	6	3	2	Trichophyton mentagrophytes	1
Feet	5	3	-	Trichophyton rubrum Candida albicans	1 1
Hand	1	1	-		-
Head	1	1	-		-
Hair	1	-	1		-
Nails	8	5	1	Trichophyton rubrum Candida albicans	1 1
Sputum	10	4	3	Candida albicans	3
Lymph node tissue	1	-	1		-
Pus	1	-	-	Candida species	1
Cerebrospinal fluid	1	1	-		-
Lung aspirate	1	1	-		-
Total	36	19	8		9

Table 3. Mycology Specimens from Miscellaneous Sources

1 April 1969 - 31 March 1970

Sources	Total Specimens Examined	Negative for Fungus	Non-Pathogenic Fungi Isolated	Positive cultures	
Dog's skin	1	1	-		-
Dog's ear	1	-	1		-
Gibbons' hair	6	-	1	Microsporum canis	5
Sheep blood	1	1	-		-
Soil	246	225	16	Microsporum gypseum	5
Water	194	163	31		-
Bats' lungs	55	42	13		-
Culture media	2	-	2		-
Human globulin immune serum	1	1	-		-
Total	507	433	64		10

Diarrheal Diseases

Coordinator:	Howard E. Noyes, Ph.D., Chief, Department of Bacteriology & Mycology
Principal Investigators:	Pongsom Atthasampunna, M.D.[1] Chiraphun Duangmani, M.D. Prakit Kasemsarn, M.D.[2] Udom Lexomboon, M.D., Ph.D.[2] Howard E. Noyes, Ph.D.
Associate Investigators:	Panyasri Benjadol, M.S. Santiparb Chaiwongkiat M.D.[4] Supanee Kethsuwan, M.D.[4] Kanchana Leelasiri, B.S. Lakshana Nagavajara, M.D.[3] Swish Rardjamroensook, M.D., M.P.H.[5] Pravit Songsaengterm, M.D.[3] Veeravat Supradish, M.D.[3] Susri Tandhavudho, M.D.[3] Pairoj Tanvichien, M.D.[3] Prapin Tugkanon, M.D.[3] Pisut Uttamote, M.D.[4] Ravi Vanichsarn, M.D.[4]
Assistant Investigators:	Yachai Ampaipast, B.S. John C. Bell, SSG Henry H. Higaki, MSG Yupa Kasemsanta, P.H.N. Tatsanee Occeño, B.S. Pavanee Prayongratana, R.N. Yongyuth Raengpradub, B.S. Chantana Ratanavaraha, B.S. Prani Ratarasarn Chanphen Srimunta, B.S. Ronald K. Stackhouse, SP5 Curtis A. Stewart, SP6 Tasna Tamaarree, Dip. Med. Tech. Sunee Yuvachitti, R.N.

1 On loan from Dept of Health, Ministry of Public Health, Thailand
2 Children's Hospital, Bangkok, Thailand
3 Samut-prakarn Provincial Hospital, Thailand
4 Ubol Provincial Hospital, Thailand
5 Health Officer, Ubol Province, Thailand

OBJECTIVE: Several related studies on diarrheal diseases include (1) a continuing enteropathogen survey of stools from Thai and American patients, (2) monitoring of antibiotic sensitivity patterns of diarrheal agents of Thai children, (3) characterization of gram negative anaerobes in stools of patients with acute diarrhea, and (4) a clinical trial of iodochlorhydroxyquinoline (ICHQ) in hospitalized cholera patients.

DESCRIPTION: (1) The enteropathogen survey study included specimens from inpatients and outpatients of both sexes from hospitals throughout Thailand. Most specimens were from patients hospitalized with diarrhea and were collected during the acute phase of the disease. The laboratory procedures were described in detail in the previous Annual Report. (2) The plate dilution technique was used to determine antimicrobial sensitivities of enteropathogens. (3) Stool specimens that were collected by a SMRL nurse from Thai children with acute diarrhea were brought to the laboratory for immediate processing. Pure cultures of obligate gram negative anaerobes were processed on the basis of colonial and microscopic morphology, 15 biochemical reactions and antimicrobial sensitivity patterns. (4) For the clinical trials of ICHQ all diarrheal patients admitted to Ubol Provincial Hospital during the cholera outbreak were considered and patients whose stool specimens were negative for agglutinable vibrios or who had received antimicrobials prior to hospitalization were deleted from the study. On admission suspected cholera patients received fluid and electrolyte replacements but no antimicrobials. Patients with odd admission numbers received drug A (ICHQ), those with even admission numbers drug B (placebo). The dosage of ICHQ was 250 mg every four hours for 72 hours in the form of tablets for adults and suspension for children 1 - 10 years of age. Rectal swabs were taken prior to initial medication and at 0600 dialy thereafter. Assessment of therapeutic efficacy of ICHQ was by duration of vibrio excretion.

PROGRESS: 1. Results of examinations of 3,448 routine specimens from 1,891 individuals are included in this report. Most of the 3,317 specimens from Thai nationals were from patients in Children's Hospital, Bangkok, Thailand. Other specimens were collected from patients at the Royal Thai Army, Royal Thai Navy, Siriraj Hospitals, Bangkok and Praputhabath Provincial Hospital. Most of the 130 specimens from U.S. personnel were from the U.S. Embassy Medical Unit, SMRL, and the 5th Field Hospital, all in Bangkok.

Isolations of enteropathogenic bacteria are summarized in Table 1. Approximately 7.7% of the specimens yielded Salmonellae; 2.3% yielded Shigellae, and 10.7% of those tested yielded Enteropathogenic Escherichia coli (EEC). The predominance of Salmonellae isolates from Thais is consistent with recovery rates found during the preceeding six years. Most of the Salmonellae isolates, representing 23 species, were Salmonella panama and Salmonella derby. There were no isolates of Salmonella javiana, the predominant organism the previous year, and isolates of Salmonella typhosa remained low, as has been the case for the

last eight years.

Eleven species were represented among the 70 Shigellae isolates. There were 24 isolates of Shigella sonnei form I followed by decreasing frequency of isolation by Shigella flexneri 2, Shigella sonnei form II and Shigella flexneri 3. Shigellae were isolated throughout the year and no specific outbreaks were noted. Serotypes 0119:B14, 025:B19:B23 and 0127:B8 were the predominant isolates of Enteropathogenic Escherichia coli in those children (less than 5 years old) checked for these organisms. No agglutinable vibrios have been isolated since September 1969.

Studies were done on the evaluation and comparison of 4 enrichment broths reputed to facilitate higher isolation rates of enteropathogens from rectal swabs or stool cultures. Overall, preliminary findings indicate that results obtained with tetrathionate broth, Hajna broth, Rappaport broth, and Selenite F broth are all inferior to results obtained by direct streaking of the specimen. Among plate media the largest number of salmonella and shigella isolates were from SS agar followed in terms of decreasing isolates by XLD, DC and MC agars. The evaluation continues.

2. In vitro sensitivities were determined for 208 isolates of Salmonellae, 157 isolates of Shigellae and 392 isolates of Enteropathogenic E. coli during this period (Tables 2-7). Overall these studies indicate that by far the most effective antimicrobials studied were colimycin and furazolidone followed in order of decreasing effectiveness by ampicillin, neomycin, oxytetracycline and chloramphenicol. All isolates of Shigellae and Salmonellae were either very sensitive or very resistant to ampicillin. All but 3 isolates of S. panama, the enteropathogen isolated most frequently during this period, were completely resistant to ampicillin. Enteropathogenic E. coli isolates tended to be more resistant than Salmonellae or Shigellae isolates. Based on these and prior in vitro studies the use of chloramphenicol or tetracycline is not indicated for therapy of most cases of acute diarrhea in Thailand.

3. The gram-negative, non-sporulating anaerobic or filamentous bacteria have been little studied and are poorly understood, even though they are known as one of the predominant - if not the predominant - members of the normal intestinal flora of man and despite their being the causal pathogen in serious diseases of man and animals. Some reports indicate they outnumber coliforms 100 to 1 in the human bowel. The theory has been advanced that they act as "stabilizers" of bowel ecology and diarrheal diseases can be associated with changes of their types and numbers in the gastrointestinal tract. Among other gaps in our knowledge of these organisms, their classification is uncertain. This portion of the study is being done to characterize these organisms preliminary to determining their roles, if any, in diarrhea of children. A total of 368 isolates representing 11 species of these obligate gram-negative anaerobes have been studied in terms of morphology and biochemical reactions (Table 8). Some of the identifications are tentative because of variable

reactions within designated species. Antimicrobial studies of these isolates indicate that most were sensitive to lincomycin and erythromycin, sensitivities to penicillin G were variable and most organisms were very resistant to polymyxin B, kanamycin, colimycin and neomycin. Correlation of these isolates to recognize enteropathogens from these patients has not been completed.

4. The trial of iodochlorhydroxyquine (ICHQ) in El Tor cholera cases at Ubol Provincial Hospital was continued until the end of the outbreak in mid-July 1969. The results of the study are summarized in Table 9. The range of duration of vibrio excretion was from less than one day to 9 days in both control and drug groups. The mean duration of excretion in the ICHQ group was 4.08 days while that of the control was 5.25. Forty-four percent of the drug group was vibrio-free within 3 days of treatment as compared with only 28% of the control groups. After one week, 28% of the control patients were still excreting vibrios but only 8% of the drug group were positive. There were 3 relapses after sodium sulfate purging in the ICHQ group and 2 in the control group.

From 2-18 July 1969 we conducted an investigation of cholera carriers among the food handlers in restaurants and food vendors in Ubol city in cooperation with the Ubol Provincial Health Office. One rectal swab was taken from each of the food handlers by the health workers, put into alkaline peptone tubes, and processed by SMRL personnel. El Tor vibrios were not isolated but there were 19 isolates of non-agglutinable (NAG) vibrios from 362 food handlers in the 97 restaurants and food shops examined. The distribution of Heiberg groups of the NAG vibric isolates are shown in Table 10.

SUMMARY: Salmonella panama and Salmonella derby were the enteropathogens isolated most frequently from Thai children with acute diarrhea. Most enteropathogens tested were sensitive in vitro to colimycin and furazolidone and resistant to kanamycin, neomycin, oxytetracycline and chloramphenicol. Stools from Thai children with acute diarrhea were cultured for obligate gram-negative anaerobic organisms. The 368 isolates represented 11 species and were resistant to most antimicrobials used to treat diarrhea. The trials of iodochlorhydroxyquinoline (ICHQ) as a vibriocidal agent in cholera patients were completed during this period. It was shown that ICHQ shortened the duration of vibrio excretion but was considered inferior to other antimicrobials such as tetracycline, chloramphenicol, kanamycin and erythromycin. No cholera carriers among food handlers in Ubol at the end of the cholera outbreak were found.

Table 1. Enterobacteriaceae Isolated from Acute Diarrhea Cases in Thailand
1 April 1969 - 31 March 1970

Month	Thai Nationals					American Nationals				
	No. of Specimens	No. of Patients	Salmonellae	Shigellae	Vibrios	No. of Specimens	No. of Patients	Salmonellae	Shigellae	Vibrios
April 1969	207	125	10	11	2	35	35	6	2	0
May	268	142	10	1	5	42	42	1	2	0
June	222	114	3	3	1	16	16	1	2	0
July	331	201	25	12	0	9	9	0	0	0
August	365	180	30	5	0	6	6	0	0	0
September	404	179	52	3	2	9	9	0	0	0
October	262	141	47	4	0	3	3	1	1	0
November	368	179	28	6	0	1	1	0	0	0
December	159	96	4	4	0	1	1	0	0	0
Jan 1970	213	127	9	5	0	5	4	0	0	0
February	225	121	24	8	0	1	1	0	0	0
March	293	156	14	8	0	3	3	0	1	0
Total	3317	1761	256	70	10	131	130	9	8	0

Table 2

SENSITIVITIES OF ENTERIC ORGANISMS TO COLIMYCIN
1 April 1969 - 31 March 1970

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Salmonella typhi	5	5	-	-	-	-	-	-	-	-	-
S. kentucky	2	2	-	-	-	-	-	-	-	-	-
S. derby	55	48	7	-	-	-	-	-	-	-	-
S. moscow	3	3	-	-	-	-	-	-	-	-	-
S. weltevreden	15	13	2	-	-	-	-	-	-	-	-
S. oslo	4	4	-	-	-	-	-	-	-	-	-
S. panama	55	50	5	-	-	-	-	-	-	-	-
S. muenchen	1	1	-	-	-	-	-	-	-	-	-
S. stanley	8	5	3	-	-	-	-	-	-	-	-
S. senftenberg	1	1	-	-	-	-	-	-	-	-	-
S. lexington	6	4	2	-	-	-	-	-	-	-	-
S. heidelberg	2	2	-	-	-	-	-	-	-	-	-
S. typhimurium	7	6	1	-	-	-	-	-	-	-	-
S. bovismorbificans	2	2	-	-	-	-	-	-	-	-	-
S. virchow	4	4	-	-	-	-	-	-	-	-	-
S. tennessee	2	1	1	-	-	-	-	-	-	-	-
S. tananarive	7	6	1	-	-	-	-	-	-	-	-
S. anatum	4	3	1	-	-	-	-	-	-	-	-
S. meleagridis	2	2	-	-	-	-	-	-	-	-	-
S. enteritidis	1	1	-	-	-	-	-	-	-	-	-
S. newlands	11	9	2	-	-	-	-	-	-	-	-
S. newport	11	9	2	-	-	-	-	-	-	-	-
S. paratyphi B	6	4	2	-	-	-	-	-	-	-	-
S. montevideo	1	1	-	-	-	-	-	-	-	-	-
S. claibornei	3	1	2	-	-	-	-	-	-	-	-

Table 2. (Cont'd)

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.26	12.5	25	50	100	200	>200
Shigella sonnei I	35	35	-	-	-	-	-	-	-	-	-
Sh. sonnei form II	7	7	-	-	-	-	-	-	-	-	-
Sh. flexneri 1	9	9	-	-	-	-	-	-	-	-	-
Sh. flexneri 2	49	48	1	-	-	-	-	-	-	-	-
Sh. flexneri 3	22	22	-	-	-	-	-	-	-	-	-
Sh. flexneri 4	7	7	-	-	-	-	-	-	-	-	-
Sh. flexneri 6	1	1	-	-	-	-	-	-	-	-	-
Sh. boydii 1	4	4	-	-	-	-	-	-	-	-	-
Sh. boydii 2	4	4	-	-	-	-	-	-	-	-	-
Sh. boydii 4	1	1	-	-	-	-	-	-	-	-	-
Sh. boydii 5	3	3	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 1	2	2	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 2	3	3	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 4	4	4	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 6	1	1	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 7	1	1	-	-	-	-	-	-	-	-	-
Aeromonas sp.	1	1	-	-	-	-	-	-	-	-	-

Table 3

SENSITIVITIES OF ENTERIC ORGANISMS TO FURAZOLIDONE
1 April 1969 - 31 March 1970

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Salmonella typhi	5	5	-	-	-	-	-	-	-	-	-
S. kentucky	2	2	-	-	-	-	-	-	-	-	-
S. derby	55	53	2	-	-	-	-	-	-	-	-
S. moscow	3	3	-	-	-	-	-	-	-	-	-
S. weltevreden	15	11	4	-	-	-	-	-	-	-	-
S. oslo	4	3	-	-	1	-	-	-	-	-	-
S. panama	55	50	5	-	-	-	-	-	-	-	-
S. muenchen	1	1	-	-	-	-	-	-	-	-	-
S. stanley	8	8	-	-	-	-	-	-	-	-	-
S. senftenberg	1	1	-	-	-	-	-	-	-	-	-
S. lexington	6	3	3	-	-	-	-	-	-	-	-
S. heidelberg	2	2	-	-	-	-	-	-	-	-	-
S. typhimurium	7	7	-	-	-	-	-	-	-	-	-
S. bovismorbificans	3	2	1	-	-	-	-	-	-	-	-
S. virchow	4	4	-	-	-	-	-	-	-	-	-
S. tennessee	2	2	-	-	-	-	-	-	-	-	-
S. tananarive	7	7	-	-	-	-	-	-	-	-	-
S. anatum	4	4	-	-	-	-	-	-	-	-	-
S. meleagridis	2	2	-	-	-	-	-	-	-	-	-
S. enteritidis	1	1	-	-	-	-	-	-	-	-	-
S. newlands	11	11	-	-	-	-	-	-	-	-	-
S. newport	11	11	-	-	-	-	-	-	-	-	-
S. paratyphi B	6	6	-	-	-	-	-	-	-	-	-
S. montevideo	1	1	-	-	-	-	-	-	-	-	-
S. claibornei	3	2	-	1	-	-	-	-	-	-	-

Table 3 (Cont'd)

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Shigella sonnei formI	34	33	1	-	-	-	-	-	-	-	-
Sh. sonnei form II	10	10	-	-	-	-	-	-	-	-	-
Sh. flexneri 1	9	6	3	-	-	-	-	-	-	-	-
Sh. flexneri 2	49	46	3	-	-	-	-	-	-	-	-
Sh. flexneri 3	22	19	-	3	-	-	-	-	-	-	-
Sh. flexneri 4	7	7	-	-	-	-	-	-	-	-	-
Sh. flexneri 6	1	-	-	1	-	-	-	-	-	-	-
Sh. boydii 1	4	4	-	-	-	-	-	-	-	-	-
Sh. boydii 2	4	4	-	-	-	-	-	-	-	-	-
Sh. boydii 4	1	1	-	-	-	-	-	-	-	-	-
Sh. boydii 5	3	3	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 1	2	2	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 2	3	-	1	-	2	-	-	-	-	-	-
Sh. dysenteriae 4	5	5	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 6	1	1	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 7	1	1	-	-	-	-	-	-	-	-	-
Aeromonas sp.	1	1	-	-	-	-	-	-	-	-	-

Table 4

SENSITIVITIES OF ENTERIC ORGANISMS TO AMPICILLIN
1 April 1969 - 31 March 1970

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Salmonella typhi	5	5	-	-	-	-	-	-	-	-	-
S. kentucky	2	2	-	-	-	-	-	-	—	-	-
S. derby	55	52	-	-	-	-	-	-	-	-	3
S. moscow	3	3	-	-	-	-	-	-	-	-	-
S. weltevreden	15	12	-	-	-	-	-	-	-	1	2
S. oslo	4	4	-	-	-	-	-	-	-	-	-
S. panama	55	2	-	1	-	-	-	-	-	-	52
S. muenchen	1	1	-	-	-	-	-	-	-	-	-
S. stanley	8	8	-	-	-	-	-	-	-	-	-
S. senftenberg	1	1	-	-	-	-	-	-	-	-	-
S. lexington	6	5	-	-	-	-	-	-	-	-	1
S. heidelberg	2	2	-	-	-	-	-	-	-	-	-
S. typhimurium	6	5	-	-	-	-	-	-	-	-	1
S. bovismorbificans	3	3	-	-	-	-	-	-	-	-	-
S. virchow	4	3	1	-	-	-	-	-	-	-	-
S. tennessee	2	2	-	-	-	-	-	-	-	-	-
S. tananarive	7	6	-	-	-	-	-	-	-	-	1
S. anatum	4	3	-	1	-	-	-	-	-	-	-
S. meleagridis	2	2	-	-	-	-	-	-	-	-	-
S. enteritidis	1	1	-	-	-	-	-	-	-	-	-
S. newlands	11	11	-	-	-	-	-	--	-	-	-
S. newport	11	11	-	-	-	-	-	-	-	-	-
S. paratyphi B	6	6	-	-	-	-	-	-	-	-	-
S. montevideo	1	-	-	-	-	-	-	-	-	-	1
S. claibornei	3	3	-	-	-	-	-	-	-	-	-

Table 4. (Cont'd)

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Shigella sonnei form I	33	21	6	2	-	-	-	-	-	2	2
Sh. sonnei form II	10	1	5	1	-	-	-	-	-	1	2
Sh. flexneri 1	9	8	-	-	-	-	-	-	-	1	-
Sh. flexneri 2	49	44	1	2	1	-	1	-	-	-	-
Sh. flexneri 3	22	18	1	-	-	-	-	-	3	-	-
Sh. flexneri 4	7	4	2	-	-	-	-	-	-	-	1
Sh. flexneri 6	1	1	-	-	-	-	-	-	-	-	-
Sh. boydii 1	3	2	-	-	-	-	-	-	-	1	-
Sh. boydii 2	4	3	-	1	-	-	-	-	-	-	-
Sh. boydii 4	1	1	-	-	-	-	-	-	-	-	-
Sh. boydii 5	3	2	-	-	1	-	-	-	-	-	-
Sh. dysenteriae 1	2	1	-	-	-	-	1	-	-	-	-
Sh. dysenteriae 2	3	-	-	-	-	-	-	-	-	3	-
Sh. dysenteriae 4	5	-	-	-	-	1	-	-	-	3	1
Sh. dysenteriae 6	1	1	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 7	1	1	-	-	-	-	-	-	-	-	-
Aeromonas sp.	1	1	-	-	-	-	-	-	-	-	-

Table 5

SENSITIVITIES OF ENTERIC ORGANISMS TO NEOMYCIN
1 April 1969 - 31 March 1970

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Salmonella typhi	5	4	-	1	-	-	-	-	-	-	-
S. kentucky	2	2	-	-	-	-	-	-	-	-	-
S. derby	55	38	11	3	-	1	-	-	-	2	-
S. moscow	3	2	1	-	-	-	-	-	-	-	-
S. weltevreden	15	6	4	1	-	-	-	-	-	1	3
S. oslo	4	-	3	1	-	-	-	-	-	-	-
S. panama	55	-	2	6	-	1	-	-	-	6	40
S. muenchen	1	-	1	-	-	-	-	-	-	-	-
S. stanley	8	3	5	-	-	-	-	-	-	-	-
S. senftenberg	1	-	-	1	-	-	-	-	-	-	-
S. lexington	6	4	2	-	-	-	-	-	-	-	-
S. heidelberg	2	-	-	2	-	-	-	-	-	-	-
S. typhimurium	7	4	3	-	-	-	-	-	-	-	-
S. bovismorbificans	3	2	1	-	-	-	-	-	-	-	-
S. virchow	4	3	1	-	-	-	-	-	-	-	-
S. tennessee	2	1	1	-	-	-	-	-	-	-	-
S. tananarive	7	2	5	-	-	-	-	-	-	-	-
S. anatum	4	-	3	1	-	-	-	-	-	-	-
S. meleagridis	2	2	-	-	-	-	-	-	-	-	-
S. enteritidis	1	1	-	-	-	-	-	-	-	-	-
S. newlands	11	8	1	1	-	-	-	-	-	-	1
S. newport	11	5	4	2	-	-	-	-	-	-	-
S. paratyphi B	6	5	1	-	-	-	-	-	-	-	-
S. montevideo	1	-	-	-	-	1	-	-	-	-	-
S. claibornei	3	-	1	2	-	-	-	-	-	-	-

Table 5. (Cont'd)

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Shigella sonnei form I	34	6	11	11	2	-	-	-	-	-	4
Sh. sonnei form II	10	1	2	2	-	-	-	-	-	1	4
Sh. flexneri 1	9	-	-	6	2	1	-	-	-	-	-
Sh. flexneri 2	48	2	6	20	10	9	1	-	-	-	-
Sh. flexneri 3	22	2	7	11	1	1	-	-	-	-	-
Sh. flexneri 4	7	-	-	3	-	4	-	-	-	-	-
Sh. flexneri 6	1	-	1	-	-	-	-	-	-	-	-
Sh. boydii 1	4	-	1	1	-	-	-	-	-	-	2
Sh. boydii 2	4	-	-	2	2	-	-	-	-	-	-
Sh. boydii 4	1	-	-	1	-	-	-	-	-	-	-
Sh. boydii 5	3	3	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 1	2	1	-	1	-	-	-	-	-	-	-
Sh. dysenteriae 2	3	-	-	-	-	-	-	-	-	1	2
Sh. dysenteriae 4	4	1	-	2	1	-	-	-	-	-	-
Sh. dysenteriae 6	1	-	1	-	-	-	-	-	-	-	-
Sh. dysenteriae 7	1	1	-	-	-	-	-	-	-	-	-
Aeromonas sp.	1	1	-	-	-	-	-	-	-	-	-

Table 6

SENSITIVITIES OF ENTERIC ORGANISMS TO OXYTETRACYCLINE
1 April 1969 - 31 March 1970

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Salmonella typhi	4	-	-	2	1	1	-	-	-	-	-
S. kentucky	2	-	-	-	-	-	-	-	-	-	2
S. derby	55	-	1	42	5	5	-	-	-	-	2
S. moscow	3	-	-	2	1	-	-	-	-	-	-
S. weltevreden	15	-	-	9	2	1	-	-	-	-	-
S. oslo	4	1	1	2	-	-	-	-	-	-	-
S. panama	55	3	7	38	1	1	-	-	-	-	5
S. muenchen	1	-	-	1	-	-	-	-	-	-	-
S. stanley	8	-	1	4	2	1	-	-	-	-	-
S. senftenberg	1	-	-	1	-	-	-	-	-	-	-
S. lexington	6	-	-	3	2	1	-	-	-	-	-
S. heidelberg	2	-	-	2	-	-	-	-	-	-	-
S. typhimurium	7	1	-	1	2	1	1	-	-	-	1
S. bovismorbificans	3	-	1	1	-	-	-	-	-	-	1
S. virchow	4	-	-	3	-	-	1	-	-	-	-
S. tennessee	2	-	-	-	1	1	-	-	-	-	-
S. tananarive	7	-	1	1	1	3	-	-	-	-	-
S. anatum	4	-	-	2	1	-	1	-	-	-	-
S. meleagridis	2	-	-	-	-	2	-	-	-	-	-
S. enteritidis	1	-	-	-	1	-	-	-	-	-	-
S. newlands	11	-	1	4	4	2	-	-	-	-	-
S. newport	11	-	-	4	2	5	-	-	-	-	-
S. paratyphi B	6	-	-	3	2	-	-	-	-	-	1
S. montevideo	1	-	-	-	-	-	-	-	-	-	1
S. claibornei	3	-	-	3	-	-	-	-	-	-	-

Table 6. (Cont'd)

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Shigella sonnei form I	34	-	1	4	-	-	1	14	5	2	7
Sh. sonnei form II	10	-	-	-	-	-	-	-	3	-	7
Sh. flexneri 1	9	-	-	-	1	-	-	1	-	-	7
Sh. flexneri 2	49	8	2	3	1	11	16	3	2	2	1
Sh. flexneri 3	22	-	-	1	-	-	1	3	-	11	6
Sh. flexneri 4	7	-	-	-	-	-	-	3	1	-	3
Sh. flexneri 6	1	-	-	-	-	1	-	-	-	-	-
Sh. boydii 1	4	-	1	1	-	-	-	-	-	1	1
Sh. boydii 2	4	-	1	-	-	-	-	-	-	-	3
Sh. boydii 4	1	-	-	1	-	-	-	-	-	-	-
Sh. boydii 5	3	-	-	2	-	1	-	-	-	-	-
Sh. dysenteriae 1	3	-	-	1	-	-	-	-	1	-	1
Sh. dysenteriae 2	3	-	-	-	-	-	-	-	-	2	1
Sh. dysenteriae 4	4	-	1	-	-	-	-	-	-	-	3
Sh. dysenteriae 6	1	-	1	-	-	-	-	-	-	-	-
Sh. dysenteriae 7	1	-	-	1	-	-	-	-	-	-	-
Aeromonas sp.	1	1	-	-	-	-	-	-	-	-	-

Table 7

SENSITIVITIES OF ENTERIC ORGANISMS TO CHLORAMPHENICOL
1 April 1969 - 31 March 1970

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Salmonella typhi	5	1	1	3	-	-	-	-	-	-	-
S. kentucky	2	2	-	-	-	-	-	-	-	-	-
S. derby	55	10	24	12	3	4	1	-	-	-	1
S. moscow	3	2	-	1	-	-	-	-	-	-	-
S. weltevreden	15	2	4	3	2	1	-	-	1	1	1
S. oslo	4	-	4	-	-	-	-	-	-	-	-
S. panama	55	1	7	21	8	12	5	-	1	-	-
S. muenchen	1	-	-	1	-	-	-	-	-	-	-
S. stanley	8	1	4	3	-	-	-	-	-	-	-
S. senftenberg	1	-	-	1	-	-	-	-	-	-	-
S. lexington	6	1	2	3	-	-	-	-	-	-	-
S. heidelberg	2	-	2	-	-	-	-	-	-	-	-
S. typhimurium	7	1	3	2	-	-	-	-	-	-	1
S. bovismorbificans	3	1	1	-	1	-	-	-	-	-	-
S. virchow	4	1	1	1	1	4	-	-	-	-	-
S. tennessee	2	-	-	2	-	-	-	-	-	-	-
S. tananarive	7	5	2	-	-	-	-	-	-	-	-
S. anatum	4	1	-	1	1	-	1	-	-	-	-
S. meleagridis	2	-	1	-	-	1	-	-	-	-	-
S. enteritidis	1	-	-	-	1	-	-	-	-	-	-
S. newlands	11	1	5	4	1	-	-	-	-	-	-
S. newport	11	1	4	6	-	-	-	-	-	-	-
S. paratyphi B	6	1	1	3	-	1	-	-	-	-	-
S. montevideo	1	-	-	-	-	-	-	-	-	-	1
S. chaibornei	3	1	-	-	2	-	-	-	-	-	-

Table 7. (Cont'd)

Organisms	No. of strains tested	Inhibited at mcg/ml									
		0.78	1.56	3.12	6.25	12.5	25	50	100	200	>200
Shigella sonnei formI	34	2	3	-	-	-	-	-	1	3	25
Sh. sonnei form II	10	-	-	-	1	1	-	1	-	-	7
Sh. flexneri 1	9	1	-	-	-	1	-	-	4	-	3
Sh. flexneri 2	49	10	1	-	-	1	-	5	25	5	2
Sh. flexneri 3	22	1	-	-		-	1	-	1	12	7
Sh. flexneri 4	7	-	-	-	-	1	-	-	-	-	6
Sh. flexneri 6	1	-	-	-	-	-	-	1	-	-	-
Sh. boydii 1	4	1	1	-	-	-	1	-	-	-	1
Sh. boydii 2	4	1	-	-	-	-	-	-	-	2	1
Sh. boydii 4	1	-	-	1	-	-	-	-	-	-	-
Sh. boydii 5	3	1	1	-	-	1	-	-	-	-	-
Sh. dysenteriae 1	2	-	-	1	-	-	-	-	-	1	-
Sh. dysenteriae 2	3	2	-	1	-	-	-	-	-	-	-
Sh. dysenteriae 4	4	1	1	-	-	-	-	-	-	-	2
Sh. dysenteriae 6	1	1	-	-	-	-	-	-	-	-	-
Sh. dysenteriae 7	1	-	-	-	-	1	-	-	-	-	-
Aeromonas sp.	1	1	-	-	-	-	-	-	-	-	-

Table 8. Obligate Anaerobes from Stools of Thai Children with Acute Diarrhea

Organism	Number of Isolates
Bacteroides fragilis	86
Bacteroides incommunis*	76
Bacteroides variabilis*	58
Dialister pneumosintes	56
NCDC group F-2*	45
Bacteroides oralis	20
Bacteroides melaninogenicus	12
Fusobacterium fusiforme	10
Bacteroides corrodens	2
Sphaerophorus necrophorus	2
Bacteroides terebrans	1

* National Communicable Disease Center Classification scheme.

Table 9. Effect or Iodochlorhydroxyquine (ICHQ) on Vibrio Excretion of Cholera Patients in Thailand 1968-1969

Treatment groups	No. of Patients Excreting Vibrios Through Day										
	Day 0	1	2	3	4	5	6	7	8	9	10
ICHQ	39	36	31	30	22	16	11	6	3	1	0
Control	29	26	25	22	21	18	15	10	8	4	0

Table 10. Distribution of NAG Vibrios Isolated from Food Handlers, Ubol City July 1969

Heiberg group	No.
I	5
II	6
III	0
IV	1
V	7
VI	0
Total	19

Acute Bacterial Meningitis in Thai Children

Coordinator: Howard E. Noyes, Ph.D., Chief, Department of Bacteriology and Mycology

Principal Investigators: Chiraphun Duangmani, M.D.
Howard E. Noyes, Ph.D.
Prakit Kasemsarn, M.D.*
Pramuan Sunakorn, M.D.*

Associate Investigators: Udom Lexomboon, M.D.*
Pethai Mansuvan, M.D.*
Pitsawas Thongphakdi, M.D.*

Assistant Investigators: Yachai Ampaipast, B.S.
Panyasri Benjadol, M.S.
Chantana Ratanavaraha, B.S.
Yongyuth Raengpradub, B.S.

OBJECTIVE: This is a continuation of a study of bacterial meningitis in children at Children's Hospital, Bangkok, Thailand. The objectives of this study are to determine the etiology of these infections, determine the antibiotic sensitivities of the causal agents, and compare 3 antimicrobial regimens for the treatment of these patients.

DESCRIPTION: All patients with suspected bacterial meningitis admitted to Children's Hospital were included in this study. Antimicrobial therapy was based on order of admission. Daily administration consisted of (1) 10 megaunits of intravenous penicillin G sodium plus 50 mg/kg of intramuscular kanamycin sulfate, or (2) the same dosage of penicillin plus 100 mg/kg of intramuscular chloramphenicol, or (3) 150 mg/kg of intravenous sodium ampicillin. All regimens were divided into 4 doses which were given at 6 hour intervals.

Diagnostic lumbar punctures were made on the first day of hospitalization and spinal fluids were examined for appearance, protein, sugar and chloride content, cell counts and differentials, and bacteria as revealed by gram stains and cultures. Other procedures included routine blood counts, urinalysis, stool examinations in patients with diarrhea, blood sugar determinations and bacteriological cultures of cerebrospinal fluid and blood.

Response to therapy was evaluated by the usual clinical criteria, including duration of fever, improvement of neurologic status, and decrease in peripheral blood leucocyte count with a return of the differential count toward normal and in improvement in C.S.F. findings (including pressure, cell count, percentage of polymorphonuclear leucocytes, and glucose and protein content). Outcome of therapy was evaluated according to the

*Children's Hospital, Bangkok, Thailand

patient's courses in the hospital, clinical status at the time of discharge and, whenever possible, a follow-up study one year after discharge.

PROGRESS: Seventeen of the 24 patients studied during this period were less than one year old and all but one were seriously ill when hospitalized. Organisms isolated from CSF specimens are shown in Table 1. Concomitant septicemias were noted in the patients infected with Escherichia coli, Candida albicans and in 2 of the 5 patients infected with Diplococcus pneumoniae. Antibiotic sensitivity tests indicated that all isolates of D. pneumoniae were sensitive in vitro to penicillin and ampicillin and all isolates of H. influenzae were sensitive to chloramphenicol and ampicillin. Including the 44 patients studied before this reporting period, the mortality rate of patients infected with D. pneumoniae was 47.6% as compared with 41.1% in patients infected with H. influenzae.

SUMMARY: A study of acute bacterial meningitis in Thai children has been continued. Results to date indicate that the causal agent is D. pneumoniae or H. influenzae in most cases. While all isolates of D. pneumoniae were sensitive in vitro to antibiotics in each regimen, the mortality rate was 47.6%. The mortality rate of patients with H. influenzae meningitis was 41.1%. There are too few patients in each group to enable meaningful comparisons of therapeutic regimens at this time.

Table 1 Purulent Meningitis in Children by Etiology and Outcome

	Clinical result		Case fatality
	Lived	Died	%
Diplococcus pneumoniae	4	1	20
Haemophilus influenzae	3	1	25
Salmonella newport	1	0	0
Escherichia coli	0	1	10
Candida albicans	1	0	0
Negative cultures	8	4	33
Total	17	7	29.1

Acute Pneumonitis in Thai Children

Coordinator:	Howard E. Noyes, Ph.D., Chief, Department of Bacteriology & Mycology
Principal Investigator:	Pramuan Sunakorn, M.D.*
Associate Investigators:	Chiraphun Duangmani, M.D. Howard E. Noyes, Ph.D. Lloyd C. Olson, MAJ, MC Suthee Vallikul, M.D.*
Assistant Investigators:	Tatsanee Occeño, B.S. Yongyuth Raengpradub, B.S. Sunee Yuvachitti, R.N.

OBJECTIVE: Earlier studies at Children's Hospital, Bangkok, Thailand showed bacterial pathogens such as Diplococcus pneumoniae and Staphylococcus aureus could be cultured from throat and nasopharyngeal swabs of many children in the absence of clinical pneumonia. Therefore the interpretation of such cultures is difficult. The objectives of this study are (1) to determine the etiology of acute pneumonitis of Thai children by culturing lung aspirates for bacteria and viruses, (2) compare laboratory findings of nasopharyngeal and throat cultures to those of lung aspirate cultures, and (3) obtain confirmatory information of diagnoses by analyses of acute and convalescent sera for antibodies to selected viruses and Mycoplasma pneumoniae.

DESCRIPTION: Excluding those with antimicrobial therapy prior to hospitalization, all patients with a clinical diagnosis of pneumonitis and with a definite shadow on their chest X-ray films were studied. Routine procedures used included AP and lateral chest X-rays, CBC including differential, tuberculin test, and bacteriologic cultures of blood, nasopharyngeal swabs and throat swabs. Lung aspirates were obtained by Thai physicians at Children's Hospital and sent immediately to the laboratory for processing. Additional X-rays were taken 24 hours later to detect possible complications.

PROGRESS: Twenty of 26 patients studied during this period were infants of 1 year or younger and 20 were males. Lung aspirates were positive for D. pneumoniae in 6 patients, alpha hemolytic streptococcus in 1 patient and Micrococcus spp. in 1 patient. One patient's lung aspirate was negative on admission but was positive at autopsy for Escherichia coli and Enterobacter aerogenes. There were 3 instances where pneumococci were cultured from blood, nasopharynx, or throat but not from lung aspirates. The only isolation of Haemophilus influenzae was from the nasopharynx of a patient whose lung aspirate was positive for alpha hemolytic streptococcus. Although all lung aspirates and blood cultures were negative for Staphylococcus aureus, this organism was isolated

*Children's Hospital, Bangkok, Thailand

from the nasopharynxes of 8 patients, throats of 2 patients and from both sites in 3 patients. Results of virus and serological studies are not available at this time.

SUMMARY: A st dy has been initiated to determine the etiology of acute pneumonitis i . Thai children by culturing lung aspirates. The bacterial pathogen isolated most frequently from lung aspirates was D. pneumoniae.

Wound Infections

Coordinator: Howard E. Noyes, Ph.D., Chief, Department of Bacteriology & Mycology

Principal Investigators: Tamrongratana Kaowkarn, LTC, MC*
Howard E. Noyes, Ph.D.
Prapat Paktongchai, MAJ, MC*

Associate Investigator: Chiraphun Duangmani, M.D.

Assistant Investigators: Panyasri Benjadol, M.S.
Pavanee Prayongratana, R.N.
Curtis A. Stewart, SP6
Sunee Yuvachitti, R.N.

OBJECTIVE: This study was designed to evaluate Neosporin(R)** as an adjunct to systemic antimicrobial therapy of infected traumatic wounds.

DESCRIPTION: Patients were Thai nationals hospitalized at Phra Mongkutklao Hospital. Specifically excluded from the study were penetrating wounds of the head, chest or abdomen. Topical Neosporin(R), a commercial mixture of neomycin sulfate, zinc bacitracin and polymyxin B, or a placebo was administered twice daily as an aerosol. Neosporin(R) and placebo were in identical canisters marked A or B and the code will not be revealed until the study is completed. Cultures for aerobic and anaerobic bacteriologic analyses were taken on the day of admission and each Monday, Wednesday and Friday for a total of 10 days. Clinical progress of patients was determined by daily observation of the wound by surgeons who assigned a number based on the scale shown in Table 1. Results were evaluated in terms of clinical changes of wounds and by quantitative and qualitative changes of the bacterial flora.

PROGRESS: A total of 23 patients have been studied. There were no differences in the clinical progress of the wounds of the two groups during the first 7 days of treatment but wounds treated with Drug B were in better clinical condition by the tenth day of treatment. Organisms isolated most frequently from both treatment groups were Pseudomonas aeruginosa, Streptococcus fecalis and coliform bacteria.

*Phra Mongkutklao Hospital, Bangkok, Thailand
**"Neosporin" Aerosol and placebo was supplied by Burroughs Wellcome & Co. (U.S.A.) inc., Tuckahoe, N.Y.

Most bacterial isolates were sensitive to one or more of the three antimicrobials contained in Neosporin(R) and there was no evidence of increased resistance of bacterial isolates during the treatment period. Contrary to results of similar studies overgrowth by Staphylococcus aureus, Ps. aeruginosa or Candida albicans has not been a serious clinical problem.

SUMMARY: A double-blind study is being carried out to determine the effect of topical Neosporin(R) on the clinical progress and bacterial flora of infected traumatic wounds. Better clinical results have been noted in one of the two groups of patients but the bacteriological results are equivocal. Emergence of bacterial isolates resistant to the antimicrobials used or overgrowth by antimicrobial-resistant organisms have not been noted.

Table 1 Numerical Scale for Designating Status of Wounds

1. Clean and closed primarily
2. Open and clean, healthy granulation tissue
3. Converted recently to clean open wound
4. Open, not clean but improving
5. Previously closed and opened for drainage
6. Periwound erythema with edema, induration and tenderness
7. Presence of foreign body
8. Serous drainage
9. Purulent drainage
10. Necrotic tissue present
    a. Skin
    b. Muscle
11. Wound deteriorating (further invasion, necrosis, increased drainage)
12. Presence of crepitus

Melioidosis

Coordinator: Howard E. Noyes, Ph.D., Chief, Department of Bacteriology and Mycology

Principal Investigators: Pongsom Atthasampunna, M.D.*
Richard A. Grossman, MAJ, MC
Howard E. Noyes, Ph.D.

Associate Investigators: Paibul Busapathamrong, MAJ, MC, RTA**
Prayoth Tanticharoenyos, DVM
Markpol Tingpalapong, DVM
Saridwongsa Wongsathuaythong, LTC, MC,RTA**
William L. Wooding, MAJ, VC

*On loan from Dept of Health, Ministry of Public Health, Thailand
**Dept of Medicine, Phra Mongkutklao Hospital, Bangkok, Thailand

Assistant Investigators: Yupa Kasemsanta, P.H.N.
Ovath Khunphol, R.N.
Yongyuth Raengpradub, B.S.
Chanphen Srimunta, B.S.
Tasna Tamaarree, Dip. Med. Tech.

OBJECTIVE: This study was designed to determine the presence and distribution of Pseudomonas pseudomallei in Thailand and to evaluate its importance as the causative agent of the disease, melioidosis.

DESCRIPTION: Previous studies determined that Ps. pseudomallei was present in the water and soil of southern and northeastern Thailand, and there was an association of serological activity of Thai people with the presence of the organism in the environs. Prior to this study no clinical cases of melioidosis in man and animals indigenous to Thailand have been described for 14 years. It was concluded that Ps. pseudomallei is saprophytic in soil and water although it must still be regarded as a potential pathogen for man.

Continuing studies during this period included analyses of water and soil for isolation of Ps. pseudomallei, attempts to find clinical melioidosis in Thai nationals, a prospective epidemiological study of melioidosis which was initiated in late August 1968 in a small village where well water and soil were found to be contaminated with Ps. pseudomallei. and determination in vitro antimicrobial sensitivities of pure cultures of Ps. pseudomallei isolated from water, soil and specimens from patients.

PROGRESS: 1. Survey of water and soil for the presence of Ps. pseudomallei. Specimens were obtained during field trips on other SMRL studies because soil from most provinces in Thailand have been studied in previous years. Samples from Songkla and Yala in the South and Maehongsorn in the North were positive for Ps. pseudomallei. None of 15 specimens from Vientiane, Laos were positive for this organism.

2. Case finding of clinical melioidosis in Thai Nationals and in animals. Attempts to find cases of clinical melioidosis were made in a Bangkok Hospital, a provincial hospital and by testing sera sent to this laboratory for other studies. In Bangkok, Phra Mongkutklao Hospital of the Royal Thai Army was selected for continuing studies because it serves civilians as well as military personnel and cases are referred there from all parts of Thailand. Those considered most likely to have melioidosis are pulmonary disease patients. Sera were tested by the indirect HA test for antibodies and sputa were collected for isolation of the organism. Ten of 448 patients tested had significant HA titers (1:80 or higher) but none of 373 sputum specimens from 140 patients were positive for Ps. pseudomallei. Five of 81 sera from patients of Songkla Provincial Hospital and sera from 4 of 234 residents of Mae Sarieng (Maehongsorn Province) had significant titers against Ps. pseudomallei. None of 67 sera submitted for febrile agglutination tests and 48 sera from Thai employees of an American military hospital in Bangkok had significant HA antibody

titers. Of 160 sera from rodents trapped at Sakaerat Research Station in Nakornrajsima Province, 30 sera from Cholburi Province, and 20 sera from rodents trapped in Kabinburi, Prachinburi Province, none had demonstrable HA titers against Ps. pseudomallei. However 3 of 10 cattle sera from the ranch of the Accelerated Development Center in Kanchanaburi Province and 33 of 117 other bovine sera submitted by the Department of Veterinary Medicine had HA titers, but all were 1:40 or lower.

3. Epidemiology of melioidosis at Ban Huay Jode, Thailand. In April 1968 Ps. pseudomallei was isolated from a water sample obtained from a shallow roadside well in Ban Huay Jode village, Wattana Nakorn district, Prachinburi. In May of that year a team returned to obtain blood specimens from the residents of the village, most of whom used the well water for all purposes. Twelve of 114 sera tested had antibody titers of 1:20 or greater against Ps. pseudomallei and at that time the organism was isolated from neighboring wells and from soil taken near the wells. These wells had been dug only 3 years earlier so it was decided that this village offered an opportunity to study the epidemiology of melioidosis in a community where the population was continously exposed to Ps. pseudomallei. Details of the procedures used and preliminary findings are described in the 1968-1969 Annual Report. The study is a joint project between the Department of Bacteriology and Mycology and the Department of Epidemiology.

Analyses of water sources and soil samples adjacent to water sources in the village and soil from designated sites of farming areas were analysed each month for Ps. pseudomallei. Twenty-six percent of the water and 20 percent of the soil samples were positive with one or more water specimens being positive each time samples were obtained. The organism was isolated from farm soils more frequently in the rainy than the dry season. An additional 21 soil samples taken from the habitats of domestic animals were negative for Ps. pseudomallei.

Sera from domestic animals were analysed for HA antibodies and it was found that water buffalo, cattle and dogs showed evidence of immunologic response to Ps. pseudomallei while, with one possible exception, none of 39 fowls did so. Sera from 2 of 43 water buffaloes, 2 of 12 cattle and 1 of 14 dogs were positive at significant titers. Stool specimens from 12 water buffaloes, 2 cattle, 4 pigs, 1 chicken and 1 dog were negative when cultured for Ps. Pseudomallei. Fifty rodents representing 5 species were trapped in the village and rice fields for serum antibody determinations and for isolation of Ps. pseudomallei from their ectoparasites, internal organs and fecal contents. All were negative.

Attempts to produce and study water-borne melioidosis in gibbons were continued. Water from a well known to be positive for Ps. pseudomallei was the sole source of drinking water for normal and splenectomized gibbons for 19 months. None of the gibbons developed clinical symptoms suggestive of melioidosis and none had measurable serum antibodies as measured by the HA procedure.

Blood specimens were voluntarily given by the residents every three month for determination of antibodies against Ps. pseudomallei by the indirect HA test. Results shown in Table 1 show that 28 percent of the 219 residents tested had HA antibodies and 18% had significant titers of 1:80 or greater. It was noted that only 4 of 84 children under 10 years of age had significant titers as compared with 16 of 49 in the 10-19 year old group. Overall more males than females had significant titers. It should be noted that these titers were not static in that 7 residents had titer rises of four-fold or greater, 3 residents had similar decreases of titers and 7 residents had rising and falling titers of four-fold or greater during this reporting period. Except for the 2 patients discussed below, no clinical illnesses suggestive of melioidosis were noted in any of the residents.

Based on clinical findings, isolation of Ps. pseudomallei from sputa and serologic conversions, two brothers were diagnosed as having clinical melioidosis. The first case was a 26 year old farmer who moved into this village with his family on 27 December 1968, but he had been working on a farm in this village for 2 years at that time. He was seen first at the village clinic on 22 January 1969 with complaint of tightness in the chest, He was given two doses of 300,000 u of crystalline penicillin G and 0.5 gm of tetracycline three times daily for 3 days. On 20 February he returned with a complaint of a productive cough in the mornings in addition to tightness in the chest. His lungs were clear on examination. A sputum specimen was taken for culture and, although direct streaking of the sputum yielded no Ps. pseudomallei, the organism was isolated from heart blood of hamsters dying after innoculation with the specimen. On 6 March he complained of anorexia and abdominal discofort. Again Ps. pseudomallei was isolated from heart blood of hamsters injected with sputum but not from the direct culture of the sputum. Indirect HA titer for Ps. pseudomallei which was less than 1:20 in January rose to 1:2,560. On 20 March he complained of fever and shaking chills in the afternoons for the previous 5-6 days but his coughing had decreased. A history of past illness indicated he had chest pains before and had been to the Prachinburi Provincial Hospital in October 1965. A physical examination at that time revealed no positive signs and he was given symptomatic treatment. No chest X-ray was taken. Since then he had experienced chest pains with or without coughing from time to time.

A physical examination carried out on 20 March 1969 revealed a 26 year old male with a temperature of 99.6 F and normal pulse and respiration rates. There was tenderness of the left anterior chest wall at the 3rd and 4th intercostal spaces. No crepitation or rhonchi were heard on ausculation. Liver and spleen were not palpable. A blood culture was negative but his serum antibody titer was 1:640. Immediate hospitalization in Bangkok was advised but the patient waited until 4 April when he was hospitalized at the Phra Mongkutklao (Thai Army) Hospital in Bangkok. A sputum specimen obtained the day before was negative for Ps. pseudomallei.

Laboratory studies were carried out on 9 April 1969. His white blood count was 7,850/cu.mm. with 28% polymorphonuclear neutrophils, 70% lymphocytes and 2% eosinophils. Hemoglobin was 11 grams %; VPC was 43 volume % and ESR was 11 mm/h. Other chemistry values and urinalysis findings were normal. A stool examination was positive for hookworm ova. Chest X-rays were normal. Sputum cultures on 9 and 10 April were negative by direct culture but positive for Ps. pseudomallei by hamster inoculation. His melioidosis HA titer on 8 April was 1:160.

While in the hospital he had no fever; the chest pain diminished; the coughing became less frequent and sputum became scanty. On 11 April he felt much better and could produce no sputum. However, he was given 0.5 g of chloramphenicol every 6 hours by mouth for a total dose of 25 g. On 16 April, another chest X-ray was taken and was normal as before. Cultures of sputum specimens obtained on 16, 17, 18 and 21 April was negative and his melioidosis HA titer on 17 April was 1:1,280. On 18 April his WBC was 7,150/cu.mm. with 42% neutrophils, 55% lymphocytes and 3% eosinophiles. Hemoglobin was 14.1 g%; VPC 44 vol.% and ESR 10 mm/h. He was discharged symptom-free on 23 April and remained free of clinical symptoms during this reporting period.*

The second case was a 16 year old brother of the first case. He was first seen at the village clinic in January 1969 with a history of chest pains after hard work and on cold days during the last year. Physical examination revealed a young man too small for his age with normal chest findings. In May 1970 he was taken to Prachinburi Provincial Hospital for chest X-rays which were normal. His HA titer of serum taken at that time was less than 1:10. On 25 July he returned to the clinic with complaints of fever and joint pain. His HA titer had risen to more than 1:10,240. On 14 August he was seen with the same complaints plus pain in the calves. He did not feel feverish although his body temperature was 100 F. His chest was clear and his liver and spleen were not palpable. There was slight enlargement of left submandibular lymph node. Hemocultures yielded no growth and blood films for malarial parasites were negative. A sputum specimen was positive for Ps. pseudomallei by hamster inoculation but not by direct culture. His HA titer vs Ps. pseudomallei was 1:2,560. On 11 September 1969 he felt weak and had a productive cough. Sputum taken then was positive by both direct culture and hamster inoculation and his HA titer was 1:20,480. Chest X-rays taken on 18 September were negative except for increased lung markings in the left upper lobe area. A CBC done on the same day revealed that he had Hgb 11 gm%, Hct 38%, WBC 7,500 with 35% PMN, 53% L and 12% E. Stool examination was positive for Taenia spp. ova. His sputum was again positive for Ps. pseudomallei. No antibiotics were given and during the next month his cough and joint pains disappeared. However his sputum was still positive and his HA titer was 1:655,360. In November he felt much better but was unable to do hard work. He had gained 5 lbs since September (from 80 to 85 lbs).

*In mid April 1970 this patient had a relapse as indicated by isolation of Ps. pseudomallei from his sputum.

He was unable to produce sputum for culture during the remainder of the study period. The result of his subsequent HA titers were as follows:

Date	HA titer
11 November 69	1:20,480
16 December 69	1: 5,120
14 January 70	1:81,920
11 February 70	1:20,480
12 March 70	1: 5,120

He has had no additional clinical symptoms and had returned to his regular farm duties at the time this report was prepared in April 1970.

4. Antimicrobial sensitivities of Ps. pseudomallei. The plate dilution procedure was used to determine antimicrobial sensitivities of 104 isolates of Ps. pseudomallei isolates from water, soil, and specimens from patients with suspected melioidosis. Overall these in vitro studies showed that the most effective antimicrobial studied was novobiocin followed in order of decreasing effectiveness by kanamycin, chloramphenicol, oxytetracycline, rifampin, and neomycin. Isolates from patients tended to be more resistant than those from soil or water. No isolates were sensitive to 0.78 mcg/ml or less of any of the antimicrobials tested and none from humans was sensitive to 3.12 mcg/ml or less.

SUMMARY: During this period the presence of Ps. pseudomallei was documented in Yala, Songkla, Prachinburi and Maehongsorn Provinces but was not detected in water soil samples from Vientiane, Laos. Fifteen of 878 Thai nationals from the north, central and southern areas of Thailand had significant (1:80 or greater) HA titers against Ps. pseudomallei. Sera from water buffaloes and cattle (but not rodents) showed evidence of immunologic response to that organism. Based on clinical and laboratory studies a second melioidosis patient was diagnosed in Ban Huay Jode, a study area where 18% of the 219 residents tested had significant HA titers against Ps. pseudomallei. In vitro studies of isolates of Ps. pseudomallei showed novobiocin was the most effective of the 6 antimicrobials tested.

Table 1. Melioidosis Epidemiology Study
HA Antibody Titers of Ban Huay Jode Residents
(1 April 1969 - 31 March 1970)

Age groups (years)	Number of residents bled		Total with positive titers		Total with titers of 1:80 or greater	
	Male	Female	Male	Female	Male	Female
Under 5	12	12	2	0	0	0
5-9	29	31	6	2	3	1
10-19	25	24	11	8	9	7
20-29	5	11	2	6	1	2
30-39	10	18	5	6	3	4
40-49	11	12	4	6	3	2
50-59	4	5	2	1	2	0
60 and over	7	3	3	1	3	1
Total	103	116	35	30	24	17

Insecticide Susceptibility in the Oriental House Fly (Musca domestica vicina) in Thailand

Principal Investigators : Michael F. Sullivan, CPT, MSC
Somkiet Vongtangswad, MPH*

Associate Investigator : Pacharee Nawarat, B.S.

Assistant Investigator : Somboon Maneechai

OBJECTIVE : The oriental house fly (Musca domestica vicina) is common throughout Thailand. Efforts to control this and other insect pests in Thailand by the widespread use of a variety of insecticides has led to the development of tolerance to these chemicals in several species. The object of this study is to determine the susceptibility level of the oriental house fly to various insecticides that are at present or may be in the future used in Thailand for its control.

DESCRIPTION : Adult oriental house fly specimens are collected in the field, taken to SMRL and colonized. Reared adult females are tested three to seven days after emergence in the F_1 generation by methods outlined in Memorandum Number 3 (Methods for Determining the Susceptibility or Resistance of Insects to Insecticides) of the U.S. Armed Forces Pest Control Board.

PROGRESS : The level of insecticide resistance in the oriental house fly has been determined for DDT, malathion and lindane in Bangkok, Cholburi, Nakhon Ratchasima, Trat, Samut Songkhram and Nakhon Sawan (Tables 1 & 2). The lethal concentration (LC) and lethal time (LT) values of the above strains are compared to that of a susceptible (USDA, Gainesville). A ratio of three or greater indicates a significant level of resistance. Significant resistance has been found to lindane in all areas tested, to DDT in Nakhon Ratchasima and Trat, and to malathion in Nakhon Ratchasima. Borderline levels of resistance were found to DDT in Cholburi and Samut Songkhram.

* Lecturer, School of Public Health, Mahidol University

Table 1. Susceptibility level (LC-50 and LC-90) of six strains of the oriental house fly from Thailand compared with a susceptible strain of the house fly (15 min. exposure)

Strain	Insecticide	LC-50*		LC-90*	
		% Concentration	Ratio to Regular	% Concentration	Ratio to Regular
Susceptible	DDT	0.054	-	0.19	-
Bangkok	"	0.13	2.4	0.47	2.5
Cholburi	"	0.17	3.1	0.35	1.8
Nakhon Ratchasima	"	0.47	8.7	1.1	5.8
Trat	"	0.44	8.1	>2.5	>13.1
Samut Songkhram	"	0.17	3.1	0.32	1.7
Nakhon Sawan	"	0.14	2.6	0.28	1.5
Susceptible	Lindane	0.0059	-	0.024	-
Bangkok	"	0.07	11.9	>1.0	>41.6
Cholburi	"	0.0061	1.0	0.135	5.6
Nakhon Ratchasima	"	0.09	15.3	>1.0	>41.6
Trat	"	>1.0	>169.6	>1.0	>41.6
Samut Songkhram	"	0.015	2.5	>1.0	>41.6
Nakhon Sawan	"	0.7	118.6	>1.0	>41.6

LC-50 & LC-90 : concentrations of insecticides required to kill 50% and 90%, respectively, of the exposed population in a specified period of time.

Table 2. Susceptibility level (LT-50 and LT-90) of six strains of the oriental house fly from Thailand compared with a susceptible strain of the house fly.

Strain	Insecticide	LT-50*		LT-90*	
		Number of minutes for knockdown	Ratio to regular	Number of minutes for knockdown	Ratio to regular
Susceptible	malathion 27 mg/m^2	31	-	42	-
Bangkok	"	15	0.5	21	0.5
Cholburi	"	21	0.7	27	0.6
Nakhon Ratchasima	"	22	0.7	>300	8.1
Trat	"	17.5	0.6	22	0.5
Samut Songkhram	"	20	0.6	32	0.8
Nakhon Sawan	"	22.5	0.7	29	0.7

LT-50 & LT-90 : length time required to kill 50% and 90%, respectively, of the exposed population at specified concentrations of insecticides.

Insecticide Tolerance Level of Fleas from Southeast Asia

Principal Investigators: Edward W. Davis, SFC
Nongnuj Maneechai
Harold E. Stark, Ph.D.

OBJECTIVE: To determine resistance in populations of fleas from Southeast Asia to commonly used insecticides.

DESCRIPTION: Testing procedures were conducted in accordance with the recommendations of the World Health Organization. Ten fleas were exposed for 1 hour to papers impregnated with varying concentrations of the insecticides, and then permitted to rest on clean paper in test tubes for 23 hours. With replicates, up to 100 fleas were tested in each exposure. One major exception to the above involved exposure to DDT. Many colonies were so resistant to this chemical that no mortality occurred after 1 hour exposure to paper impregnated with 4 per cent DDT [the highest concentration provided by WHO]. Rather than increase the concentration, exposure time to 4 per cent DDT was increased to 2,4,8,16 and 32 hrs.

Pesticides tested during this period included DDT, lindane, dieldrin, malathion, fenthion and carbaryl. Impregnated papers were prepared according to WHO standard techniques and were supplied by WHO and US Army Environmental and Hygiene Agency. Papers impregnated with lindane and carbaryl were prepared by SEATO Medical Research Laboratory in accordance with WHO standard techniques.

PROGRESS: Preliminary tests in Bangkok and Nha Trang indicated existence of high resistance of the Xenopsylla cheopis populations in those two cities to chlorinated hydrocarbons, especially DDT. Colonies of Xenopsylla cheopis from the Vietnamese provinces of Darlac [Pleiku] Khan Hoa [Nha Trang], Tuyen Duc [Dalat], and Binh Dinh [Qui Nhon] and from the provinces of Phranakhon [Bangkok], Surat Thani [Koh Samui], Nakhon Ratchasima [Korat] and Chieng Mai in Thailand were established in the SMRL insectary. In addition, a second species, Stivalius klossi, from Pak Thong Chai, Nakhon Ratchasima province, was tested for resistance/susceptibility to DDT, lindane, malathion, fenthion, and carbaryl. A strain of Xenopsylla cheopis from Florida [Gainesville], considered by USDA and the USPHS Technical Development Laboratories to be susceptible to DDT, was used in these tests as a standard for comparison.

Separate colonies of X. cheopis, from the same site in Bangkok, were initiated from different hosts [Rattus norvegicus and R. exulans]. Extensive tests [at least 15 each] were carried out with lindane and carbaryl. The LC_{50} and the mortality at different concentrations were so similar that the colonies were combined without regard to host of origin. Comparisons of deaths of X. cheopis from exposure to all insecticides were made on the basis of original collections from different hosts in Vietnam also, but not necessarily from the same site, and no significant differences were observed. The hosts were house shrew [Suncus murinus], house rat [Rattus exulans], roof rat [R. rattus] and Norway rat [R. norvegicus].

Of particular interest is the fact that no change in resistance was evident on comparison of colonies of X. cheopis initiated before and after a massive plague control dusting program was conducted in Vietnam during 1968 and 1969 in which the principal insecticide used was 10 per cent DDT dust. Fleas were collected from Pleiku, Dalat and Qui Nhon and tested both before and after the program, and no difference in mortality to DDT or any other pesticide tested was noted in pre-and post-control program samples. It is possible that a change in level of resistance takes place over a period of time, though this could not be demonstrated with Vietnam colonies collected a few weeks apart following the plague control program.

DDT: All X. cheopis from Vietnam tested were so resistant to DDT that the standard 1 hour exposure to DDT-impregnated paper was inadequate to demonstrate any significant mortality. In hopes of demonstrating mortality and variation between collection sites, exposure times to 4 per cent DDT were increased to 1,2,4,8,16 and 32 hours. A few deaths [4 per cent-16 per cent mortality], resulted following 4 hours exposure in 3 of 10 colonies and at 32 hours exposure some deaths [12-54 per cent of the fleas] occurred among all colonies. The LC_{50} could not be calculated since exposure to all other pesticides was for 1 hour. However 54 per cent of fleas from a colony established from Binh Dinh province [Qui Nhon, Tuy Hoa] died after 32 hours exposure to 4 per cent DDT. The mortality rate, following this exposure time, was less than 50 per cent of fleas for the other Vietnamese strains of X. cheopis tested.

Xenopsylla cheopis from Thailand were highly variable in response to exposure to DDT. Those from Koh Samui were as resistant as any from Vietnam; after 4 hours exposure to 4 per cent DDT only 2.1 per cent of the fleas died, and after 32 hours exposure only 27 per cent died. The only colonies of X. cheopis susceptible enough to calculate an LC_{50} [based on 1 hour exposure] came from Klong Toey in Bangkok and from Gainesville, Florida. The LC_{50} for the Gainesville strain was 3.4 per cent. The WHO has established that the LC_{50} for a susceptible strain of X. cheopis, unsexed, ranges from 0.35-0.7 per cent and the LC_{100} from 2.0-4.0 per cent. On longer exposure, Gainesville fleas are clearly more susceptible than any from Vietnam or Thailand. For

these fleas the mortality was 51 per cent after 1 hour, 54 per cent after 2 hrs., 92 per cent after 4 hrs., 98 per cent after 8 hrs and 100 per cent after 16 and 32 hours, respectively, exposed to paper impregnated with 4 per cent DDT. A sylvan flea, Stivalius klossi, which occurs in Thailand in dry evergreen forest on Rattus surifer, was included in these for comparison since this species has probably never been exposed to DDT. It was completely susceptible to DDT, for the LC_{50} for this species was 1.4 per cent. The mortality of S. klossi exposed for 1 hour to 2 per cent DDT was 67 per cent [90 fleas tested]. After a 1 hour exposure to 4 per cent DDT mortality in S. klossi was 97 per cent, and a complete kill was obtained following more than an hour's exposure.

LINDANE AND DIELDRIN: These insecticides were quite effective in the concentrations tested. The LC_{50} for lindane with X. cheopis from Vietnam ranged between 0.3 and 0.9 per cent and a mortality rate of 77-100 per cent was obtained after a 1 hour exposure to papers impregnated with 2 per cent lindane. Fleas from Thailand were a little more susceptible. The LC_{50} was between 0.2 per cent and 1.0 per cent when they were exposed to lindane impregnated papers. Mortalities of 94-100 per cent occurred following a 1 hour exposure to paper impregnated with 2 per cent lindane, while 75-100 per cent of X. cheopis died after a 1 hour exposure to 1.6 per cent dieldrin. The LC_{50} for dieldrin ranged between 0.4 and 1.4 per cent.

There appears to be some correlation between resistance of X. cheopis to DDT and resistance to lindane and dieldrin. This was evident in colonies from Pleiku and Nha Trang, Vietnam and from Koh Samui, Thailand [Table 1]. Dieldrin was tested against as many colonies as lindane. Stivalius klossi was not resistant to lindane impregnated papers [LC_{50} 0.3 per cent lindane].

MALATHION AND FENTHION: The organophosphates malathion and fenthion appear to be satisfactory pesticides for controlling X. cheopis. Results were quite uniform among the various colonies of X. cheopis tested. The LC_{50} for malathion varied between 2 per cent and almost 5 per cent. When X. cheopis was exposed to paper impregnated with 5 per cent malathion, mortality was about 60-100 per cent. One hour's exposure of X. cheopis to paper impregnated with 2.5 per cent fenthion was highly lethal [between 84 and 100 per cent mortality]. Less than 8 per cent of 40 Stivalius klossi exposed to paper impregnated with 2.5 per cent fenthion died. The mortality rate of 80 S. klossi exposed to paper impregnated with 5 per cent malathion was only 30 per cent. Thus, S. klossi is apparently highly resistant to these two pesticides.

CARBARYL: Mortality from exposure to papers impregnated with varying concentrations of Carbaryl [Sevin] was erratic and sometimes difficult to correlate with a series of concentrations for a given colony. The LC_{50} calculated for various strains of fleas varied from about 0.4 per

cent to more than 4 per cent. Since a regular progression of mortality was not always obtained, estimates of the LC_{50} were not considered reliable. Furthermore, it was observed that impregnated papers deteriorated rapidly during testing procedures. Thus further variation was thrown into the accumulating results. When the above difficulties with the bioassay were encountered, a chemical assay of our test papers was undertaken on request of the USPHS Technical Development Laboratories in Savannah. They determined that concentrations in our freshly prepared test papers were very close to stated values.

Another unexpected but interesting problem was that of quick knock down. Fleas which appeared dead on removal from impregnated papers often recovered after the 23 hour observation period. An even more confusing fact was that often at higher concentrations different fleas were dead after the 23 hour period than were first knocked down. Table 2 shows per cent knock down and mortality of fleas from all colonies from all areas combined. The knock-down effect [first column] appears to be negligible below a concentration of 0.25 per cent carbaryl. In many individual tests involving 10 fleas the initial knock down from higher concentrations exceeded final mortality. In the third column the values are really averages so these individuals are not revealed. The knock down effect is not increased beyond a concentration of 1 per cent carbaryl.

When added together and averaged, the erratic results of each test become surprisingly uniform. When fleas were exposed to 4 per cent carbaryl [the highest concentration prepared] the mortality averaged 57 per cent for fleas from Vietnam and 60 per cent for fleas from Thailand. The LC_{50} averaged 1.2 per cent for Vietnamese strains and 1.7 per cent for Thai fleas. However, the mortality within individual colonies varied between 32 per cent and 90 per cent. In view of the difficulty in finding differences, and the apparent unreliability of data obtained because of reasons discussed above, existence of resistance or susceptibility to carbaryl remains undetected. Variation between areas cannot be demonstrated.

Values for X. cheopis from Gainesville, Florida, and for Stivalius klossi [Table 1] are within the range of values discussed above.

Table 1. Preliminary Results of Pesticide Tests on Colonies of Xenopsylla cheopis and Stivalius klossi Reared from Indicated Collection Sites, Vietnam

Pesticide / Location	LC_{50}	4% DDT Percent Mortality Time Exposure				LINDANE		DIELDRIN		MALATHION		FENTHION	CARBARYL	
		4	8	16	32	LC_{50}	P.M.* at 2.0%	LC_{50}	P.M. at 1.6%	LC_{50}	P.M. at 5.0%	P.M. at 2.5%	LC_{50}	P.M. at 4%
Darlac, Pleiku [Post Dust]	*	2	6	10	12					[4.2]	59	85	[>4]	32
Darlac, Pleiku [Pre Dust]	*	0	13	30	60	0.34	99	[1.0]	85	4.7	[60]		[0.5]	89
Khanh Hoa, Nha Trang Xon Bon [Post]	*	16	14	33	21	0.4	100	0.8	84	3.7	78	97	0.96	57
Khanh Hoa, Nha Trang Phuong Cui [Post]	*	0	0	17	20	0.34	100	0.4	100	3.0	[75]		0.38	100
Tuyen Duc, Dalat Vo Thanh [Pre]	*	0	11	20		[0.6]	100	[0.6]	90	2.5	[75]		[0.5]	90
Binh Dinh, Qui Nhon Nguyen Do [Pre]	*		0	10						2.0	[80]		[1.0]	74
Binh Dinh, Qui Nhon Nguyen Do [Post]	*	7	4	29	54					1.2	100	100		
Binh Dinh, Qui Nhon Tuy Hoa [Post]	*	0	0	10	31	[0.9]	77			[4.2]	59			
Binh Dinh, Qui Nhon Cuong De [Post]	*	0	0	2.4	21.4	[0.7]	93							

Table 1. Continued; Thailand and U.S.A.

Location \ Pesticide	LC_{50}	4% DDT Percent Mortality Time Exposure 4	8	16	32	LINDANE LC_{50}	LINDANE P.M.* at 2.0%	DIELDRIN LC_{50}	DIELDRIN P.M. at 1.6%	MALATHION LC_{50}	MALATHION P.M. at 5.0%	FENTHION P.M. at 2.5%	CARBARYL LC_{50}	CARBARYL P.M. at 4%
Bangkok, Phayathai Din Dang Road	*	30	30	88	79	0.24	100			[1.6]	95	100	[1.0]	54
Bangkok, Klong Toey 1969	3.1	42	66	73	81	[0.25]	100			[1.6]	100	100	4	48
Bangkok, Klong Toey 1966	3.6	35	72	95	98	[0.21]	100	0.4	100	[2.9]	83		[0.4]	53
Surat Thani Koh Samui	*	2	0	4	27	[1.0]	94	1.4	75	[0.96]	100	100	[1.5]	86
Nakhon Ratchasima Korat	*	14	22	48	29					[0.8]	100	100	[1.5]	61
Chiengmai										[3.0]	83	84		
Florida Gainesville	3.4	54	92	98	100	[0.2]	100	[0.6]	100	[2.5]	99	100	[1.3]	67
Stivalius klossi Nakhon Ratchasima Pak Thongchai	1.4	100	100	100	100	0.3	100			7.6	31	8	4	50

P.M. = Percent Mortality at concentration listed
* = LC_{50} beyond measure
[ ] = Estimated on probit scale-not calculated
LC_{50} = Lethal concentration expressed as percent concentration of pesticide

Table 2. Percent Knock Down and Percent Mortality of 900 Xenopsylla cheopis Exposed for 1 Hour to Paper Impregnated with each Indicated Concentration of Carbaryl.

CONCENTRATION [%]	Percent "down" of 900 fleas after 1 hour exposure	Percent Mortality of 900 fleas after 23 hours rest	Percent of down/dead Total:
4.0	47	54	47/53
2.0	42	47	47/53
1.0	41	45	48/52
0.5	20	31	39/61
0.25	20	30	41/59
0.125	6	15	27/73
0.0625	2	10	14/86
0.03125	0.1	5	2/98

Distribution and Ecology of Ectoparasites of Vertebrates in Southeast Asia

Coordinator: Douglas J. Gould, Ph.D., Chief, Department of Medical Entomology

Principal Investigators: Carleton M. Clifford, Ph.D.[1]
Panita Lakshana, B.Sc.
K.C. Emerson, Ph.D.[2]
Harry Hoogstraal, Ph.D.[3]
Joe T. Marshall, Ph.D.
Harold E. Stark, Ph.D.

Associate Investigators: M. Nadchatram[4]
Edward W. Davis, SFC
Inkam Inlao
Nongnuj Maneechai

Assistant Investigators: Nittaya Klaimanee
Prachong Punthusiri

OBJECTIVE: To assemble information on the geographic and seasonal distribution and the host-parasite relationships of the ectoparasites of vertebrates in Southeast Asia.

DESCRIPTION: Ectoparasites are removed from mammals, birds and other vertebrates collected in selected study sites and in connection with various disease studies in Thailand and elsewhere in Southeast Asia. The ectoparasites are preserved, sorted into major groups and identified at SMRL or submitted to specialists abroad for identification. Aliquots of collections used for inoculations of test animals are given priority in these identifications. Studies on the taxonomy and ecology of the various vertebrate hosts are also conducted.

PROGRESS: During this period ectoparasites were collected from small mammals trapped in three habitats representative of a major part of Thailand-dry evergreen forest, deciduous dipterocarp forest and

---

1 Rocky Mountain Laboratory, Hamilton, Montana
2 Office of the Assistant Secretary Research and Development, Dept of the Army, Washington, D.C. 20310
3 Head, Medical Zoology, US Naval Medical Research Unit No.3 Cairo, Egypt
4 Institute for Medical Research, Kuala Lumpur, Malaysia

land under agricultural use-to determine the types of ectoparasite faunas associated with these habitats. Grids of 81 trap sites have been established in each of the first two types of habitat at the Trend site at Sakaerat in Nakhon Rachasima province, and collections of mammals have been made on four consecutive nights each month to measure the response of ectoparasite populations to seasonal changes. A total of 432 collections of mammals have been made in the three areas to date. Analysis of data on the relative abundance, habitat, host and seasonal distribution of ectoparasite species from these areas is underway.

The immature stages of four species of ticks collected during this period, Dermacenter auratus, Haemaphysalis bandicota, Rhipicephalus sanguineus and Ixodes granulatus were reared through to the adult stage at SMRL. Eggs have been obtained from the first three species, and it is anticipated that colonies of these species will be established for use in insecticide susceptibility tests and possible virus and rickettsial transmission studies. Host and distribution records on ticks collected in Thailand by SMRL and from other sources have been compiled and a summary of these data for the 48 species recorded from the kingdom is given in Table 1.

Three species of trombiculid mites described from Thailand, but not previously represented in SMRL collections, were identified during this period. These species were 1] Ascoshongastia (Laurentella) kittii from Rattus rattus, R. sabanus and R. fulvescens in Chieng Mai, 2] Sasatrombicula siamensis from Rhinolophus luctus in Chieng Mai, and 3] Whartonia prima from Hipposideros armiger in Chantaburi province. A fifth species, Leptotrombidium (L.) nakatae collected in Chiengmai, constitutes a new record for Thailand.

Table 1. A Checklist of the Ticks Recorded from Thailand

	Locality	Host
Suborder IXODOIDEA Family IXODIDAE Subfamily IXODINAE IXODES		
Ixodes [Lepidixodes] kopsteini [2] [4] Ref: Kohls and Clifford 1968	Saraburi	Tadarida plicata, Taphozous theobaldi
Ixodes granulatus	Chiengmai, Chieng Rai, Nan, Udon Thani, Ubon Ratchathani, Nakhon Ratchasima, Prachinburi, Surat Thani, Trang, Song Khla, Pattani, Yala, Narathiwat, Phuket	Tupaia glis, Suncus murinus, Homo sapiens, Tamiops macclellendi, Callosciurus erythraeus, C. finlaysoni, C. notatus, Dremomys rufigenis, Menetes berdmorei, Mus nitidulus, Bandicota sp., R. rattus, R. exulans, R. surifer, R. sladeni, R. cremoriventer, R. mulleri, R. sabanus, R. niviventer, R. berdmorei, Felis bengalensis
Ixodes spinicoxalis	Chiengmai	Pitta oatesi, Muscicapa grandis, Tupaia glis
Ixodes paradoxus [4]	information not available	
Ixodes radfordi	Chiengmai	Rattus surifer
Subfamily RHIPICEPHALINAE BOOPHILUS		
Boophilus microplus	Chiengmai, Narathiwat	"cow"

Table 1 [Continued]

	Locality	Host
RHIPCEPHALUS Rhipicephalus sanguineus	Kanchanaburi, Nakhon Ratchasima, Prachinburi, Pra Nakhon	Hylobates lar, Homo sapiens, Rattus rattus, Bandicota bengalensis, B. indica, Canis familiaris
Rhipicephalus haemaphysaloides	Chiengmai, Udon Thani, Ubon Ratchathani, Nakhon Ratchasima, Chon Buri, Surat Thani	Macaca irus, Homo sapiens, Bandicota indica, Rattus rattus, Rattus berdmorei, Mus caroli, Mus cervicolor, Lepus siamensis, Felis domesticus, Canis familiaris, "Goat", "Cattle"
Subfamily AMBLYOMMINAE HAEMAPHYSALIS		
Haemaphysalis [Haemaphysalis] capricornis[2] Ref; Hoogstraal 1966	Extreme southern provinces	Capricornis sumatraensis
Haemaphysalis [Haemaphysalis] atherurus[4] [2] Ref: Hoogstraal et al 1965a	Loei	Atherurus macrourus
Haemaphysalis [Haemaphysalis] formosensis [4] group	Information not available	
Haemaphysalis bispinosa spp[4]	Information not available	
Haemaphysalis canestrinii [Supino][3,4]	Chiengmai, Chieng Rai, Chonburi	Bandicota indica, Melogale personata, Viverricula indica

Table 1. [Continued]

	Locality	Host
Haemaphysalis indica group[4] Haemaphysalis near indica group [4]	Information not available	
Haemaphysalis [Kaiseriana] anomala Warb. 1913 [H. cornigera anomala Warb. 1913][4][2] Ref: Hoogstraal et al 1967	Chiengmai, Loei	Homo sapiens, Bubalus bubalus
Haemaphysalis [Kaiseriana] papuana	Surat Thani, Yala	Copsychus saularis, Rattus bowersi, R. rattus, R. surifer
Haemaphysalis [Kaiseriana] nadchatrami [2,4] Ref: Hoogstraal et al 1965b	Satun, Trang	Homo sapiens, Tapirus indicus
Haemaphysalis [Kaiseriana] kinneari[4]	Information not available	
Haemaphysalis [Kaiseriana] hystricis [2,4] Ref: Hoogstraal et al 1965c	Chiengmai	Homo sapiens, Arctonyx collaris
Haemaphysalis [Kaiseriana] obesa [4] [2] Ref: Hoogstraal et al 1966	Sakhon Nakhon, Nakhon Ratchasima, Satun	Sus scrofa, Homo sapiens, Tapirus indicus
Haemaphysalis [Kaiseriana] semermis [2] Ref: Hoogstraal et al 1965b 1966	Satun [holotype]	Tapirus indicus
H. [K] lagrangei	Thailand	Unknown

Table 1. [Continued]

	Locality	Host
Haemaphysalis [Rhipistoma] asiaticus [Supino] [H. dentipalpis Warb & Nutt1909]	Chiengmai, Loei	Civet, Felis bengalensis
H. [Rhipistoma] near heinrichi	Nakhon Ratchasima, Phra Nakhon	Canis familiaris, Melogale personata
Haemaphysalis [Rhipistoma] bandicota	Chiengmai, Saraburi, Chonburi	Bandicota indica, Bandicota sp.
Haemaphysalis [Rhipistoma] doenitzi[4]	Bang Phra, Chonburi	Acridotheres tristis
Haemaphysalis [Rhipistoma] koningsbergeri [4]	Malaya [Berak]	Arctogalidia trivirgata
Haemaphysalis [Rhipistoma] ornithophila [2] Ref: Hoogstraal and Kohls 1959	Information not available	
Haemaphysalis megaleimae[4]	Information not available	
Haemaphysalis traguli[2,4] Oudemans 1928 [=H. monospinosa Krijgsman & Ponto 1932] Ref: Hoogstraal 1964a	Khon Kaen	Tragulus javanicus
Haemaphysalis wellingtoni	Chiengmai, Bang Phra, Nakhon Ratchasima, Saraburi	Gallus gallus, Centropus sinensis, Glaucidium cuculoides, Pitta moluccensis
Haemaphysalis [Haemaphysalis] probably darjeelingi	Prachinburi	Melogale personata

Table 1. [Continued]

	Locality	Host
DERMACENTOR Koch 1844		
Dermacentor auratus	Nakhon Ratchasima, Saraburi, Trang, Song Khla, Yala, Narathiwat	*Tupaia glis*, *Homo sapiens*, *Mus cervicolor*, *R. sladeni*, *R. rattus*, *R. bowersi*, *R. niviventer*, *R. surifer*, *R. sabanus*, *Cannomys sp.* *Melogale personata*
AMBLYOMMA Koch 1844		
Amblyomma sublaeve Neumann 1899 [=*A. javanense* Supino 1897]	Chiengmai, Udon Thani, Nakhon Ratchasima	*Homo sapiens*, *Manis javanica*
Amblyomma helvolum	Samut Prakan, Chonburi, Surat Thani, Narathiwat	*Varanus bengalensis*, *Varanus salvatar*, *Varanus* sp., *Python reticulatus*
Amblyomma geoemydae	Chanthaburi, Nakhon Si Thammarat	*Lacedo pulchella*, *Pitta caerulea*, *Zoothera marginata*
Amblyomma cordiferum[2]	Information not available	
Amblyomma testudinarium[1]	Information not available	
Amblyomma cyprium?	Surin	Turtle
APONOMMA		
Aponomma gervaisi	Ratchaburi, Surat Thani	*Varanus* sp.
Aponomma lucasi	Chiengmai, Khon Kaen, Surat Thani	*Varanus* sp., *Python reticulatus*, *Naja naja*, *Rattus rattus*
Aponomma pattoni?	Nakhon Ratchasima, Khon Kaen	*Naja naja*

Table 1. [Continued]

	Locality	Host
FAMILY ARGASIDAE ARGAS Argas [Carios] pusillus Kohls	Chiengmai	Pelecanus philippinensis
Argas [Persicargas] persicus	Samut Prakarn	Bat
Argas [Persicargas] robertsi [2] Ref: Hoogstraal et al 1968	Pathumthani	Anastomus oscitans
ORNITHODOROS Ornithodoros sp., [Reticulinosus batuensis group]	Chiengmai, Prachinburi	Rousettus leschenaulti
Ornithodoros [Alectorobius] capensis	Chohburi, Rayong	Sterna sumatrana, Sterna anaethetus, Rousettus leschenaulti
Ornithodoros sp. [Fainigroup] 9L [3 retained]	Saraburi	Scotophilus heathii, Rousettus leschenaulli
Ornithodoros kelleyi	Thailand	

[1] Reported only in SMRL Annual Progress Report of 1964
[2] Reported in Literature
[3] Represented in SEATO Museum Collection
[4] Represented in RML Museum Collection from SEATO specimens

Plague Occurrence in Wild and Domestic Rodents and their Fleas.

Principal Investigator: Harold E. Stark, Ph.D.

Associate Investigators: Jung Hyun Cho, MD*
Choon Hyun Hwang, MD*
H. Kim D.V.M.*

Assistant Investigators: Edward W. Davis, SFC
Edward Hubster, SFC**
Sung Chan Lee*
Sang Chan Nam*

OBJECTIVE: To assess the relative importance of domestic rodents and their fleas as related to plague in South Vietnam.

DESCRIPTION: Data concerning the occurrence of plague, fleas and urban and rural pest mammals were made available from the plague laboratory at the Institute Pasteur in Saigon [sponsored by Walter Reed Army Institute of Research Medical Team]. Data were obtained from ongoing routine small mammal collections [surveys to detect plague] by the 20th Preventive Medicine group and the Ministry of Health. A large amount of data was also provided by the Korean Preventive Medicine Team. These data are the outgrowth of a project commenced by SEATO Medical Research Laboratory when a plague laboratory was set up at the Institute Pasteur in Nha Trang. The work of receiving small mammal collections in region II in coastal and highland areas has continued. Completion of the tabulation of these data is now underway at SEATO.

RESULTS: During the period from September 1967 through September 1969 a total of 14, 064 hosts belonging to the species Suncus murinus, Rattus norvegicus, R. rattus, R. exulans, Bandicota indica and B. bengalensis were submitted to the plague laboratory at Saigon. Occasional captures of other hosts [85 specimens] include such species as Tupaia glis, Herpestes javanica, Rattus rajah, R. diardi, R. jalorensis and R. argentiventer. From the period January 1967 through April 1969 a total of 1012 fleas from the 6 principal hosts submitted to the Saigon laboratory were identified. From the collections submitted to the plague laboratory in Nha Trang from October 1968 through January 1970 a total of 2463 hosts were examined and

---

* Korean Preventive Medicine Team, Region II, Nha Trang
** US Army Medical Research Team [WRAIR] Saigon

the majority of these belonged to the same 6 principal host species. During the same period a total of 5026 fleas were identified in the plague laboratory at Nha Trang.

Of the 1012 fleas identified at Pasteur Institute in Saigon 99.5% were Xenopsylla cheopis and 0.5% were other species [Ctenocephalides felis, C. canis and wild rodent fleas]. The list of flea species other than X. cheopis is larger from data collected at the Nha Trang laboratory and the proportion of X. cheopis is less [nearly 90%]. Wild rodent fleas continued to be identified and species are the same as were presented in the annual report of 1969. Published accounts indicate the proportion of X. cheopis to other species in Hanoi is only 68% so there appears to be a decrease in relative prevalence of X. cheopis from south to north.

The mean number of X. cheopis per infested host [6 common hosts] is 1.50 for Saigon data. The preferred hosts for X. cheopis are probably Rattus norvegicus and Rattus rattus. For these rats the mean number of X. cheopis per infested host averaged 2.5 and 2.6 respectively, according to Saigon data. R. exulans had only 0.55 fleas per infested host which suggests R. exulans is less suitable host for X. cheopis. Suncus murinus had 1.2 fleas per infested host and Bandicota indica and B. bengalensis seldom had fleas. In a few rural areas bandicoots had numerous X. cheopis. In these places they averaged 2.0 and 4.6 fleas per host. From Nha Trang, mean numbers of fleas per host were nearly twice those from Saigon data indicating fleas to be more numerous farther north. In rural Delta areas the flea index was very low. This is consistent with the low human plague incidence in the Delta area as opposed to higher incidence in the Central Highlands and coastal area of south central Vietnam.

Populations of the 6 common host species fluctuate greatly, but no seasonal correlation was evident with this phenomenon. It is assumed that the animals replaced one another over periods of time. In Saigon the populations were more constant, with R. norvegicus predominant. In Cam Ranh Bay, R. norvegicus was replaced almost entirely by R. rattus over a long period of time starting in January 1969. The remaining 4 species [R. exulans, S. murinus, B. indica and B. bengalensis] also increased in proportion. In Tay Ninh, which is more rural, R. norvegicus appeared only occasionally and bandicoots assumed the predominant role, their numbers fluctuating in relation to populations of other mammals.

Pasteurella pestis Infection in Humans.

Principal Investigator: James H. Rust, Jr., Ph.D.

Associate Investigators: Jung Hyun Cho, MD*
Choon Hyun Hwang, MD*
H. Kim D.V.M.*

Assistant Investigators: Edward W. Davis, SFC
Edward Hubster, SFC**
Sung Chan Lee*
Sang Chan Nam*

OBJECTIVE: To determine extent of inapparent Pasteurella pestis infection in humans.

DESCRIPTION: According to description given for this report for the period 1 April 1968 to 31 March 1969 residents of Nha Trang in high risk areas who had been bled in January 1969 indicated they would submit to a second bleeding the following May.

PROGRESS: During December 1968 and January 1969, 113 sera were collected, mostly from school children aged 13 through 18, in the city of Nha Trang. Of 28 sera from non-immunized individuals, 9 positives [32.1%] were demonstrated [hemagglutination test] while 26 positive sera [36.1%] were demonstrated from 72 individuals who had received immunization. Thirteen individuals were uncertain as to their immunization status. Sera from 18 of the original 113 individuals were obtained 4 to 8 weeks later. On testing these 18 sera for plague antibody, the one positive sera remained positive, while two individuals converted from sero-negative to sero-positive in the absence of overt clinical disease or immunization.

These observations suggest that naturally occurring, sub-clinical plague infections might be one mechanism for the decline of apparent urban plague, by the process of natural immunization.

---

*Korean Preventive Medicine Team. Region II, Nha Trang
**US Army Medical Research Team [WRAIR] Saigon

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 045, Bacterial and mycotic diseases of man and animals

Literature Cited.

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OB 6467	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D, Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	046
b. CONTRIBUTING				
[redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)*

(U) Parasitic Infections of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002600 Biology; 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

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	5	300
FISCAL YEAR CURRENT 71	5	300

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* US Army Medical Component, SEATO

ADDRESS:* Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Altstatt, LTC L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Diggs, LTC C. L.

NAME: Johnsen, MAJ D. O. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Parasitic Diseases; (U) Malaria; (U) Gnathostomiasis; (U) Immunity in Parasitism; (U) Drug Resistance (U) Host-Parasite Relationships

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by Precede text of each with Security Classification Code.)

23. (U) To define the ecology of parasites of military importance in Southeast Asia providing estimates of the risk and consequences of infections with these parasites and describing effective methods of control.

24. (U) Prevalence estimates for a given parasite are made in populations of interest by serological techniques and/or by isolation and identification of the organism in suitable preparations. The disciplines of clinical medicine, veterinary medicine, medical entomology, epidemiology and parasitology are used to identify life cycles and the variables which influence the life cycles.

25. (U) 69 07 - 70 06 A promising test for in vitro antimalarial antibody activity has been developed employing re-penetration of fetal cells using falciparum malaria in vitro. A new, heretofore unidentified, species of Phaneropsolus has been identified in northeast Thailand. Dirofilaria immitus was successfully transmitted to the gibbon. Clinical symptoms were produced and an antibody response was detected by SAFA technique. For technical reports see SEATO Annual Progress Report, 1 Apr 69 - 31 Mar 70.

*Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 046, Parasitic infections of man and animals

Investigators.

Principal: Carter L. Diggs, LTC, MC

Associate: Warren Y. Brockelman, CPT, MSC, Tanongsak Bunnag, M.D., Damri Chawalitrutiwong, Tan Chongsuphajaisiddhi, M.D., Ph.D., Nipon Chuanak, Dhira S. Comer, M.D., Svasti Daengsvang, Med.D.*Kanjika Devakul, M.D., Ph.D., Robert Gentzel, SSG, Douglas J. Gould, Ph.D., Dhawee Guname, B.Sc., Bruce A. Harrison, CPT, MSC, D.O. Johnsen, MAJ, VC, Kanokwan Kanakakorn, B.Sc., Ratanaporn Kasemsuthi, B.Sc., Angoon Kiettivuti, M.S., Pravet Lertprasert, Rapee Machimasatha, B.Sc., George S. Manning, CPT, MSC, Ronald E. Marshall, SP5, E-5, Aumpon Na-nakorn, Tongchai Papasarathorn, M.D., M.P.H., S.M. (Harvard), Katchrinnee Pavanand, M.D., Barnyen Permpanich, R.N., Pirom Phisphumvidhi, B.Sc., Sajee Pinnoi, Sompone Punyagupta, LTC, MC, Thamma Sakulkaipeara, Boonsiri Sermswatsri, Phaibul Sirichakwal, B.Sc., Prasit Sookto, Vivat Suthivong, DVM, Prayot Tanticharoenyos, D.V.M., Terdpone Tesprateep, DVM, Stanley W. Theune, MSG, Chirapan Vinagupta, M.T., Vithoon Viyanant, B.S., Sopon Vuthara, Kitti Watanasirmkit, B.S., William L. Wooding, MAJ, VC, Paisal Yingyourd, B.Sc., Pichit Youngyi, B.Sc. Vithune, Yuthasastr-Kosol, M.D.

Pathology of Domestic Animals of Thailand

Principal Investigators: William L. Wooding, MAJ, VC
Vivat Suthivong, DVM[1]
Terdpone Tesprateep, DVM[2]

---

1 Bangkok Abattoir.

2 School of Veterinary Medicine, Chulalongkorn University.

Assistant Investigators: Sopon Vuthara
Damri Chawalitrujiwong

OBJECTIVE: A study of grossly visable parasites and pathological conditions in animals in Thailand.

DESCRIPTION: Parasites and pathology specimens were collected, photographed and prepared for microscopic examination. Animals from which specimens were taken included water buffalo, cattle, pigs, dogs, wild cats, and domestic cats. Because records of parasite or pathology incidence were not kept, a rough impression of incidence was all that was possible.

PROGRESS: Conditions occurring in pigs, buffalo, and cattle at the abattoir will be listed in decreasing order of incidence according to the records of the Bangkok Municipal Abattoir.

Pigs,
Macracanthorynchus hirudinaceous, the thorny-headed worm, an acanthocephalid, lodges in the small intestine and often results in enteritis or peritonitis. The incidence of this parasite is 3.7%.

Cysticercus cellulosa, the larval form of the tapeworm, Taenia solium occurs in about 1.3% of the animals and is commonly seen in pectoral and thigh muscles and the heart.

Stephanurus dentatus, the kidney worm which encysts in the perirenal area and tunnels into the ureter, is seen in about 1% of the pigs. This parasite has not been reported in Thailand according to Segal's comprehensive review[1].

Metastrongylus apri, the lung nematode of swine, occurs in 0.9% of the pigs and will also infect cattle, sheep, dog and man.

Hog cholera, or swine fever, is an acute viral disease of swine with 100% mortality. Although the occurrence in the slaughter house is .01%, one of the animals necropsied came from a neighboring pig farm that had lost 200 pigs in one week.

Cattle And Buffalo.

Paramhistoma, or rumen flukes are not reported in Thailand by Segal(1) or Mohan(2). However, according to abattoir records, 100% of the cattle and buffalo are infected with this parasite. These are twenty-four members

of the paramphistome but after attempting to classify, we agree with Soulsby who says "classification of the Paramphistomes should be left to the experts"[4].

Sarcosporidia, a muscle inhabiting protozoal parasite is present in 82% of the buffalo at the Bangkok Abattoir. This unusual parasite has been classified as a fungus and protozoa but current toxonomists place it in the Protozoa in an order with the toxoplasma. Although the parasite occurs in all warm blooded animals, birds and many poikilothermic animals, it is usually seen only on microscopic examination. However, many of the infected buffaloes contain cysts visibly grossly and up to 2 inches in length.

Elaeophora poeli, a large nematode residing in the wall of the aorta is present in 65% of the buffalo at the abattoir. A record is not kept of this parasite in cattle, but during the past year we have found a number of cattle affected also. Neither Segal[1] or Mohan[2] list this as occurring in Thailand. The male of this parasite is embedded in the aorta wall with the head of the female often resulting in a large necrotic mass frequently 1-3 inches in diameter in the intima of the aorta. The rest of the female extends out the lumen of the aorta. The female has been described as varying from 4 inches to 10 feet long, but the ones I have seen are never longer than 2 feet.

Eurytrema pancreaticum, the pancreas fluke, has been described as occurring in many countries of South East Asia including Thailand. In cattle at the Bangkok slaughter house, 25% are infected but less than 1% of the buffalo are affected. This difference may be due to a difference in feeding habits or to a less complete examination of the buffalo on the part of the inspector. The pancreatic ducts appear blocked in the affected animals but no microscopic evidence of atrophy or necrosis has been seen.

Fasciola hepatica, or liver fluke is seen in 23% of the cattle and 17% of the buffalo in Bangkok. As in other parts of the world, the livers are filled with fibrous tracts which marks the migrating parasites. In severe infections, the lining of the bile ducts and gall bladder are lined by a hard gritty crystalline material embedded in the mucosa. We have had a chemical analysis of these crystals and have found that they are pure calcium carbonate.

Syngamus laryngeus, is an unusual nematode that embeds its mouth in the mucosa of the larynx of cattle and buffalo. At the abattoir it is seen in 22% of the cattle and 10% of the buffalo. There seems to be little reaction

on the part of the host but on microscopic examination, the mouth parts of the parasites contain laryngeal epithelium. This has not been reported in Thailand according to Segol, [1] Mohan [2] and two current parasitology texts Soulsby[4] and Levine[5].

Thelazia rhodesii, the eye worm, is easily missed on cursory examination of the eye. Two different surveys in the last two years at the abattoir have found 20% of the buffalo and 10% of the cattle affected with this parasite. We have found this parasite on many of the buffalo heads by lifting the third eyelid (nictitating membrane) and flushing them out of the medial canthus. A conjunctivitis may result, but the eyes we have examined were not affected.

Onchocerca gibsoni, is a nematode that forms hard nodules consisting of fibraus tissues which frequently become calcified in the muscles and skin of the pectoral region or brisket. This occurs in 20% of the cattle at the slaughter house but only 0.1% of the buffalo. The intermediate host, the midge (culicoides), bites the skin and transfer the microfilaria to the blood where it circulates and lodges in the pectoral region. The predisposition of the buffalo for mud and water probably explains the difference in incidence in these animals. The female has been described up to 20 inches long but the fibrous nodule with fibrous tissues and parasites has made it impossible for us to extract an entire worm to measure.

Setaria digitata is a slender nematode 4-6 cm long that is often seen wandering around in the abdominal cavity. In the abattoir, 20% of the cattle and 9% of the buffalo are affected with this parasite. Microfilaria of this parasite in the blood are ingested by a mosquito and after migrating to the salivary glands are reinjected into other cattle. We have found sheathed microfilaria 180 microns long in peripheral blood of many of these cattle. We believe they are the microfilaria of Setaria. Segal[1] and Mohan[2] do not mention this parasite or any Setaria in Thailand.

Tuberculosis seems to be present all over the world and Thailand is no exception. One percent of the cattle at the abattoir are infected but only 0.3% of the buffalo are infected. The organ most commonly involved in cattle infection is the lung. A few of these cases that involved practically the entire lung have been cultured as Mycobacterium bovis,

Hydatid cyst, the larval from of Echinococcus granulosus occurs in 0.25% of the cattle in the abattoir and in none of the buffalo. The cases of

this we have seen have been present in the lung.

A number of other parasites and pathological conditions were seen but we have no idea of the incidence because records have not been kept on these conditions. These include:

1. Onchocerca armillata, a small nematode reported by different authors between 2 and 29 inches long. Grossly, the intima of aortas affected with this were thickened, contained multiple tortuous tracts and were often covered with yellow atheromatous plaques. On section, the aorta was seen to contain many parasites lying entirely in the wall, many containing microfilaria. We have not been able to extract an entire parasite to measure its length.

2. A schistosome, was seen in several buffalo livers that grossly appeared as white spots through the liver intermingled with congested areas. On microscopic examination, we have found large dilated arteries with massive exuberant proliferation of the vascular intima and media with sections of larva but with no eggs evident. We have not identified this species.

3. A number of tumors were seen, primarily in the buffalo. They were microscopically typical of those seen in other countries. They included: papilloma and rhabdomyosarcoma of the rumen, mesathelioma of the peritoneum and liver capsule and phaeochromocytoma of the adrenal gland. A small white focus in a cow heart was sectioned and was found to consist of glandular tissue. It was surrounded by smooth muscle and fibrous tissue. There were no other foci in the animal and it was diagnosed as a "Choristoma", or normal tissue in an abnormal location. A buffalo liver taken from the abattoir contained multiple diffuse white foci some as large as 4 inches across. Microscopically the cell type and pattern were similar to the argentaffinoma or carcinoid in man. The cells stained weakly for silver in our case, but in man, these tumor cells stain from an intense positive to a negative. According to Smith and Jones[6], there is one confirmed case of this tumor in an ox jejunum but there are no instances of a metastasis as ours evidently was.

We have had the opportunity to examine a number of nasal tumors in large animals from farms in the vicinity of Bangkok. Two sheep had nasal tumors which completely blocked the posterior nares and caused suffocation. Although they appeared grossly similar, one was an undifferentiated sarcoma and the other was an adenocarcinoma. A horse with a mild unilateral nasal discharge suddenly died. On opening the turbinates, we found a large mass in the posterior turbinate invading the cribriform

plate and extending into the brain. Microscopically this tumor was a squamous cell carcinoma. A number of nasal tumors have been seen from a farm northeast of Bangkok. Microscopically they have consisted of a mosaic of morphologic types. We have seen three olfactory neuroblastoma, a carcinosarcoma, a leiomyosarcoma, a schwannoma, a squamous cell carcinoma, an undifferentiated sarcoma, and an embryonal rhabdomyosarcoma. We have not studied the epidemiology of this outbreak but the etiology and epidemiology warrant further study.

Canine distemper and infectious canine hepatitis are quite common in the street dogs in Bangkok and we have seen many cases of these diseases. As would be expected in an area with many mosquitoes, the heartworm, Dirofilaria immitis is present in most of the dogs. Although it was surprising to find such a high incidence of Spirocerca lupi in the esophagus and aorta of dogs at the dog pound, on some days as many as 90% of the animals were affected. The cecum of most of the dogs necropsied contained Trichuris vulpis and a few of the blood slides contained Babesia canis.

Many of the domestic and wild cats that we have seen from the Veterinary School and animal handlers in Bangkok have had panleukopenia or "feline infectious enteritis". This disease appears to be prevalent because many of the wild cats were direct from the jungle. An unusual parasite was seen in the heart muscle of the wild cats. Slides were sent to Dr. Beaver of the School of Public Health and Tropical Medicine, Tulane University. He felt that they were protozoal but on study of other wild cats and re-examination of the original cases, we believe that they were sections of migrating lungworm, Aleurostrongyius abstrusus.

A number of conditions were seen in gibbons at SEATO Laboratory: These included; a natural outbreak of lymphosarcoma, fungal (microsporum canis) infection of the skin and severe Strongyloides stercoralis of the lung and intestine.

Regional ileitis, similar to that described in the literature, was seen in the colony hamsters. We obtained no better elucidation of the etiology of this condition than have the authors of any of the articles in print.

We have seen chronic respiratory disease in rats and mice similar to that described in the literature. As many authors claim the pathological results seem to be due to more than one agent but definitive work in our laboratory has not proved this as fact. The dwarf tapeworm, Hymenolepis nana, has been seen in mice in the animal screening project.

A Search for the Snail Hosts of Schistosomiasis in Southern Thailand.

Principal Investigator: George S. Manning, CPT, MSC

Associate Investigators: Vithoon Viyanant, B.S.
Pravet Lertprasert
Kitti Watanasirmkit, B.S.

OBJECTIVE: The objective of this study was to obtain evidence relevant to the possibility of transmission of schistosomiasis in southern Thailand.

DESCRIPTION: The study area was in Nakorn Sri Tammaraj Province. A number of proven cases of schistosomiasis have been found by other workers but there is no evidence of transmission.

The approach was to collect snails from all available habitats for examination for cercariae. Species not usually considered as vector candidates were not excluded since it was deemed possible that an unusual vector might be involved.

It was planned to examine as many stools as possible from people in the villages in which schistosomiasis was previously found. In addition to concentration methods for ova the miracidium hatching technique was also employed.

PROGRESS: The results of the snail examinations are presented in Table 1. No human pathogens were found.

In spite of encouragement by local health authorities, the populace in the involved areas were reluctant to cooperate, possibly because of earlier studies involving rectal biopsy by another research group. However, stools from a total of 345 people were obtained and examined; none were found to contain schistosome ova.

This is the final report on this study.

SUMMARY: No evidence of schistosomiasis transmission was found during the examination of 4086 snails and stools from 345 people in Nakorn Sri Tammaraj.

Table 1. Results of examinations of Nakorn Sri Thammaraj snails for schistosome cercariae.

Snail species	No. Exam.	No. Pos.	Remarks
Radix rubiginosa	2475	2	Orientobilharzia harinasuti
Brotia laevis	220	0	
Wattebledia siamensis	550	0	
Bithynia sp.	440	0	
Clea sp.	115	0	
Melanoides tuberculata	37	0	
Trochorbis and Helicorbis spp.	250	0	
Total:	4087	2	

A Study of Simian Malaria in Animals Treated with Serum from Chronic Plasmodium coatneyi and Plasmodium inui Infections.

Principal Investigator: Carter L. Diggs, LTC, MC

Associate Investigators: Barnyen Permpanich, R.N.
Stanley W. Theune, MSG
Robert Gentzel, SSG

Assistant Investigator: Nipon Chuanak

OBJECTIVE: The objective os this study was to determine the effect of serum from monkeys chronically infected with Plasmodium coatneyi or Plasmodium inui on homologous and heterologous infections.

DESCRIPTION: A total of 18 cynamolgus monkeys were divided into six groups so that animals of differing weights were distributed approximately equally among the groups. Three groups were infected with P. coatneyi ($6X10^5$ parasitized erythrocytes per kg. body weight intravenously) and at the same time given either pooled normal monkey serum (group 1) or pooled serum from monkeys with chronic coatneyi (group II) or inui (group III) malaria (10 ml per kg, subcutaneously). Similarly, nine animals were infected with P. inui ($6X10^5$ parasitized erythrocytes per kg.) and simultaneously treated with one of the three serum preparations.

Parasitemia was monitored by thick and thin blood films. The number of parasitized erythrocytes per 50 oil immersion fields of the thin films was determined and an estimate of the percentage of cells parasitized calculated assuming 10,000 erythrocytes per 50 fields. When parasitemia was detected

on the thick but not the thin film, the observation was arbitrarily assigned a value of 0.001% parasitized cells for purposes of data reduction and analysis.

In some other host-malaria systems (1,2), serum mediated passive immunity is effective primarily prior to peak parasitemia. Therefore, this portion of the course of the infection was isolated for analysis. The parasite burden (area under the plot of per cent erythrocytes parasitized vs. time) prior to peak parasitemia was measured by planimetry of data plots sufficiently large to allow duplicate measurements which differed by less than 2%. Since the P. coatneyi infections showed a marked tertian periodicity for the first 42 post infection day, only the values obtained on alternate (high) days were included in the analysis during this period for these animals.

PROGRESS: The prepatent periods and parasite burdens during early parasitemia for each animal are presented in Tables I and II. Parasitemias were quite low and variation within groups was great. In each case, animals given homologous serum had lower parasite burdens and longer prepatent periods than the normal serum controls, but the differences in the case of the P. inui infections were very small. With heterologous serum, the results are even more difficult to interpret; however, the mean parasite burden were again lower than in the normal serum controls, and in the case of P. inui infected animals lower than in the homologous serum treatment group.

The course of parasitemia in each animal for the entire observation period is depicted in Fig. 1 and Fig. 2. There are no obvious differences between groups during the post-peak period of parasitemia.

Although clearly inadequate for a firm conclusion, we view these data as suggestive of an inhibitory effect of serum on parasitemia. The wide variation between groups observed would dictate much larger group sizes in further experiments; at present no serum is available for such studies.

This is the final report on this study.

SUMMARY: The effect of serum from monkeys with chronic P. coatneyi and P. inui malaria on homologous and heterologous infections was studied. Individual variation in the response of the animals precluded clear evaluation. However, group mean parasite burdens were lower in all chronic malaria serum treated groups than in the appropriate normal serum treated groups. In the case of the P. inui infections, the mean parasite burden in the P. coatneyi serum treated group was lower

Table 1. Summary of *P. coatneyi* infections prior to peak parasitemia in serum treated monkeys.

Serum Source	Monkey No.	Weight (Kg)	Prepatent period (days)	Parasite burden * (% days)
Normal	81	2.8	10	4.2
monkeys	68	2.8	14	0.3
	82	3.3	20	0.4
	Mean	3.0	14.7	1.6
Monkeys infected	74	2.4	8	3.1
with *P. inui*	72	2.8	10	0.2
	73	3.2	10	0.5
	Mean	2.8	9.3	1.3
Monkeys infected	77	2.0	16	0.2
with *P. coatneyi*	78	2.8	16	0.02
	76	3.2	14	0.2
	Mean	2.7	15.3	0.1

* Area under the curve of per cent erythrocytes parasitized vs. time (days).

Table II. Summary of P. inui infections prior to peak parasitemia in serum treated monkeys.

Serum Source	Monkey No.	Weight (Kg)	Prepatent period (days)	Parasite burden* (% days)
Normal	86	2.9	1	2.5
monkeys	85	2.7	2	1.7
	79	2.3	5	1.1
	Mean	2.6	2.7	1.8
Monkeys infected	84	2.8	7	1.5
with P. coatneyi	90	2.7	7	0.3
	89	2.3	2	0.4
	Mean	2.6	5.3	0.7
Monkeys infected	83	2.8	2	2.8
with P. inui	69	2.3	6	1.1
	80	1.5	7	0.5
	Mean	2.2	5.0	1.7

* Area under the curve of per cent erythrocytes parasitized vs. time (days).

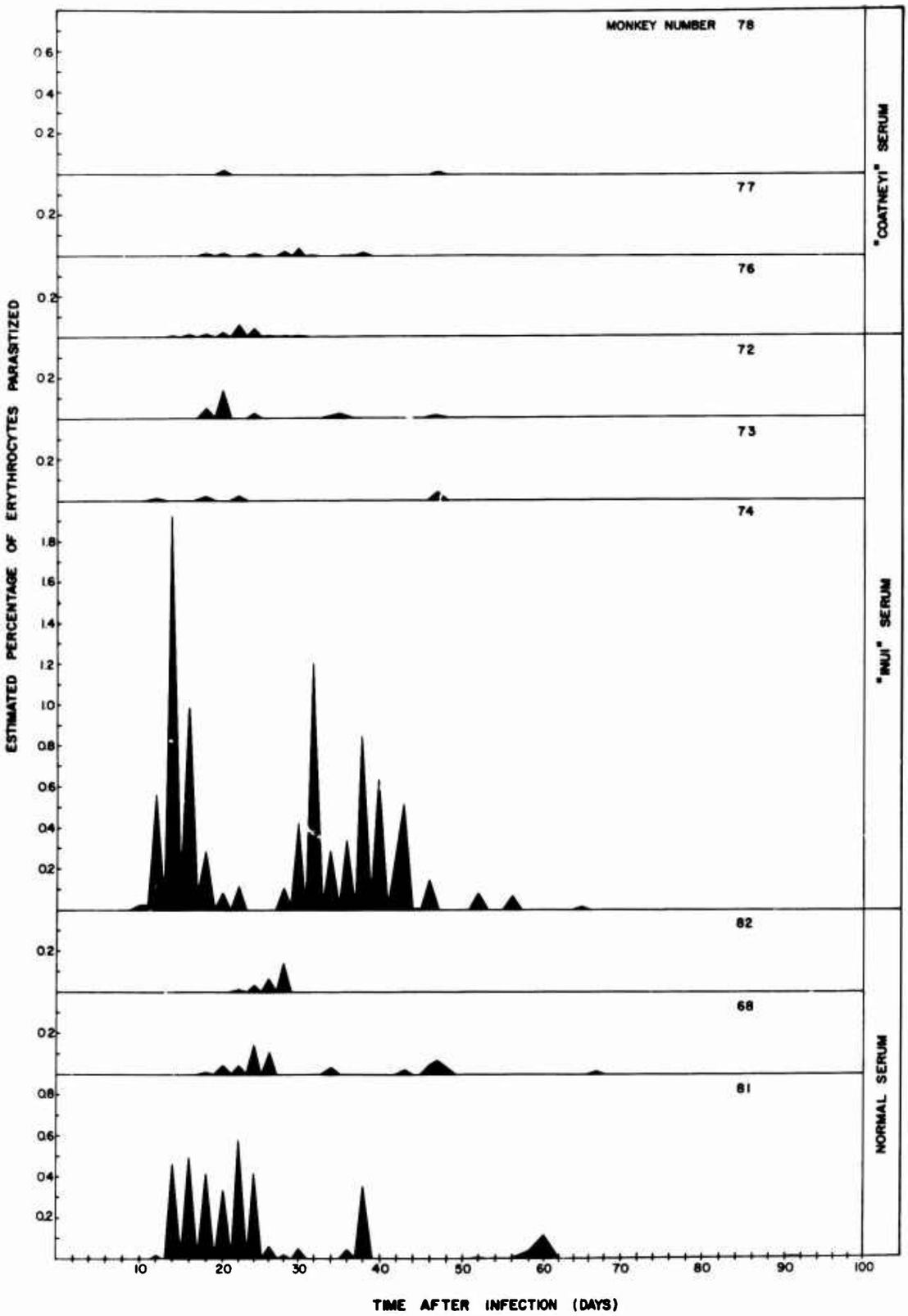


Fig 1. Parasitemia in serum treated monkeys with Plasmodium coatneyi infections

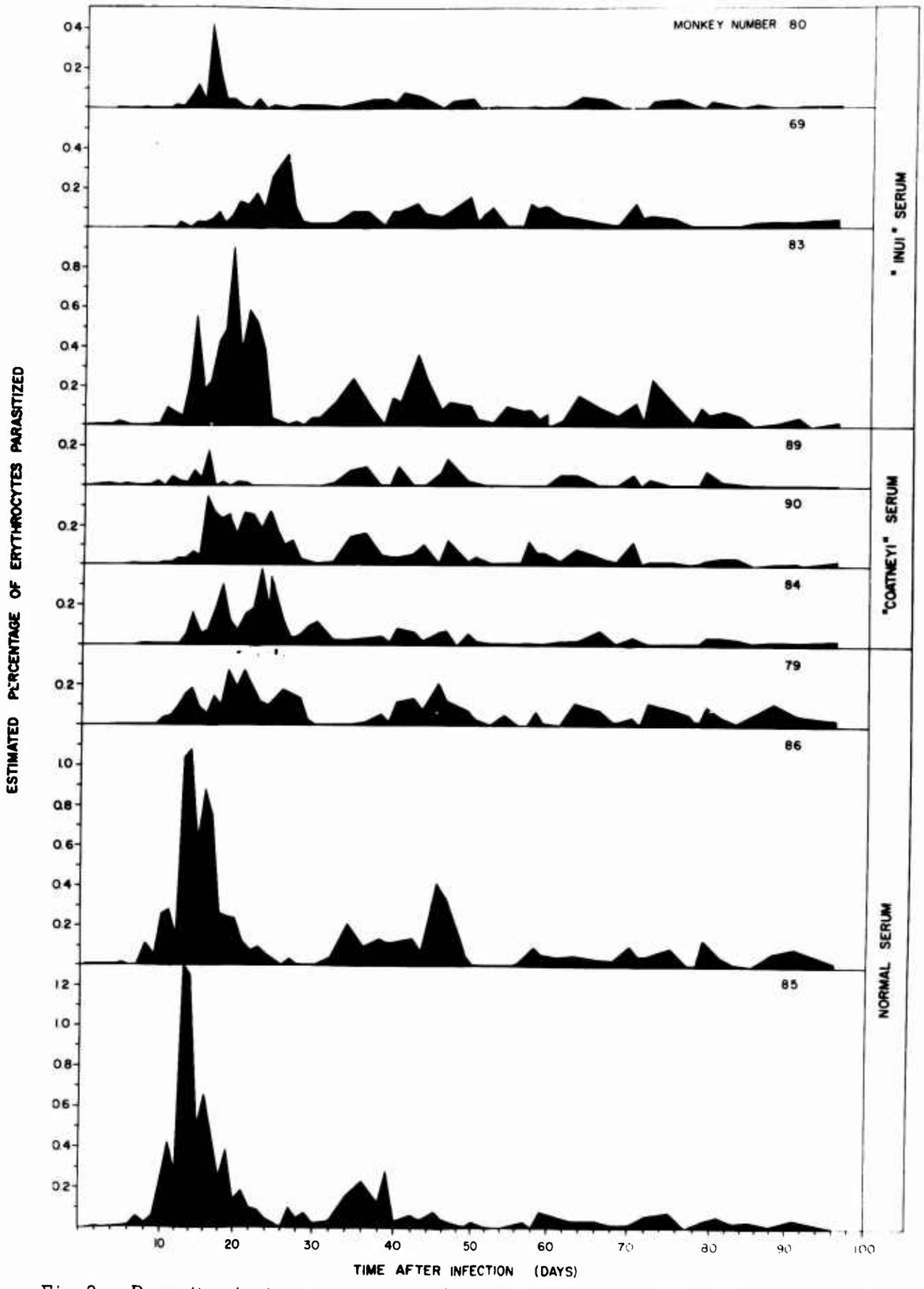


Fig 2. Parasitemia in serum treated monkeys with Plasmodium inui infections

than in the P. inui serum treated group.

Increased Susceptibility for Rats to Plasmodium berghei after Treatment Designed to Induce Immunologic Tolerance.

Principal Investigator: Carter L. Diggs, LTC, MC

Associate Investigator: Barnyen Permpanich, R.N.

Assistant Investigator: Nipon Chuanak

OBJECTIVE: Immunity to Plasmodium berghei infections in rats is conferred by prior experience with the infection, but little information is available concerning the antigenic substances which provoke the protective immune response. Most antigenic materials can produce immunologic tolerance when administered under appropriate conditions. The present experiment was designed to determine whether or not susceptibility to infection could be enhanced by techniques used to induce tolerance.

DESCRIPTION: Tolerance to heterologous erythrocytes can be induced by the simultaneous administration of large doses of antigen (erythrocytes) and the nitrogen mustard derivative cyclophosphamide.[1] This technique was adopted for use with P. berghei antigens by allowing parasitemia to build up, thus providing a large antigenic mass, and then administering the drug. A single experiment was performed using weanling albino rats randomly assigned to three groups (8 rats per group). Group I and II were infected intravenously with $1X10^7$ P. berghei parasitized erythrocytes per 100 gm body weight and the parasitemia monitored by thin blood films. Eleven days later the animals in group I were given 27 mg of cyclophosphamide per 100 gm body weight intraperitoneally, white rats in groups II and III received saline. On days 15, 16 and 17 all rats received 10 mg sulfadiazine per 100 gm body weight. Reticulocyte counts were performed until the three groups were at comparable levels; all rats were then injected intravenously with $4X10^7$ parasitized erythrocytes per 100 gm body weight, and the course of parasitemia monitored by thin blood films.

PROGRESS: Death of all but three of the group I rats (malaria plus drug treatment) between day 15 and day 22 limited the amount of data obtained. However, the results on the survivors are striking. Figure I summarizes the course of parasitemia in all three groups. With the exceptions cited below, each symbol represents the mean parasitemia in eight rats. It can be seen that cyclophosphamide

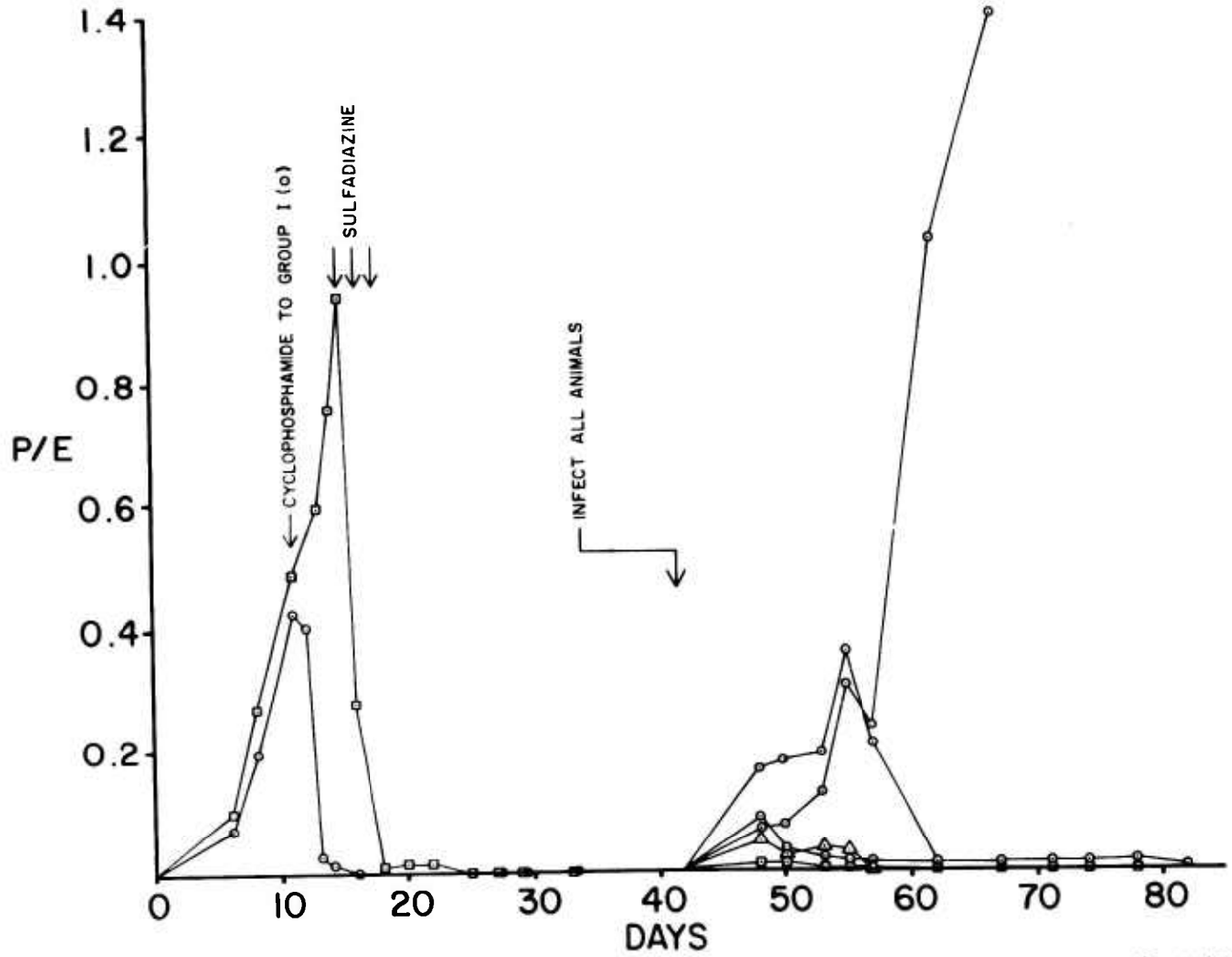


Fig 1. Plasmodium berghei parasitemia in rats expressed as ratio of total parasites to erythrocytes during drug treatment and rechallenge (see text for explanation)

quickly suppressed parasitemia (circles); further sulfadiazine abolished the infection in the remaining infected rats (squares). The animals recovered quickly, the group I rats showing a reticulocytosis of 67-89% as compared with 7-26% in the group II animals on day 25. By day 36, six days prior to challenge, reticulocyte counts were 7-9% in group I, 4-6% in group II, and 5-7% in group III (normal controls). Individual reticulocyte counts in the group III animals ranged from 3-8% during the observation period. On rechallenge, little parasitemia developed in the previously uninfected rats (group III, triangles; maximum individual parasitemia, 9 parasites/100 erythrocytes) and even less in the sulfadiazine treated animals (squares; maximum individual parasitemia, 0.3 parasites/100 erythrocytes). These results were to be expected in view of the relatively low susceptibility of rats of this age (greater than 3 months) to the infection and to the well known phenomenon of acquired immunity. The three rats which had received cyclophosphamide, however, (plotted individually, circles) sustained higher parasitemias and, in fact, one animal died with an overwhelming infection.

Although no evidence is offered relavant to the specificity of the enhanced susceptibility, there was no gross evidence of generalized debility and erythropoiesis was essentially normal as judged by the pattern of reticulocyte counts. We therefore feel that the phenomenon is best explained in terms of induction of tolerance in the drug treated animals. Obviously, more work is needed to explore this hypothesis. This is the final report on this project.

SUMMARY: 1. Rats treated by a combined P. berghei malaria cyclophosphamide, regimen exhibited abnormally high susceptibility to rechallenge with P. berghei. It is suggested that immunologic tolerance to protective antigens was induced.

2. Incidental to the experimental was the observation that cyclophosphamide rapidly depresses P. berghei parasitemia in rats.

Studies on the Effect of a Serum Permeability Substance Produced During Malaria Infection on Platelets in vitro.

Principal Investigators: Katchrinnee Pavanand, M.D.
Carter L. Diggs, LTC, MC

Assistant Investigators: Chirapan Vinagupta, M.T.
Bamyen Permpanich, R.N.

OBJECTIVE: Previous studies in this laboratory have revealed that during the course of a malaria infection, the sera of infected gibbons (Plasmodium jeffreyi) and monkeys (P. inui) produce increased vascular permeability when injected intradermally into the skin of white rabbits (see 1967 and 1968 Annual Reports). This reaction was completely abolished by antihistaminic drugs. The factor was stable to heating at 56°C for thirty minutes. Attempts at characterization of the substance were unsuccessful (see 1969 Annual Report) in part because of its apparent lability during fractionation.

Since thrombocytopenia is associated with P. inui infections (1969 Annual Report), the notion that the permeability factor induced rabbit platelet lysis with accompanying histamine release was entertained. Rabbit platelet lysis can be induced in vitro by immune complexes and the phenomenon detected by measurement of the resulting histamine in the fluid phase.[1] The possibility that the permeability factor could also induce platelet lysis in vitro was therefore explored.

DESCRIPTION: The assay system has been previously described (see Annual Report 1969). Briefly, sera containing activity by rabbit skin tests and sera from normal monkeys were incubated at 37°C for 60 min. with rabbit platelets in the presence of fresh autologous rabbit plasma, Ca^+ and Mg^+. The preparations were centrifuged and the supernatants deproteinized and extracted for histamine. Histamine was converted to a fluorescent derivative and assayed fluorometrically; fluorescence intensities were related to histamine concentrations by the use of a curve prepared from a set of histamine diphosphate standards extracted and otherwise treated identically with the unknowns.

PROGRESS: The results of two experiments are summarized in Table 1. Histamine release is expressed in terms of the total available histamine (determined by assay of an aliquot of platelets) found in the fluid phase. Spontaneous release from mixtures without serum is indicated (cell blank). Release of histamine from platelets in the presence of serum from infected monkeys (8-16%) was not greater than from control monkeys (9-23%).

These results do not preclude the possibility of platelet lysis as a mechanism of enhanced vascular permeability in rabbit skin by malarial monkey sera. However, it is considered that further studies at this time would not likely yield additional information. This is the final report on this project.

SUMMARY: Serum from P. inui infected monkeys which enhanced vascular permeability in rabbit skin failed to induce the release of histamine from rabbit platelets in vitro.

Table 1. Histamine release from rabbit platelets in the presence of serum from P. inui infected monkeys. Lesions produced in rabbit skin by the same sera are shown for comparison.

Experiment No.	Test preparation		Rabbit skin lesion diameter (mm.)	% platelet histamine released
	Serum origin (Monkey #)	Days post infection		
1	MS-41	preinfection	0	9
Total histamine	PK-36	preinfection	0	20
available	PB-1	normal	0	23
1.9 μg/ml	cell blank	-	-	18
2	MS-41	prior to infection	0	10
Total histamine	MS-41	29 days	14	8
available	PK-36	prior to infection	0	20
3.6 μg/ml	PK-36	15 days	14	8
	PK-36	29 days	10	16
	PB-1	normal	0	15
	SP-2	chronic malaria	N.D.	15
	cell blank	-	-	10

N.D. = Not done.

Red Cell Survival in Simian Malaria after Chemotherapy.

Principal Investigators: Vithune Yuthasastr-Kosol, M.D.
Carter L. Diggs, LTC, MC

Assistant Investigator: Thamma Sakulkaipeara

OBJECTIVE: Previous studies in this laboratory demonstrated shortening of red cell survival time in monkeys with chronic malaria due to Plasmodium coatneyi or Plasmodium inui. Erythroid hyperplasia of the bone marrow accompanied the accelerated erythrocyte destruction. The animals had very scanty parasitemias and it was suggested that the degree of red cell destruction was too great to be explained on the basis of direct destruction by parasites. However, since it is not possible to predict the degree of erythrocyte loss from the parasitemia data in quantitative terms, and because of the possibility of sequestered parasites not detectable in peripheral blood samples, the possibility of direct erythrocyte destruction by parasites could not be completely ruled out. The present study is designed to determine how long such accelerated destruction persists in the absence of parasitemia after chemotherapy; an abrupt return of erythrocyte survival time to normal values coincident with cessation of parasitemia would suggest direct destruction by parasites as a likely mechanism of red cell loss. On the other hand, persistence of accelerated destruction after cure would suggest a host mediated mechanism.

DESCRIPTION: Three chronic P. coatneyi infected rhesus monkeys, SP8, MS59, and PK21, and one chronic P. inui infected rhesus monkey, SP6, were studied for red cell survival before chemotherapy with chloroquine, 25 mg. base per kg. bodyweight intramuscularly over a three day period. Daily examinations for parasitemia were performed on each animal, and routine blood examinations were done on each animal once weekly. After the parasites completely disappeared from the peripheral blood smears, red cell survival studies were repeated periodically. (The techniques employed were the same as described in the SMRL Annual Report, 1968). Red cell survival was also studied in four normal control monkeys.

PROGRESS: Parasitemia disappeared after chemotherapy and daily blood films have remained negative up to the present. It is therefore assumed that radical cure was achieved.

The erythrocyte half-survival times (t 1/2) in all animals at various

times after cure are shown in Fig. 1. It can be seen that the values do not return to normal abruptly, but rather increase slowly over an extended period of time. Red cell survival is still abnormal in monkey MS59 at this time.

Other hematological changes were consonant with those in the erythrocyte survival times. Thus in the four animals, hematocrits ranged from 18-24% prior to therapy and range from 39-43% at present; hemoglobin rose from a range of 6.6-7.2 gms% to 13.3-13.6 grams% at present; reticulocytes, 7.5-14.2% prior to therapy, now range from 0.2-1.6%.

The red cell survival data (Fig. 1) suggested that survival times do not increase continuously after therapy, but that secondary decreases occurred during the observation period. The animals will therefore be studied further (until 200 post treatment days have elapsed) to determine whether or not the parameters measured are stable once normality is achieved.

These studies strongly suggest that the observed abnormal red cell destruction is not due to direct lysis by parasites but through some host mediated mechanism.

SUMMARY: Erythrocyte survival in four monkeys with chronic coatneyi or inui malaria returns to normal only gradually (130 days or more) after drug cure. It is suggested that this red cell destruction is not due directly to the intracellular parasite.

The Fate of Plasma Hemoglobin in Macaca mulatta Infected with Plasmodium coatneyi.

Principal Investigators: Kanjika Devakul, M.D., Ph.D.
Tan Chongsuphajaisiddhi, M.D., Ph.D.

Assistant Investigators: Kanokwan Kanakakorn, B.Sc.
Ratanaporn Kasemsuthi, B.Sc.

OBJECTIVE: The objective of this project is to study the fate of plasma hemoglobin in terms of the rate of removal from the plasma, the distribution in the liver, spleen, and bone marrow, and the urinary excretion in normal monkeys and in animals with Plasmodium coatneyi malaria.

DESCRIPTION: Rhesus monkeys (Macaca mulatta) were used in these experiments. The hemoglobin was labelled with Fe-59 or Cr-51; 120 uc.

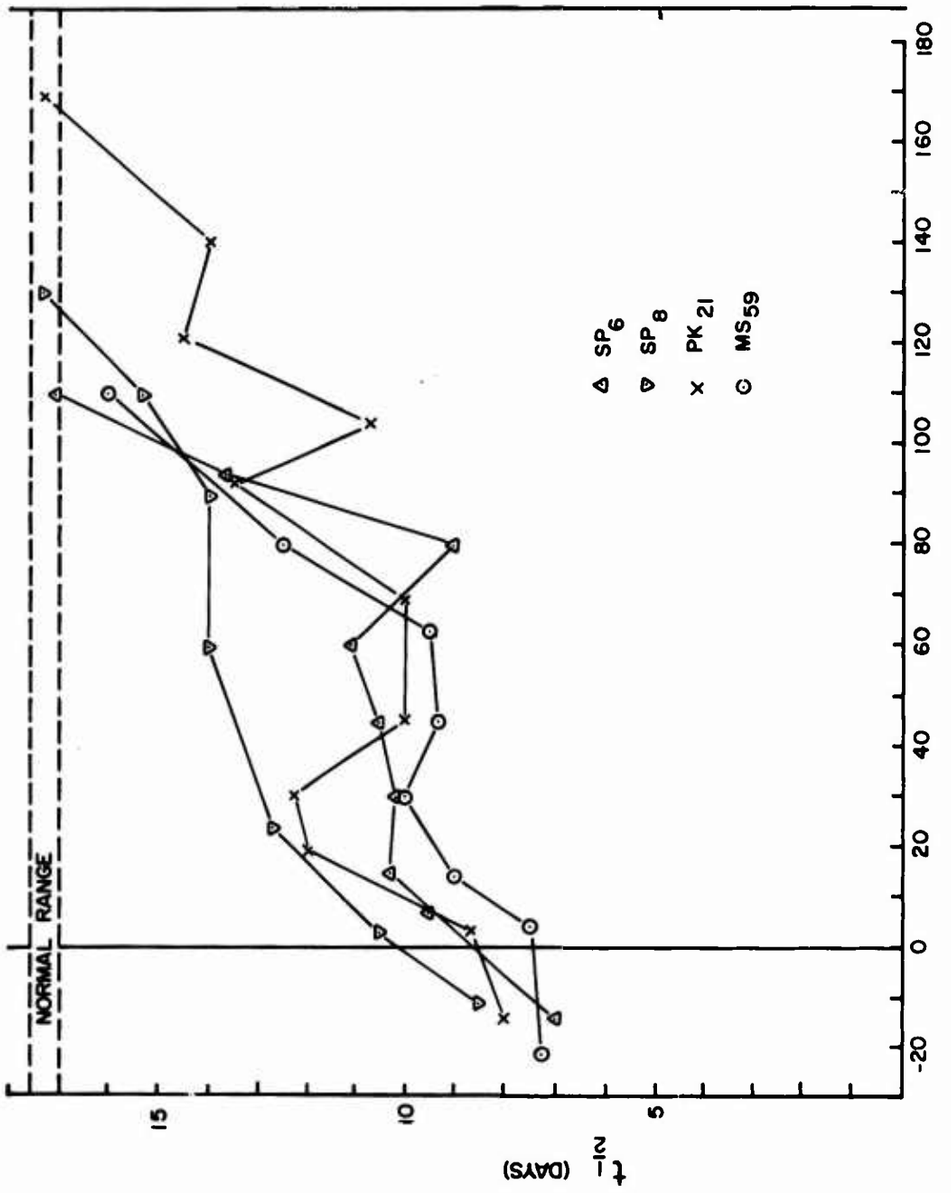


FIG.1 ERYTHROCYTE SURVIVAL FOLLOWING CHEMOTHERAPY OF MALARIA IN RHESUS MONKEYS

of Fe-59 were injected intravenously into a donor monkey. After a suitable interval, 10-15 ml of blood were drawn and hemolyzed in distilled water. In the case of Cr-51, 10-15 ml of the recipient animal's blood was labelled with 50-80 uc. of Cr-51 in vitro; after lysis, the hemoglobin concentration and the volume of hemoglobin solution were recorded. The labelled hemoglobin solution was injected intravenously to simulate intravascular hemolysis. Plasma radioactivity was measured at 10 minutes, 20 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and 24 hours after injection. The radioactivity of the spleen, the liver, the bone marrow and the urinary bladder was recorded by surface counting at 1/2 hours, 2 hours, 4 hours, 24 hours, 48 hours, 72 hours and 96 hours. Animals were kept in metabolic cages and urine was collected for 24-48 hours after the injection of labelled hemoglobin. The activity of the isotope excreted in the urine was calculated and expressed as the percentage of the dose given.

The monkeys were infected with P. coatneyi. Hemoglobin, hematocrit, red cell count and parasitemia in thick or thin blood smears were recorded daily. The study of hemoglobin metabolism was made when the hemoglobin and hematocrit decreased to about half of the initial value, usually about 20 days after the infection.

PROGRESS: The study was carried out in 9 rhesus monkeys (Macaca mulatta), 7 of which were normal and the other 2 infected with P. coatneyi. Two of the normal monkeys were injected with Fe-59 hemoglobin and all the others with Cr-51 hemoglobin.

It was found that in normal monkeys disappearance curves of Cr-51 and Fe-59 hemoglobin were similar. T 1/2 varied from 71 to 136 minutes. The activity of hemoglobin in the plasma 6 hours after injection was 6-11% of the administered dose and at 24 hours was 0-3.5% of the dose.

In two infected monkeys the study of hemoglobin metabolism was made at 19 and 21 days after infection when the parasitemia was 0.9% and 0.1% and hemoglobin was 5.5 and 6.9 gm% respectively. It was found that labelled hemoglobin seemed to leave the circulation more rapidly than in the normal animals (T 1/2 values were 63 and 69 minutes respectively). At 6 hours radioactive hemoglobin in the plasma was 10% and 5.9% of the administered dose and at 24 hours 3.7% and 2.5% respectively.

In the normal monkeys the activity over the liver and spleen rose rapidly

during the first few hours after injection of hemoglobin and reached a maximum within 4 hours in every monkey; it then stabilized at about the same level up to 24 and 48 hours in two monkeys but decreased after 6 hours in five monkeys. There was not much activity in the bone marrow. Very high activity was detected over the urinary bladder at 6 hours but none could be detected at 24 hours.

In one infected monkey the result was similar to that observed in normal monkeys except that no activity could be detected in the liver at 48 hours. In the other infected animal there was some difference from the normals. The activity over the spleen reached a maximum at 6 hours. The activity over the liver increased until 24 hours and then decreased to only about one-fifth of the 24 hour activity at 48 hours. The activity over the urinary bladder was low in comparison with that observed in the normal monkeys but activity could still be detected at 24 hours.

Studies on additional numbers of infected monkeys are in progress.

SUMMARY: Plasma hemoglobin clearance was compared in seven control normal and two P. coatneyi infected monkeys. The half survival times in both animals were greater than in any of the controls.

Studies of the Soluble Antigen Fluorescent Antibody (SAFA) Test for Filariasis.

Principal Investigator: Carter L. Diggs, LTC, MC

Assistant Investigators: Stanley W. Theune, MSG
Robert Gentzel, SSG
Prasit Sookto
Nipon Chuanak

OBJECTIVE: In spite of extensive investigation, serologic reactivity in human filariasis is not sufficiently well understood to allow meaningful interpretations of serologic tests. (For Review, see 1). One of the difficulties is the possibility of serologic reactivity induced by other helminths including filarial worms not ordinarily pathogenic in man. Recently a soluble antigen fluorescent antibody (SAFA) test for bancroftian filariasis and onchocerchiasis has been described[2]. The test employs a soluble antigen derived from the dog heartworm, Dirofilaria immitis. The possibility has been raised that humans exposed to D. immitis might become seroreactive in tests employing antigens derived from the parasite.[3] The present study was designed

to evaluate the SAFA test for reactivity in filariasis due to Brugia malayi and to attempt to gain some insight regarding serologic reactivity in a population exposed to D. immitis infected mosquitoes. In addition, some technical characteristics of the test system were studied.

DESCRIPTION: Some parts of the test procedure were different from that previously described[2]. Rabbit antiserum to human globulin was prepared using commercial immune serum globulin as antigen. Based on preliminary qualitative precipitin tests, pools were prepared, the globulin fraction isolated and labelled with fluorescein isothiocyanate under standard conditions. Details of these procedures will be presented in a later report. Free fluorescein was removed by chromatography on G-25 Sephadex and the antibody content of the labelled antiglobulin estimated by quantitative precipitin tests.

In performing the SAFA tests the fluorometer was zeroed by exclusion of light from the photocell. For each serum tested, a disc impregnated with antigen and a control disc without antigen were exposed to the serum. In this way nonspecific fluorescence for each serum sample was measured; antigen dependent fluorescence was taken as the difference between the control and experimental discs. The procedure allowed estimates of variation among sera in control as well as in infected populations.

Instrumental sensitivity, stability and linearity were evaluated by the use of discs treated with known amounts of fluorescein isothiocyanate.

PROGRESS: Instrumental stability, as determined by repeated measurements of the intensity of fluorescence of standard fluorescein isothiocyanate impregnated discs, was judged to be good. Linearity of response with concentration, however, was limited to about 40 fluorescence units, a value much lower than that obtained with many samples in the serologic test. Assuming that a similar nonlinearity exists for the serologic test, these results suggest that fluorescence intensities can be quantitatively related to antibody concentration only in a limited range of measurements.

Three groups of sera have been studied: (1) 128 sera from patients with B. malayi microfilaremia; (2) 69 sera from American soldiers recently arrived in Southeast Asia; and (3) 97 sera from Bangkokians (blood donors and patients receiving prenatal care). This latter group comprises an appropriate population for study in connection with the question of seroreactivity induced by exposure to D. immitis since there is active transmission of the parasite in Bangkok. Examination

of the distribution of fluorescence intensities obtained revealed fairly extensive deviations from normality. The distributions are presented in Fig. 1, in which the percentage cumulative frequencies of given fluorescent intensities have been plotted on log-probit paper. It is apparent that the distribution of the transformed data approximates normality as judged from these plots. The data clearly indicate that the patients with Brugia malayi infections have a higher reactivity as a group than do either the American troops or Bangkokians. However, it is also apparent that approximately 50% of the sera from infected individuals have seroreactivities which overlap the values of the control group. Thus the usefulness, for diagnostic purposes, of the test as performed is limited. This circumstance might be related to the stage of the disease or age of the individual patient.[2] Alternatively, greater discrimination might be obtained through the use of higher concentrations of antigen and/or antiglobulin in the test. These possibilities are being explored.

The mean reactivity of Bangkokians was higher than that of American troops and the difference was statistically significant ($P < 0.001$, test). However, the reactivity is not striking when compared with the filariasis group. Determination of whether or not the difference reflects antibody in the Bangkok population will require further study.

The results indicate, however, that living in an area endemic for canine heartworm does not necessarily confer seroreactivity to D. immitis antigen.

SUMMARY: The reactivity of sera from patients with Brugia malayi infections in a soluble antigen fluorescent antibody (SAFA) test using Dirofilaria immitis antigen has been studied. Approximately 50% of the patients' sera gave greater reactivity than any of the control sera obtained from Americans or Bangkok residents. The mean reactivity of the Bangkok sera was higher than that of Americans, but not markedly so. The question of seroreactivity to D. immitis antigens in human populations at risk of exposure to this parasite is therefore not resolved, but the data indicates that such seroreactivity, if it exists, does not necessarily interfere markedly with serologic testing for human filariasis in populations at risk with respect to infection by D. immitis.

The Gibbon As A Host For The Canine Heartworm

Principal Investigators: D.O. Johnsen, MAJ, VC
Douglas J. Gould, Ph.D.
William L. Wooding, MAJ, VC
Carter L. Diggs, LTC, MC
Prayot Tanticharoenyos, D.V.M.

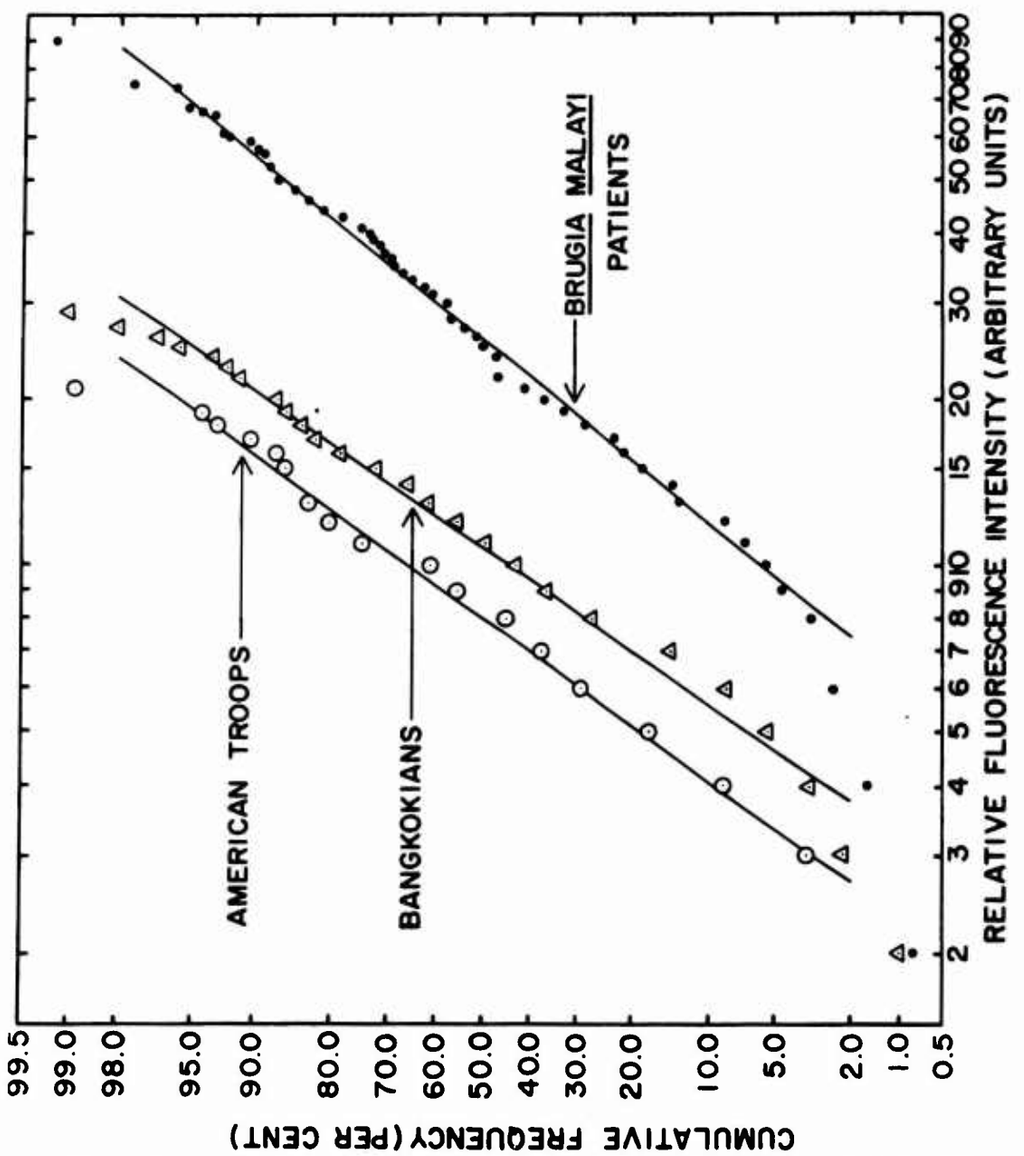


Fig 1. Log-probit plot of the cumulative frequency (probit scale) of fluorescence intensities (log scale) obtained with three groups of sera tested by the SAFA test using D. immitis antigen

Assistant Investigators: Bruce A. Harrison, CPT, MSC
Ronald E. Marshall, SP5, E-5

PURPOSE: Human infections with Dirofilaria immitis, the canine heartworm, has been the subject of a number of reports. United States and Japan, confirmed infections have occurred where Dirofilaria immitis was found in the heart, lungs, subcutaneous tissue, conjunctival, or periorbital area of humans. The possibility that this parasite is the etiological agent for tropical eosinophilia has also been suggested because humans with this disease have a positive skin test to Dirofilaria antigen and respond to treatment with the vermicide diethylcarbamazine. Dirofilaria immitis occurs widely in dogs throughout Southeast Asia and in some areas of Thailand may infect 100% of the adult dog population. Because Dirofilaria is transmitted between dogs by Culex quinquefasciatus and Aedes aegypti, mosquitoes that commonly feed on man too, there may be a great opportunity for human infections to occur where heartworms are endemic. There is evidence, both in the literature and from this laboratory, that indicates that subhuman primates may be good animals to study heartworm infections as they occur in man. Dirofilaria has been found in the hearts of two orangutans and was tentatively identified as an incidental finding in the adrenal vein of an SMRL gibbon. Since Dirofilaria immitis may become increasingly recognized as a significant human parasite, this study was initiated to determine the suitability of the gibbon as a model for studying transmission as well as the clinical and pathological features of this disease in primates.

DESCRIPTION: Three gibbons and one one dog were inoculated in November with between 35 and 50 Dirofilaria immitis larvae which were obtained through dissection of mosquitoes. The mosquitoes had fed approximately three weeks earlier on a dog with a high microfilaremia which, at necropsy, was confirmed to be infected with Dirofilaria immitis. The following laboratory examinations have been performed on the experimentally infected animals during the report period:

a. a complete blood count and serum sample collected for examination by the fluorescent soluble antigen test each week;

b. skin testing with the Takeda (Japanese) and Parke-Davis Dirofilaria antigens and blood examinations for microfilaria using the Knott's concentration technique each month;

c. thoracic radiographs taken each month in those gibbons that become

either reactors to the skin test or develop abnormally high eosinophil counts.

PROGRESS: The data collected during the report period is summarized in Table 1 through 4. Serum samples collected for examination by the fluorescent soluble antigen technique are being stored until the study is completed so that they may be run together. Only one gibbon, V-165, and the dog, Pup 4, have experienced an eosinophilia. Each of the inoculated animals has turned positive on either or both of the skin test antigens. In the case of the gibbons, uninoculated control animals have not converted, but as least one of the uninoculated control dogs have developed positive reactions to the skin test. This unexpected conversion in the dog may reflect the problem experienced in controlling mosquitoes in the dog holding area. Consultation with the radiologist at 5th Field Hospital is being obtained to interpret the thoracic radiographs of V-165, V-166, and V-169.

Difficulties experienced in obtaining adequate numbers of infective larvae from the mosquitoes have restricted experimental inoculations to only three gibbons and one dog. The yield from Aedes aegypti was exceedingly low and the high yield in Culex quinquefasciatus was offset by a high mortality in this species following feeding. In the latter case we feel the high mortality was due to the adverse physical effects of heavy microfilarial infections. Further attempts will be made to obtain microfilaria for the purpose of infecting one more gibbon and dog by using a different species of mosquito and feeding mosquitoes at the time of day when microfilaremias are at lower levels.

Eosinophilic Meningoencephalitis in Thailand.

Principal Investigator:	Sompone Punyagupta, LTC, MC
Associate Investigators:	Thanongsak Bunnag, M.D. Dhira S. Comer, M.D.
Assistant Investigators:	Aumpon Na-nakorn Sajee Pinnoi

OBJECTIVE: Continuation of epidemiologic, clinical, neuropathologic and experimental studies on eosinophilic meningoencephalitis.

DESCRIPTION: Epidemiologic study was terminated in May 69 and the data obtained from the previous years were analyzed. The clinical and pathologic studies were carried out only in Bangkok. Four experimental

Table I. Gibbon # V - 165

White Count x 10^3	7.6	Inoc.	8.1	13.8	10.7	7.9	11.3	9.3	16.7	10.2
Eosinophils (%)	0	50 micro-	0	0	5	9	2	6	14	23
Skin test (J)	-	fil.	-	N.T.	-	N.T.	N.T.	N.T.	+	N.T.
Skin test (PD)	-	14 Nov	-	N.T.	-	N.T.	+	N.T.	+	N.T.
Knott's Technique	-		-	N.T.	-	N.T.	-	N.T.	-	N.T.
	10 Oct		15 Dec	5 Jan	19 Jan	2 Feb	16 Feb	2 Mar	12 Mar	30 Mar

Table II. Gibbon # V - 166

White Count x 10^3	8.3	Inoc.	10.7	9.6	9.8	15.3	14.1	8.6	9.5	12.9
Eosinophils (%)	2	35 micro-	2	4	4	4	3	1	1	1
Skin test (J)	-	fil.	-	N.T.	+	N.T.	N.T.	N.T.	+	N.T.
Skin test (PD)	-	17 Nov	-	N.T.	+	N.T.	-	N.T.	-	N.T.
Knott's Technique	-	69	-	N.T.	-	N.T.	-	N.T.	-	N.T.
	10 Oct		15 Dec	5 Jan	19 Jan	2 Feb	16 Feb	2 Mar	17 Mar	30 Mar

Table III. Gibbon # V - 169

White Count x 10^3	11.4	Inoc. 50	11.6	11.4	20.9	18.5	16.7	14.4	11.0	12.0
Eosinophils (%)	1	micro-	2	3	2	4	1	0	1	4
Skin test (J)	-	fil. 19 Nov 1969	-	N.T.	+	N.T.	N.T.	N.T.	+	N.T.
Skin test (PD)	-		-	N.T.	+	N.T.	+	N.T.	+	N.T.
Knott's Technique	-		-	N.T.	-	N.T.	-	N.T.	-	N.T.
	10 Oct		15 Dec	5 Jan	19 Jan	2 Feb	16 Feb	2 Mar	17 Mar	30 Mar

Table IV. Dog 4

White Count x 10^3	24.1	Inoc. 50	25.7	35.1	24.5	24.5	23.9	19.8	27.8	25
Eosinophils (%)	1	micro-	15	52	14	15	8	14	27	12
Skin test (J)	-	fil. 14 Nov 1969	+	N.T.	+	N.T.	-	N.T.	+	N.T.
Skin test (PD)	-		N.T.	N.T.	+	N.T.	-	N.T.	+	N.T.
Knott's Technique	-		-	N.T.	-	N.T.	-	N.T.	-	N.T.
	10 Oct		15 Dec	5 Jan	19 Jan	2 Feb	16 Feb	2 Mar	17 Mar	30 Mar

studies were performed to clarify the pathogenesis of eosinophilic myeloencephalitis caused by Gnathostoma spinigerum, based on some findings in the study of human cases.

PROGRESS: A. Clinical and Pathologic Study.

During the period 21 cases of eosinophilic meningitis were studied; 19 cases belonged to the typical or and two cases were myeloencephalitis. Of the 19 typical cases, 11 patients were male and 8 were female. In one case, a fifth stage A. cantonensis larva was seen in the posterior chamber of the eye and was subsequently surgically removed from the anterior chamber. This is the first case demonstrating the association between ocular angiostrongyliasis and eosinophilic meningitis. Both cases of eosinophilic myeloencephalitis were female and were admitted with cerebral hemorrhage. Eye swelling was noted in a case. One case died and the post-mortem findings were characteristic of Gnathostoma invasion of the central nervous system but no worm was recovered.

B. Laboratory Studies.

Experiment 1: To confirm the study that the early third stage larva of G. spinigerum in copepods are infective to mice, chicken and cats.

Background: Epidemiologic evidence on eosinophilic myeloencephalitis due to Gnathostoma indicated that patients may be infected by drinking contaminated water. Prof. Daengsvang has shown that mice may be infected by infected copepods. This study is designed to learn more about this and to study other animals including the definitive host.

Materials and Methods: Laboratory bred copepods were infected with first stage G. spinigerum larvae and were kept for 5, 10 and 16 days before being fed to 30 mice, 20 chickens, and 1 cat in each group. Each animal received 10 larvae from the copepods and were sacrificed at 6 hrs., 24 hrs., 5, 10, 15 and 30 days post-infection and examined for the third stage larva.

RESULTS: 1. In mice five-day-old infected copepods were non-infective but in 10-day and 15-day-old infected copepods the infection with third stage larvae was found in 40% and 80% respectively.

2. None of the 20 chickens infected with 15-day-old infected copepods yielded positive results.

3. Two cats infected with 20 and 50 larvae from 15-day-old copepods sacrificed on the 15th day post-infection were negative for

gnathostomes. Another three cats infected with 20; 50 and 100 larvae each were still negative for Gnathostoma ova in stools 9-12 months post-infection.

4. Infected copepods can be kept alive in the laboratory for as long as 64 days. Gnathostome larvae had 2 molts in the copepods. The heaviest infection in one copepod was 22 larvae. In most instances a copepod was infected with 1-3 larvae.

Experiment 2: To study the morphological changes and development of the third stage infective G. spinigerum larva following repeated infections in transport hosts.

Background: It is suspected that the third stage G. spinigerum may develop to a more advanced stage after many passages in various transport hosts and these advanced stage larvae may cause the disease in man.

Materials and Methods: Third stage G. spinigerum larvae obtained from experimentally infected mice were divided into two groups, those from the liver and those from muscles; ten of each were separately infected into mice which were sacrificed on the 5th and 15 th day post-infection. Sites of organs of recovery of larvae were recorded. The recovered larvae were used for reinfection until no larva was found. Tissues where larvae were found were sectioned and studied for pathological changes.

RESULTS: 1. After infection, third stage larvae may or may not enter the liver, no matter whether they have previously migrated to the liver or not. This finding is contrary to the other reports.

2. There were no morphological changes in these larvae even after as many as 15 reinfections. However, the non-encysted form of larvae can occasionally be seen in the musculature.

3. Frequency of organs involved where 400 larvae were recovered are as follows: muscle of back, 35%; muscle of hind legs, 22%; muscle of chest wall, 12%; muscle of abdominal wall, 10%; mesenteric fat, 8%; muscle of fore leg, 6%; muscle of neck, 2%; heart, 1%; diaphragm, 0.7%; kidney, 0.7%; stomach wall, 0.4%; and spleen, 0.2%. This may explain why the portal of entry of Gnathostome larvae into the central nervous system in man occurs mainly in the lower part of the spinal cord.

4. Sections from various tissues were made and will be studied.

Experiment 3: To prove experimentally that the chicken may serve as a transport host of G. spinigerum and to observe any morphological changes of the larvae.

Background: Chickens are noted to be a source of human infection and may be important in eosinophilic myeloencephalitis.

Materials and Methods: Third stage larvae were fed to chickens which were sacrificed on the 15th and 30th day post-infections.

RESULTS: Infection was complete in 25 per cent. Both encysted and non-encysted third stage larvae were found but without any morphological changes.

Experiment 4: To study the pathogenicity of the 4th and 5th stage Gnathostome larvae in mammals.

Background: All G. spinigerum recovered from man were fifth stage larvae or immature adult worms. We have shown in other studies that the spinal cord of the mouse is invaded by the 5th stage larva of G. spinigerum, recovered from man, following ingestion. This experiment was designed to confirm the finding.

Materials and Methods: 15 cats were infected with varying numbers of third stage larvae; 7 cats were sacrificed on the 5th, 9th, 12th, 20th, 34th, 60th, and 120th days after infection. The recovered larvae, all third stage, were injected into mice. The mice were subsequently sacrificed and third stage larvae were found in the muscle. In two instances, the larvae had 4 rows of hooklets and were longer than the ordinary third stage. None involved the CNS. This experiment is in progress.

Serologic Response in Human Fasciolopsiasis.

Principal Investigators: Vithune Yuthasastr-Kosol, M.D.
Carter L. Diggs, LTC, MC
George S. Manning, CPT, MSC

Assistant Investigator: Thamma Sakulkaipeara

OBJECTIVE: In most helminthic infections in which serologic responses have been demonstrated, there is considerable invasion of host tissues

by the parasite. The extent to which such tissue involvement is necessary for the production of antibody is not well understood. It is therefore of interest to study antibody production in helminthiases in which there is little tissue invasion. Fasciolopsis buski ranks low among intestinal parasites with respect to the degree of tissue invasion accompanying infection. The organism has no extra-intestinal developmental forms and is believed to maintain its position in the bowel by the muscular acetabulum alone, feeding on intestinal contents rather than host tissue. We therefore examined sera from patients with F. buski for antibody reactive with antigens of the worm.

DESCRIPTION: Sixty-two sera were collected from individuals previously shown to be infected with F. buski. Control sera were obtained from Americans and Thais with no history of parasitic infection and from people infected with the liver fluke Opisthorchis viverrini. The latter specimens were employed to investigate reactivity of F. buski antigen with sera from people infected with another species of trematode which has a greater potential for the induction of an immune response.

A crude antigen was prepared from whole adult F. buski collected from infected pigs and stored at -70°C. The frozen worms were thawed and weighed, added to 4 ml of saline for each gram of worm and extracted in a high speed steel bladed homogenizer for 10 minutes in an ice bath. The preparations were centrifuged for 30' at 11.500 X g and the supernatant stored at -70°C.

The sera were studied by a quantitative complement fixation test essentially as described by Levine[1]. Antigen concentration was varied and the serum dilution kept constant at 1/400. This high serum dilution was necessary to minimize anticomplementary effects noted with some sera in preliminary experiments. The extent of lysis was determined by absorption measurements at 412 mu. (Soret band of hemoglobin) on supernatants from reaction mixtures. The difference in absorption (ΔA_{412}) between experimental mixtures and complement controls (i.e. mixtures without serum or antigen) was taken as an estimate of complement fixation. The mixtures were prepared so that complete lysis gave an absorption reading of 1.5 units and the complement controls gave 80-90% lysis. Sera which gave ΔA_{412} values of greater than 0.15 in the absence of antigen were considered anticomplementary. All measurements were performed on duplicate reaction mixtures.

PROGRESS: Six experiments were performed comparing individual sera in each of the four categories described above. A representative

experiment is summarized in Fig. 1. Complement fixation was observed with all four sera, but was much greater with the F. buski serum than in the controls. These experiments are summarized in Table 1; the figures are for the highest antigen concentration employed. In all but one case, greatest fixation was observed at the highest antigen level. The exception was a serum from an American which gave strong fixation ($\Delta A_{412} = 0.595$) at a relative antigen concentration of 0.005 ml/ml but essentially none at 0.05 ml/ml.

Tests of a total of 62 F. buski sera, including those employed above, were also tested at four antigen concentrations up to and including 0.1 ml/ml. At least one serum from a normal Thai was tested in each experiment, and a total of 14 such control sera were studied. All F. buski sera induced a progressive increase in complement fixation with increasing antigen. However, 10 sera showed significant anti-complementary activity so that estimates of complement fixation might be unreliable. The remaining 52 sera gave ΔA_{412} values ranging from 0.748-1.389 with the 0.1 ml/ml level of antigen. Seven of the 14 controls also showed some fixation, but the maximum ΔA_{412} was 0.371.

The fact that many of the control sera have some reactivity is not surprising in view of the complexity of the worm extract used as antigen. Since the lowest degree of fixation observed with any F. buski serum was higher than that obtained with any of the controls, the data indicate that there is a serologic response to the infection. Serum dilution titrations are in progress.

SUMMARY: Tests on 52 sera from individuals with F. buski infections showed greater complement fixation than in any of 32 control sera from normal individuals (26 sera) or people with O. viverrini infections. (6 sera).

Table 1. Complement fixation by human sera and crude F. buski antigen.

Serum	ΔA_{412}, Range in six experiments
F. buski	0.667-1.260
O. viverrini	0.066-0.553
Normal Thais	0-0.372
Normal Americans	0-0.257

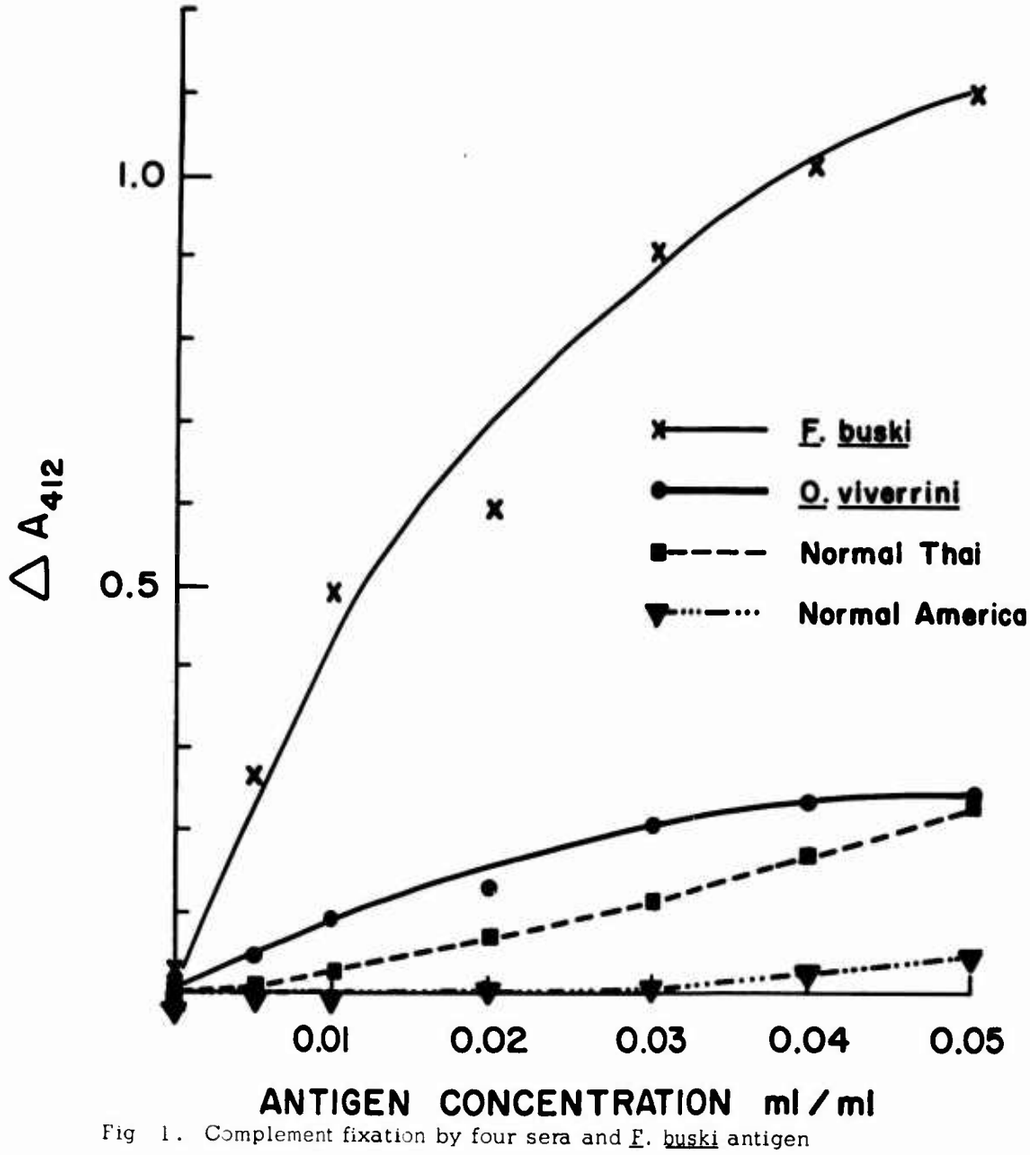


Fig 1. Complement fixation by four sera and F. buski antigen

Phanerosolus bonnei

Principal Investigators: George S. Manning, CPT, MSC
Carter L. Diggs, LTC, MC

Assistant Investigators: Vithoon Viyanant, B.S.
Pravet Lertprasert
Kitti Watanasirmkit, B.S.

OBJECTIVE: To study the life history, prevalence and pathology of this newly discovered human trematode infection.

DESCRIPTION: A survey of helminthic infections was recently conducted (August, 1969) in the village of Ban Phran Muan, Udornthani Province. Study of the morphological characteristics of Opisthorchis viverrini like eggs, which were found in abundance in the stool specimens, revealed the presence of two distinct types. In addition to the typical eggs of O. viverrini, eggs similar in size, but differing in morphology and staining characteristics, were found in 156 of 413 of the specimens examined. These findings were interpreted as evidence for the presence of a trematode infection heretofore undetected in Thailand, and efforts were instituted to obtain adult worms.

Fifteen residents of Ban Phran Muan, who had previously been found positive for the new trematode eggs, were given hexylresorcinol (Merck, Sharpe & Dohme) and the total stool output collected for three days and examined for adult worms.

On 17 November 1969, an autopsy was performed at the Udornthani Provincial Hospital on a 43 year old woman. The contents of the colon were examined microscopically for parasite eggs and larvae. The liver and bowel were examined grossly for adult helminths. The bowel was then carefully washed and the washings examined for the smaller species of parasites. All adult helminths were first examined unstained and then stained with acid carmine and mounted for identification.

PROGRESS: Adult trematodes were found in the stools of three of the fifteen persons treated with hexylresorcinol, but all were partially digested making study of the internal morphology unsatisfactory. The post-mortem examination revealed many O. viverrini adults in the liver. Several hookworms (Necator americanus), pinworms (Enterobius vermicularis) and one tapeworm (Taenia saginata) were found in the bowel. Microscopic examination of the contents of the colon revealed both the eggs of O. viverrini and the newly discovered trematode eggs. The

intestine was carefully washed and in excess of 100 small (less than 1 mm.) pyriform flukes were recovered in the washings, most of them from the duodenum and upper jejunum. A description of the fluke follows:

Adult Worm: a small fluke, with pyriform to oval body, covered anteriorly with minute pointed spines; the spines gradually become smaller posteriorly and become fine hair-like projections on the posterior one-fifth of the worm; body measures 0.5-0.7 mm. in length, 0.33-0.45 mm. in width; the oral sucker is slightly subterminal and well developed; a muscular pharynx is present immediately posterior to it; esophagus, short; ceca short, extending to the level of the acetabulum; acetabulum median, well developed, in middle third of body; genital pore at or slightly anterior to the level of the pharynx, submedian, prebifurcal; vitellaria prominent, extracecal, forming clusters of follicles (approximately 8) on either side in forebody; ovary, more or less ovoid and situated just lateral to acetabulum; uterus, well developed, and irregularly winding in hindbody, occupying most of hindbody. Eggs numerous, small (see description below); testes, ovoid, symmetrical, at acetabular or pre-acetabular level; cirrus pouch, curved, pre-acetabular, although over-lapping acetabulum slightly; excretory bladder, Y-shaped; but the extent of the development of the side arms in the body could not be traced due to the extensive development of the uterus.

Eggs: oval, rather thin shelled, with no distinct shoulder at operculum; Operculum, distinct or indistinct but not clearly differentiated as in O. vivirrini; length, 27 microns (23-33); width, 15 microns (13-18); no distinct abopercular excrescence; may have a thin albuminous coat adhering to the shell. When stained with iodine, the eggs appear tan or brownish, not wine red as do O. viverrini, and the contents do not appear fully developed but have a vacuolated appearance.

These characteristics conform closely to those of specimens kindly made available by Dr. Lie Kian Joe as discussed below.

The features described above clearly place this trematode into the Genus Phaneropsolus[1,2]. To our knowledge, there is only one previous report of human infection with a trematode of this genus; Phaneropsolus bonnei Lie Kian Joe, 1951, was described as a new species from a single autopsy in Djakarta[3]. Examination of some of the type specimens revealed only minor variations from the above describtion. We therefore identify our specimens as Phaneropsolus bonnei. Since over 150 cases have been detected, one of which was at a distance of 40 kilometers from the others, P. bonnei is established as a naturally

occuring parasite of man possibly widespread in northeast Thailand. Studies are now underway to elucidate the life cycle of the parasite and estimate the prevalence of infection.

Description of Fasciolopsis buski Infections in Thailand by use of a Catalytic Model.

Principal Investigators: George S. Manning, CPT, MSC
Warren Y. Brockelman, CPT, MSC
Vithoon Viyanant, B.S.

OBJECTIVE: Since many parasitic diseases exist at steady state levels of prevalence, it is possible to learn a great deal about the pattern of acquisition and loss of infection by analysis of age-specific prevalence data. By substituting age for time, attempts to fit exponential rate functions to the data are feasible. The application of these techniques has been described by Muench.[1] Previous Fasciolopsis buski surveys[2,3,4] have suggested that the prevalence typically reaches a peak in the 10-15 year age group and drops with older age groups. The purpose of the present study is to apply the new analytic approach to F. buski prevalence data in order to derive statistics describing the transmission of the parasite in quantitative terms.

DESCRIPTION: Study Populations: Pak Hai, Ayuthaya Province. The study area consisted of a small village along a tributary of the Noi River a few kilometers from the town of Pak Hai. No sanitary facilities are available in the village and the people usually defecate into the standing water beneath the houses (see 1969 Annual Report on Fasciolopsis buski). There is little evidence that the way of life of the people in the village has changed significantly during recent history, so that fundamental assumption of this application of catalytic models, the interchangeability of age and time, seems justified.

Kho Kho Tao, Suphanburi Province. The village of Kho Kho Tao, unlike Pak Hai, is situated at the edge of the basin in Central Thailand where the annual flood waters inundate the land. Approximately one-third of the village is situated over the water and the remainder on dry ground. No sanitary facilities are available, but the chance of parasite eggs reaching the water is considerably less than at Pak Hai, where the ground is almost completely convered by water for several months each year.

METHODS: A house-to-house survey was carried out in Pak Hai, with all houses along a 1 km stretch of the river being visited. Stool cups

were distributed to all members of each family. Name, sex and age were recorded, and usually, whether or not the youngest members had begun to eat uncooked green vegetables. Stools were collected on successive days and returned to the laboratory for processing (see 1969 Annual Report on F. buski). The response rate was approximately 65%. All stools were concentrated by the formalin-ether technique and examined microscopically for F. buski eggs by three examiners at Pak Hai, and two at Kho Kho Tao.

The Kho Kho Tao data were not originally intended to be subjected to detailed age-prevalence analysis, so that the sampling was not uniform. Approximately 30% of the sample was composed of 5-15 year old children surveyed in the school. The remainder was obtained by house-to-house surveying as at Pak Hai. We place less importance on conclusions reached from these data.

The two stage catalytic model described by Muench[1] assumes that newly exposed people become infected at rate a, become negative at rate b. and remain negative. The rate of change in prevalence is

$$\frac{dP}{dt} = ae^{-at} - bP$$

where e^{-at} is the proportion of people at time (or age) t that have never been infected, and P the proportion at that age actually positive. The solution is

$$P = \frac{a}{a - b}(e^{-bt} - e^{-at}).$$

The rates a and b can be found to roughly two decimal places by calculating two moments from the data and entering them on a nomogram supplied by Muench. The solution can be tested for goodness of fit with the X^2 statistic with k-2 degrees of freedom, where k is the number of age groups, reduced by the number of constants (two in this case) derived from the data.

The survey data were divided into age groups as uniform in width as possible, consistent with each having approximately 50 people. The younger people were divided into 5 and 2 year groups because they were more numerous and in order to increase the resolution of this critical region of the prevalence curve.

One additional assumption was made prior to analysis; risk of infection

was taken as beginning at two years of age and the curve was fitted so that t = 0 at age two. This decision was made on the basis of information obtained during the interviews which suggested that few babies under two years of age had begun eating vegetables.

PROGRESS: Pak Hai. An excellent fit to the data is obtained with the two stage catalytic curve, shown in Figure 1. In the test for fit, X^2 = 7.70, DF = 9, $.75 > p > .50$. The exponential rates, a = 0.23 and b = 0.024, can be converted into finite rates from:

$$a(\text{finite}) = 1 - e^{-a} = .205 \text{ per year}$$
$$b(\text{finite}) = 1 - e^{-b} = .023 \text{ per year}$$

The peak prevalence is predicted to be at age 13 by the equation. If everyone in the population is at risk, virtually everyone will have been infected by 20 years of age.

Kho Kho Tao. The prevalence over age 30 is significantly lower than the prevalence under age 30(X^2 with 1 DF, $p < .005$), indicating that a curve that rises to a horizontal asymptote is not appropriate for these data. The fitted two-stage model is shown in figure 2; the hypothesis that the data conform satisfactorily is strongly rejected (.005 p .001). The data which deviates most markedly from the model was obtained in the 2-8 year age groups. The actual rate of acquisition seems to be about double the 3.2% per year required to produce the prevalence observed for the older age groups, if the model provides an adequate description of transmission.

Mere reversibility of stool positivity and negativity is not sufficient to produce a decline in prevalence with age and, therefore, cannot be considered in interpretation of the results. As least three possible types of explanations for decline in prevalence with age can be considered; (a) nonreversible loss of parasites due to immunity; (b) unsuitability of older hosts for parasite survival to maturity (excluding immune mechanisms); and (c) decreasing risk of ingestion of metacercariae with increasing host age. Information gathered from personal interviews and questionnaires, which were distributed to many of the villagers, suggest that the ingestion of infection-carrying vegetables was not related to age in adults, so that assumption (c) alone cannot likely explain so marked a decline in prevalence as seen in Pak Hai. Our data do not permit discrimination between assumptions (a) and (b); further, we do not, at present, have any particular a priori model for non-immune host unsuitability. The first explanation conforms exactly to the two-stage model and is obviously an attractive one, but since there is little

evidence for immunity to fasciolopsiasis, we suggest both explanations (a) and (b) as being possible. Preliminary findings (see 1970 Annual Report on Immunologic response of F. buski) indicate that there is a serologic response in man to infections with F. buski, suggesting the possibility that acquired immunity is also operative.

We cannot provide a definite explanation for the poorer fit of the Kho Kho Tao data, though an increase in the infection rate during the past 8-10 years is one possibility.

Additional information about the physiology of the host-parasite interaction and quantitative information about diet are necessary before generality of applicability of the two stage model for F. buski infections can be claimed.

Analysis of the age-specific prevalence of several of the parasites common in northeastern Thailand is now underway.

Proteolytic Activity in Trichinella-Spiralis Larval Secretions.

Principal Investigators: Carter L. Diggs, LTC, MC
Tongchai Papasarathorn, M.D., M.P.H., S.M. (Harvard)
Chief, Department of Parasitology
Faculty of Public Health
Mahidol University

Associate Investigators: Pirom Phisphumvidhi, B.Sc.
Angoon Kiettivuti, M.S.
Department of Parasitology
Faculty of Public Health
Mahidol University

OBJECTIVE: One of the prevailing hypotheses concerning immunity in trichinosis is that secretions and/or excretions (here referred to as "secretions") of adult Trichinella spiralis contain antigen(s) which provoke(s) protective immunity in infected experimental animals[1,2]. It has been postulated that these antigen(s) consist of (or include) enzymes which are involved in the digestion of helminth nutrients, although there is no evidence for this at present. However, protease activity has been found in esophageal extracts of Ancylostom caninum and this activity was inhibited by immune serum[3]. Furthermore antibody inhibitory to larval lactic dehydrogenase has been obtained from rabbits infected with T. spiralis[4] and anti-enzymes have been described in other nematode infections. The objectives of this project are

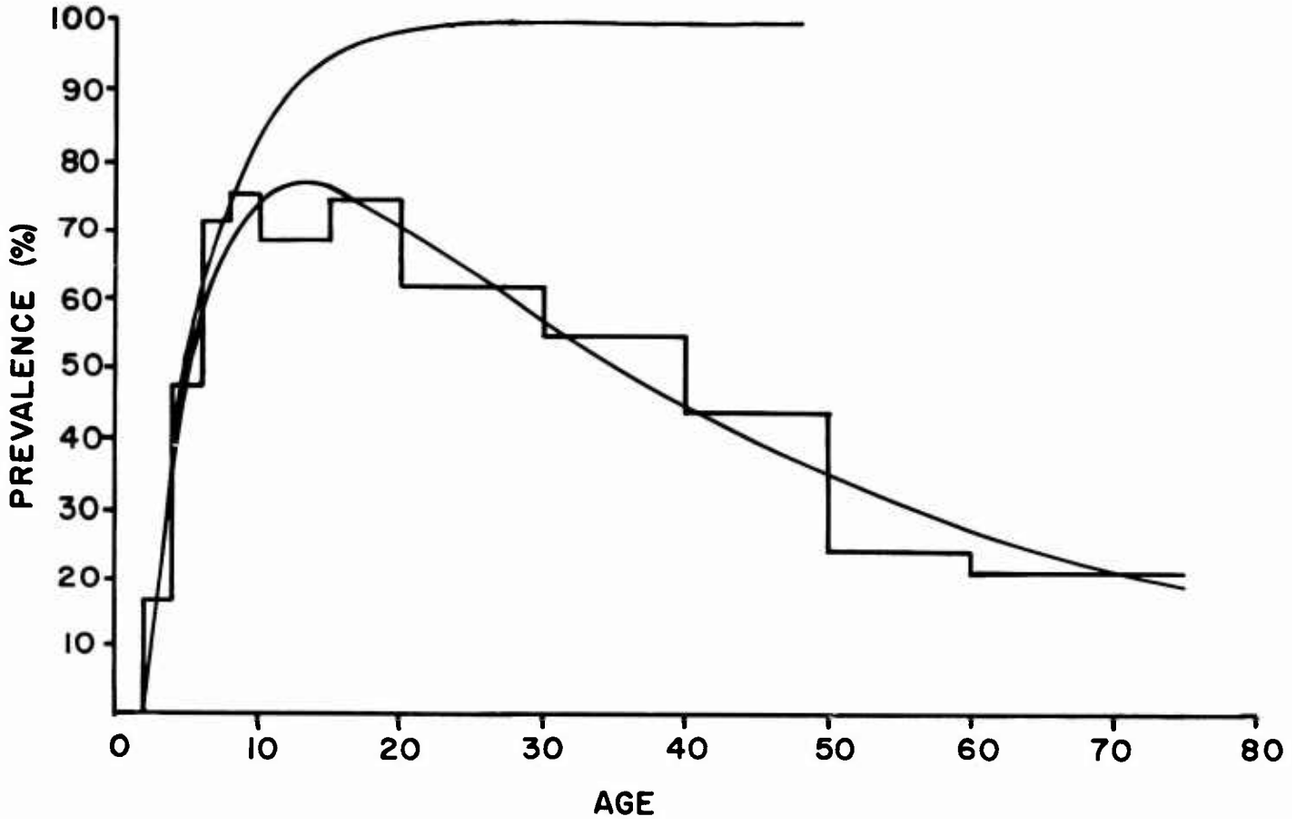


Fig 1. Age specific prevalence of F. buski infections at Pak Hai. Data indicated by horizontal lines; the fitted curve by the lower smooth line. The upper curve represents the acquisition of infection predicted by $P = \frac{a}{a-b}(e^{-bt}-e^{-at})$ where b=0.

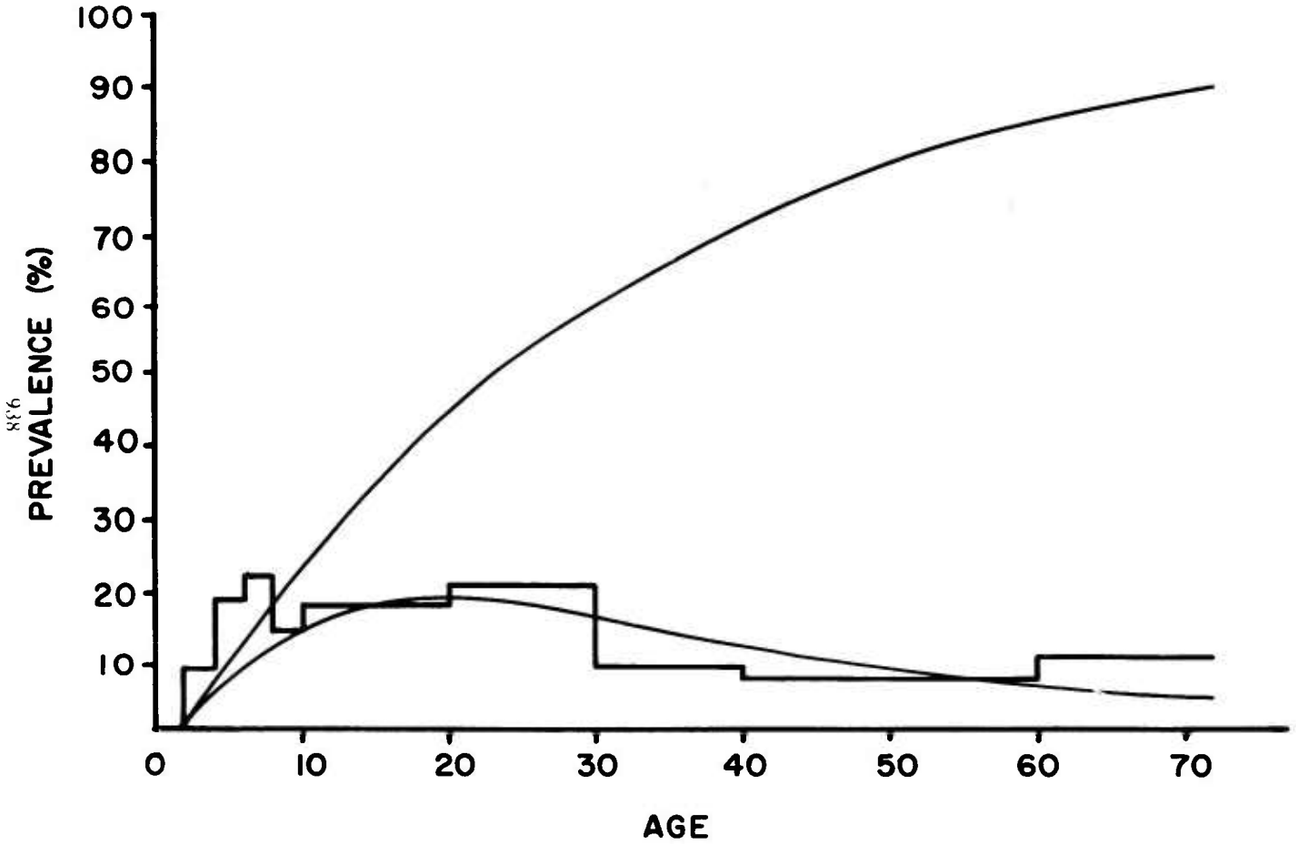


Fig 2. Age specific prevalence of F. buski infections at Kho Kho Tao. The various parts of the figure have meanings as described for Fig 1.

to (a) evaluate Trichinella spiralis secretions for protease activity and, if found, to (b) assess the ability of antisera raised in response to infection to inhibit this activity.

DESCRIPTION: T. spiralis larvae are isolated from the muscle of rats infected four weeks previously by a pepsin digestion technique and are incubated at 37°C in saline. Viability of larvae is assessed by microscopic sampling; larvae that are neither tightly coiled or motile are taken to be non-viable. Production of secretion is monitored by spectrophotometry at 280 and 260 mμ. The saline incubates are routinely cultured on blood agar and fluids showing bacterial growth are discarded and any data already obtained using such material rejected. Protease assays are performed using casein as substrate; the difference in absorbance at 280 mμ. between a trichloroacetic acid (TCA) extract of an unincubated control and that of the experimental reaction mixture is taken as an estimate of proteolytic activity. All protease determinations are performed in duplicate. Serum or serum fractions from rabbits infected with T. spiralis are tested for effect on protease activity by incubation with culture fluid for 30 minutes at 37°C prior to protease assay.

Gel diffusion tests are performed by standard techniques using both culture fluid and a somatic extract as sources of antigen.

PROGRESS: Essentially complete survival of the larvae is observed for the first 35 hours of incubation; after this, increasing numbers are found dead at each sampling. A significant amount of material absorbing at 260 and 280 mμ. can be detected as early as 15 hours and increases in an approximately linear fashion up until at least 35 hours. Routinely, culture fluid is harvested at about forty hours, when only a few larvae are nonviable. Protease activity has been found in both the culture fluid and in homogenates of the worms. TCA soluble material increases in the reaction mixtures for up to three hours, although linearity is not maintained. Since the activity is low, assays are routinely performed utilizing the three hour incubation period. The response obtained in this way is approximately linear with concentration of culture fluid (Fig. 1). The activity is abolished by boiling and appears to deteriorate on freezing and thawing. For this reason, most experiments are conducted with fresh material. Dialysis of the preparations in an effort to reduce the amount of TCA soluble material absorbing at 280 mμ. also resulted in loss of activity. Determination of the pH optimum for the proteolytic reaction revealed a biphasic dependence of activity of pH (Fig. 2). One optimum, at pH 2, is very close to that of pepsin and may represent a contaminant retained from the larval isolotion procedure, although washing of the larvae before culture is rigorous. The second optimum is at

pH 6; at this pH, there appears to be no contribution from the pepsin or pepsin like material. Studies of pH-activity curves of the pepsin preparation employed in these studies gave no evidence of activity at pH 6 (Fig. 3). These findings provide assurance that the activity detectable at pH 6 in T. spiralis secretions is in fact a product of the organisms.

Gel precipitin tests on sera from rabbits infected with T. spiralis show a brisk serologic response. Preliminary to study of the effect of antibody an protease activity, the influence of normal serum was explored; whole normal serum inhibits protease, but this inhibitory effect can be circumvented by the use of the 50% ammonium sulfate insoluble fraction of the sera (Table 1). A preliminary experiment with immune ammonium sulfate precipitated globulins suggested partial inhibition of protease, but attempts to confirm this finding with other sera have not been successful. Serially collected rabbit sera are now being titrated for antibody by passive hemagglutination and will be systematically studied for inhibition of protease activity.

SUMMARY: Proteolytic activity, as measured by production of trichloroacetic acid soluble material absorbing at 280 mu. during incubation with casein, has been demonstrated in saline in which T. spiralis larvae have been incubated. The activity appears to be a linear function of concentration and is abolished by boiling. The pH optimum is approximately 6. The activity is completely inhibited by normal rabbit serum but not by its 50% saturated ammonium sulfate insoluble fraction. Preliminary experiments suggest partial inhibition of activity by immune globulin.

Susceptibility of Laboratory Animals to Brugia tupaia.

Principal Investigators: George S. Manning, CPT, MSC
William L. Wooding, MAJ, VC
Carter L. Diggs, LTC, MC
Bruce A. Harrison, CPT, MSC

OBJECTIVE: These experiments were undertaken to test the susceptibility of laboratory animals to the tree shrew (Tupaia glis) filarial worm Brugia tupaia. If maintainance of the parasite in the laboratory were possible, a useful model for studies of host pathophysiology would be provided.

DESCRIPTION: Monthly trappings of tree shrews were made to provide a source of microfilaria and to determine whether or not seasonal

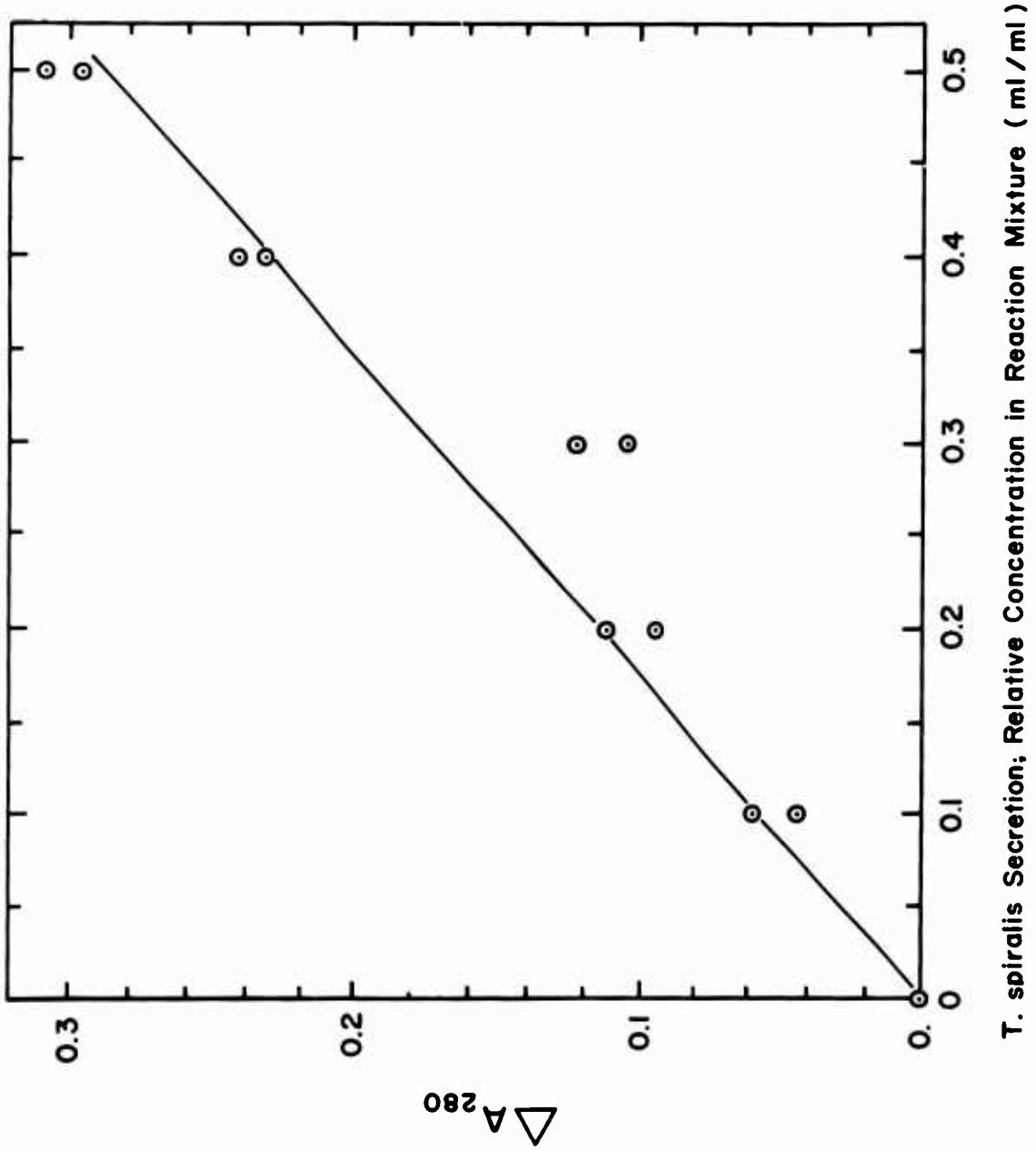
Table I. Effect of normal rabbit serum and its 50% saturated $(NH_4)_2SO_4$ precipitate on T. spiralis protease activity

	Mean Δ A_{280}
Saline	0.091
Whole serum	0
Heated serum	0.027
Dialyzed serum	0.027
$(NH_4)_2SO_4$ precipitate	0.124

Table II. Effect of the 50% saturated $(NH_4)_2SO_4$ precipitate of immune rabbit serum on T. spiralis protease activity

	Mean Δ A_{280}	
	Exp. No. 1	Exp. No. 2
Normal serum	-	0.100
Saline	0.107	-
Rabbit #1	0.089	0.069
Rabbit #2	0.071	0.070
Rabbit #3	0.085	0.080

Fig 1. Effect of concentration of T. spiralis secretion on production of TCA soluble material absorbing at 280 mu on incubation with a casein substrate. Each point represents the difference in absorbance between the TCA extract of a single reaction mixture and its unincubated control.



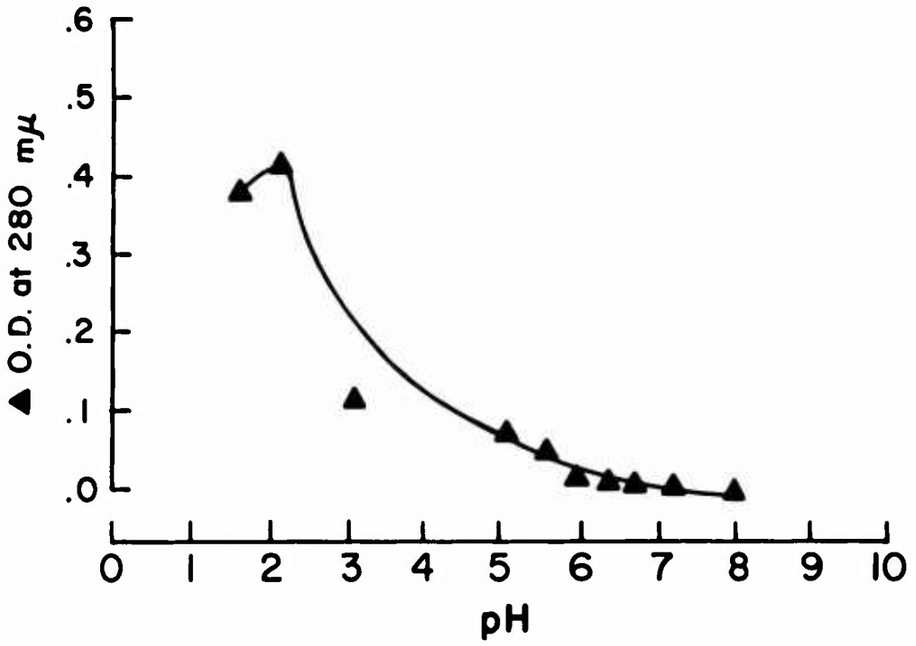
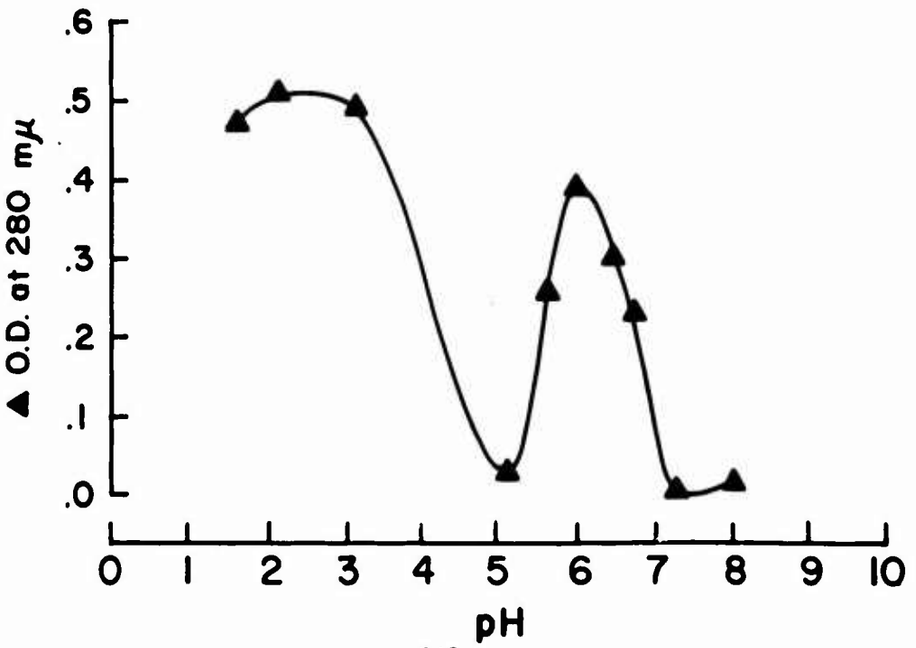


Fig 2. Effect of pH on protease activity of T. spiralis secretion. Each point represents the mean of duplicate determinations.

Fig 3. Effect of pH on protease activity of the pepsin preparation used for isolation of T. spiralis larvae. Essentially no activity is detectable at pH 6.



fluctuations in prevalence occurred in nature.

Infective larvae were inoculated subcutaneously (see 1969 Annual Report) into 21 rats (10-20 larvae each), 15 hamsters (10-20 larvae each), 3 rabbits (20-36 larvae each) and 2 gibbons (40-48 larvae each). Weekly complete blood counts were made on 10 rats and 5 hamsters, with peripheral blood examinations for microfilaria made periodically on all inoculated animals.

Two autopsies were performed on infected tree shrews to investigate the pathology of the infection in the natural host.

PROGRESS: The results to the field study suggest a peak in the tree shrew population occurring between September and January, as judged from the rise in the number of animals captured during this period. No trend suggestive of seasonal variation in prevalence of infection was observed.

The rats and hamsters have been studied for 60 and 48 weeks respectively; none have developed a patent infection. The rabbits and gibbons were bled monthly for 10 and 12 months respectively and have also remained negative for microfilaria. No remarkable changes in blood counts have been observed, except for a slight and transient elevation of eosinophils in some rats shortly after inoculation (see 1969 Annual Report).

Autopsies on the two infected tree shrews revealed numerous pathologic changes of the kidneys, liver, and certain lymph nodes. However, few if any specific changes could be directly attributed to the parasite. In one of the specimens, serial sections of adult worms, suspected to be B. tupaia, were found associated with lymph nodes in the abdominal cavity. This is the first time adult worms have been found.

When an animal remains negative for more than 12 months, it is assumed that the infection was not successful and the animal is then sacrificed If none of the experimental animals develop a patent infection, the project will be terminated.

SUMMARY: Attempts to infect laboratory animals with Brugia tupaia have, to date, been unsuccessful.

The Morphology of Gnathostoma dolcresi Advanced Third-stage Larvae.

Principal Investigator: Professor Svasti Daengsvang, Med.D.*

Assistant Investigators: Boonsiri Sermswatsri[1]
Pichit Youngyi, B.Sc.[1]
Dhawee Guname, B.Sc.[1]
Phaibul Sirichakwal, B.Sc.[2]
Paisal Yingyourd, B.Sc.[2]
Rapee Machimasatha, B.Sc.[2]

OBJECTIVE: This study was initiated to determine the numbers of hooklets in the cephalic area of the larvae of G. doloresi to allow, if feasible, differentiation from other species of gnathostomes already studied.

DESCRIPTION: Additional numbers of G. doloresi advanced third-stage larvae were obtained from 12 experimentally infected white mice. Measurements were made using the ocular micrometer; a microscopic study of the numbers and distribution of hooklets on each cephalic hooklet row after separating the head of each larva from the body was performed.

PROGRESS: The study was made on the number and distribution of cephalic hooklets in each row of 23 G. doloresi advanced third-stage 120-225 day old larvae from white mice. The size of each larva was also measured.

The results are shown in Table 1; the average body size of 23 larvae were 2.96 x 0.41 mm (range 1.83-3.99 x 0.34-0.49 mm). Two larvae were discovered to have developed a fifth row of hooklets (row V) on the cephalic bulb with a total of 37 and 19 cephalic hooklets respectively (Figure 1). This is the first observation of a fifth cephalic hooklet row in this organism. Including the present results, the body size and

---

* Retroactive in previous reports.
[1] Worked for some months during the year before resignation for further education.
[2] Replacement for 1.

Table 1. Distribution of cephalic hooklets of 23 advanced third-stage larvae of G. doloresi discovered in 12 experimentally infected white mice during the reporting period 1 April 1969 - 31 March 1970.

Row No. / Larva No.	I	II	III	IV	IV-I	Age (Days)	Size (m.m.)
1	36	37	31	34	-2	127	2.16x0.42
2	30	38	36	36	6	140	2.64x0.41
3	34	39	32	36	2	140	2.50x0.40
4	35	37	40	38	3	140	3.08x0.40
5	39	39	40	38	-1	140	3.14x0.42
6	36	38	35	36	0	119	2.68x0.37
7	40	39	37	39	-1	119	2.15x0.43
8	39	35	35	40	1	120	3.83x0.37
9	41	39	39	38	-3	120	2.07x0.36
*10	39	39	37	41	2	120	3.36x0.34
11	42	35	36	36	-6	120	2.85x0.36
12	38	39	39	38	0	120	2.89x0.36
13	42	40	39	40	-2	120	2.17x0.37
14	39	37	37	39	0	120	1.83x0.34
15	39	36	37	38	-1	218	3.16x0.46
16	37	36	37	33	-4	218	3.56x0.46
17	36	37	35	34	-2	218	3.49x0.49
*18	33	38	38	45	12	218	3.16x0.46
19	39	41	38	37	-2	225	3.56x0.46
20	35	34	35	32	-3	224	3.99x0.39
21	41	43	41	31	-10	224	3.16x0.46
22	39	41	36	35	-4	224	3.06x0.43
23	33	36	34	38	5	168	3.66x0.40
Average	37.1	38.0	36.7	36.8	-0.43	160.95	2.96x0.41

* Each larva has row V on its cephalic bulb showing 37 and 19 cephalic hooklets.

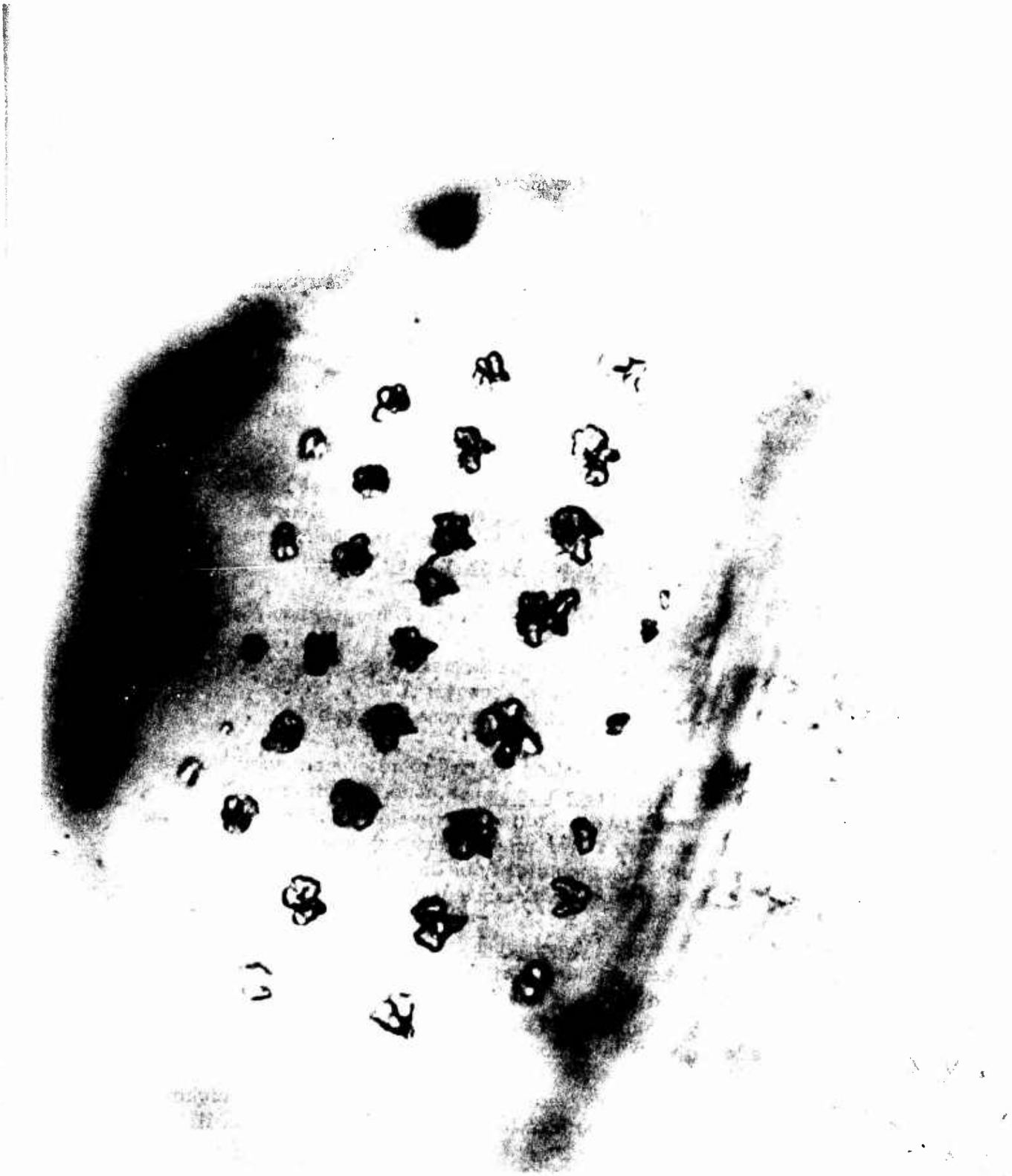


Fig 1 Photomicrograph of a cephalic bulb of G. doloresi advanced third-stage larva obtained from an experimentally infected white mouse developing 5 cephalic hooklet rows.

hooklet distribution of 38 larvae have been studied (Annual Progress Reports of 1968 and 1969). Twelve more larvae of this stage will be investigated to complete the study of the body size and of the distribution of the hooklets in 50 worms.

SUMMARY: Study of body size and cephalic hooklet distribution in 23 G. doloresi advanced third-stage larvae showed an average body measurement 2.96 x 0.41 mm; each row of cephalic hooklets had on average of 36.7 to 38.0 hooklets. Every cephalic bulb studied showed 4 cephalic hooklet rows with the exception of two larvae which had 5 hooklet rows on each cephalic bulb. Twelve more larvae will be studied before completion of the project.

A Study of the Morphology and Life Cycle of Gnathostoma vietnamicum and of the Prevalence of Infection and Renal Pathology in the Definitive Host, the River Otter (Aonyx cinerea Illiger).

Principal Investigator: Professor Svasti Daengsvang, Med.D.*

Assistant Investigators: Boonsiri Sermswatsri
Pichit Youngyi, B.Sc.
Dhawee Guname, B.Sc.

OBJECTIVE: The objectives of this study are to determine morphological characters of Gnathostoma vietnamicum useful for its differentiation from other gnathostomes, to determine the prevalance of the infection, to characterize the changes in the infected urinary organs in the definitive host, and to explore the possibility of the worm's transmission to and development in other vertebrates especially food animals of man.

DESCRIPTION: River otters (Aonyx cinerea Illiger) obtained from a southern province (Nakornsrithammarat) by a local animal dealer in Bangkok were autopsied within a few days after death. All organs were examined by using an examination box and the identification of worms and their eggs found were later confirmed microscopically.

PROGRESS: Three river otters were studied. An adult female weighing 1.7 kilograms with a body length of 18 inches and a tail length of 11.5

---

*Retroactive in previous reports.

inches was found infected with a total of 9 G. vietnamicum of which one was a larva and the others were 6 adult females and 2 adult males. The locations of the worms found were as follows: in the left kidney, 1 adult male was found in the pelvis; in the right kidney, 6 adult females and 1 adult male were found located in the pelvis and the upper part of the ureter. The measurements made on the adult worms showed a range of 31-47 mm x 1.8-2.8 mm for 5 adult females and of 35 mm and 45 x 2.0 mm for the 2 adult males (1 adult female was not measured). The cephalic hooklet rows on each head bulb were found to vary from 13-15 rows. One larva found in the lumen of the upper part of the right ureter measured 14.0 x 0.7 mm and had only 4 cephalic hooklet rows on its head. The gross pathological changes of the infected organs consisted of marked fibrous tissue thickening of the right pelvis and the upper part of the right ureter. The left kidney showed only slight fibrous tissue thickening at the pelvis. Many eggs of G. vietnamicum were found in the urine obtained from the bladder. The worms and infected kidneys were kept for further study.

The other two river otters were negative. These measured 16.5 and 17.0 inches for the body length and 10.5 and 9.5 inches for the tail length and weighed 1.3 kilograms and 1 kilogram respectively.

Previous studies on G. vietnamicum were reported in the SMRL Annual Reports for 1968 and 1969.

This study had to be suspended for some time during this reporting period due to the resignation of assistant investigators for further education. The study will be continued.

SUMMARY: A river otter obtained from Nakornsrithammarat Province, was found infected with 8 adult and 1 larva G. vietnamicum. Two other otters from the same area were negative.

Chemotherapy of Ganthostomiasis

Principal Investigator: Professor Svasti Daengsvang, Med.D.*

Assistant Investigators: Boonsiri Sermswatsri[1]
Pichit Youngyi, B.Sc.[1]
Dhawee Guname, B.Sc.[1]
Phaibul Sirichahwal, B.Sc.[2]
Paisal Yingyourd, B.Sc.[2]
Rapee Machimasatha, B.Sc.[2]

OBJECTIVE: The objective of this study is to determine the effect of multiple subcutaneous doses of Ancylol Disophenol (2, 6-diiodo-4-nitrophenol) on the immature and larval stages of G. spinigerum in the definitive hosts (dogs and cats). Since immature worms are the stages responsible for human gnathostomiasis, the study has pertinence to human as well as domestic animal infections.

DESCRIPTION: In the last Annual Progess Report (1969) a study of the chemotherapy of mature adult G. spinigerum infections in the stomach of 7 cats was reported. The adult worms were successfully removed by one subcutaneous injection of Ancylol, but the larval stages in the tissue seemed to resist the drug. A study of the effect of the same chemotherapeutic agent on the larval stages and immature adults migrating in the tissue of various organs of dogs and cats by more than one subcutaneous dose of the drug has now been initiated. The study was undertaken on 5 adult animals (3 dogs and 2 cats) 1 to 3 months after being infected transcutaneously with different numbers of advanced third-stage larvae. The drug given at a dosage of 0.1 ml per pound body weight according to the directions given by the manufacturer for treatment of canine hookworm. The experimental animals were sacrificed and examined for the presence of the worms in the tissues of various organs 7-15 days after the last dose of the drug was given.

PROGRESS: 1969: A negative adult female dog (#15) weighing 15 lbs was successfully inoculated in 45 minutes with 100 advanced third-stage larvae (97% successful penetration via skin). Thirty-three days later the animal was given 3 subcutaneous doses of 1.5 ml each of Ancylol at

---

* Retroactive in previous reports.
[1] Worked for some months during the year before resignation for further education.
[2] Replacement for 1.

7-day intervals. Autopsy one week after the last dose of the drug showed 5 living immature males and females in the muscles of the chest (1 female), back (1 female), abdominal wall (1 male), the peritoneal fat (1 female) and right lung (1 male). In addition, there were 7 living unencysted advanced third-stage larvae; one each in the flesh of back, anterior abdominal wall, right front leg, peritoneum, diaphragm and the wall of the small intestine and the rectum. Twelve dead immature worms were found in the muscles of the anterior abdominal wall (3 males, 2 females), back (2 females), right front leg (1 female), right hind leg (1 female), left lung (1 male) and liver (1) and left lung (1), Sex could not be determined in two worms.

Dog #16 was treated with 6 subcutaneous injections of Ancylol at one-week intervals. The first dose of 1.6 ml was given about 3 months after successful skin penetration with 47 G. spinigerum larvae in 1/2 hours (89.0% successful penetration); 5 doses of 1.2 to 1.7 ml of the drug according to the animals weight were then given regularly at one week intervals. The animal was sacrificed 18 days after the sixth dose and an atuopsy showed 1 living immature adult male G. spinigerum located in the tissue at the junction of the esophagus and the stomach measuring 10.7 x 1.0 mm with 7 cephalic hooklet rows. Ten growing larvae measuring 5.0-6.7 mm x 0.6 mm were found in the diaphragm; 8 were degenerate and 2 were alive with active movement when removed from the tissue. No adverse effects of the drug were noted and there was little change in body weight recognized during the course of treatment.

Dog #17 was also scheduled to be given 6 subcutaneous doses of Ancylol beginning about 1 1/2 months after being transcutaneously infected with 63 larvae (100% successful penetration). This animal unfortunately died of unknown causes 5 days after being subcutaneously injected with the third dose of 2.3 ml of the drug. During the course of treatment (about two weeks) the animal became anorexic and its body weight was reduced by about 6 lbs on the day of its death. On autopsy 2 days after its death, 11 growing larvae of the worm were found in the diaphragm; 6 showed much degeneration of the esophagus and intestine but the other 5 larvae were still alive. The measurements made on 10 larvae were 4.5-6.0 mm x 0.5-0.6 mm, slightly longer and larger than when applied to the skin.

The findings in these three treated dogs suggest that 3 to 6 doses of the chemical kills many larvae and immature worms located in the tissue when the treatment is started 1-3 months after infection.

Cat #85 was penetrated by 49 larvae in 2 hours (rate of successful penetration, 96%). Beginning 31 days after infection, the animal was treated with 4 doses of Ancylol. The first dose was injected subcutaneously (0.5 ml) and subsequent doses were given at 7-day intervals. Fourteen days after the last treatment (67 days after skin penetration) an autopsy showed a total of 5 living advanced third-stage larvae of the worm in the muscles of the back (1) abdominal wall (1), left hind leg (2) and diaphragm (1). The rate of recovery of living worms was still 10%.

Cat #94 was exposed to a total of 54 larvae in 1/2 hour (successful rate of penetration, 95%). It was given 6 subcutaneous doses of Ancylol at 7-day intervals beginning on 18 July 1969, one month after the infection, and ending on 3 September. On autopsy 15 days after the last dose of the drug on gnathostomes were found in the tissues; no macroscopic changes were noted in the organ examined. The animal remained normal during the course of treatment. Thus, in this cat, 6 subcutaneous doses of Ancylol apparently cured the animal of the infection by larval stages located in the tissues. The results of the study on the chemotherapy of G. spinigerum in cats and dogs is summarized in Table 1. The study is to be continued.

SUMMARY: Multiple subcutaneous doses of Ancylol Disophenol (2, 6-diiodo-4-nitrophenol) were used in this study of the chemotherapy of G. spinigerum infections in dogs and cats. A dosage of 0.1 ml per pound of body weight was given at weekly intervals beginning 1 to 3 months after exposure of the experimental animals. In 3 dogs, both living and dead larvae were recovered following as many as 6 subcutaneous injections of the drug.

One of 2 cats was negative for gnathostome larvae after 6 doses. These results are inconclusive and further investigation is under way.

Table 1. Chemotherapy of G. spinigerum larval and immature stages infecting 5 definitive hosts (3 dogs and 2 cats) by multiple subcutaneous doses of Ancylol Disophenol (2, 6-diido-4-nitrophenol) on the basis of 0.1 ml per pound of body weight per dose at 7-day intervals.

Animal No.	No. third-stage larvae penetrated thru skin/ per cent	Age of the worm in host before treatment (days)	Total weekly doses of Ancylol	Autopsy findings 7-15 days after treatment		Remarks
				No. /stages of worm	Organs sound infected	
Dog#15	100 (97%)	33 (1 month)	3	5 living immature	Muscles of chest, back, abdomen. Lung and peritoneum	sacrificed 7 days after the last dose
				12 dead immature	Peritoneum, diaphragm, intestinal wall	
				7 living larvae	flesh of back, abdomen, leg and lung	
Dog#16	47 (89%)	93 (3 months)	6	1 living immature male 2 living larvae 8 dead larvae	Esophagus and stomach junction, diaphragm	sacrificed 2 weeks after the last dose
Dog#17	63 (100%)	46 ($1\frac{1}{2}$ months)	3	11 growing larvae (6 dead)	diaphragm	died 5 days after the third dose of unknown causes
Cat#85	49 (96%)	31 (1 month)	4	5 living larvae	abdominal wall, leg, back, diaphragm	sacrificed days days after the last dose
Cat#94	54 (95%)	30 (1 month)	6	negative	none	sacrificed days after the last dose

Transcutaneous Infection by Gnathostoma spinigerum.

Principal Investigator: Professor Svasti Daengsvang, Med.D.*

Assistant Investigators: Boonsiri Sermswatsri[1]
Pichit Youngyi, B.Sc.[1]
Dhawee Guname, B.Sc.[1]
Phaibul Sirichakwal, B.Sc.[2]
Paisal Yingyourd, B.Sc.[2]
Rapee Machimasatha, B.Sc.[2]

OBJECTIVE: This study is designed to characterize experimental gnathostomiasis induced by the recently discovered transcutaneous route.

DESCRIPTION: Weekly stool examinations, by the formalin-ether concentration method of cats and dogs previously infected by skin penetration with G. spinigerum advanced third-stage larvae, were undertaken to determine the time required for the first ova positive stools (prepatent period) and the period of ova positive stools (patent period) of the infected animals. Quantitative estimates of the numbers of ova in the stools of the positive animals were also made by Stolls' egg counting technique. Autopsies were performed on cats and dogs to examine the migration and development of G. spinigerum larvae in various organs of the animals at various times after being transcutaneously infected with larvae of the worm.

PROGRESS: Observations of the skin penetration of third-stage larvae of G. spinigerum were continued on 7 cats and 7 dogs (see 1969 Annual Report), and the study was expended to include 2 additional adult cats and 1 adult dog. The results were as follows:

Cat #73 died of unknown causes on 22 July 1969 with the stool still positive for ova (144 + days of patent period). Autopsy showed a small round gastric tumor of about 1.0 cm in diameter containing 2 mature male G. spinigerum measuring 13.0 mm x 1.2 - 1.3 mm and 1 mature male attached to the omentum near the greater curvature of the stomach measuring 14.0 x 1.4 mm. Since the animal had been inoculated with

---

* Retroactive in previous report.
[1] Worked for some months during the year before resignation for further education.
[2] Replacement for 1.

42 larvae, the rate recovery of worms was only 7.1%. No female worms were found in the gastrointestinal tract and had the animal lived, the next stool examination would have been negative for ova. In this case probably many adult males and all females passed out with the stools before death (spontaneous elimination by the cat).

Cat #74 died on 1 April 1969 (probably as a result of vaccination against distemper), 195 days after the initiation of the experiment, and was still in a period of ova negative stools. On necropsy, the animal showed 17 immature male and female G. spinigerum in the diaphragm and anterior chest wall and 9 growing third-stage larvae in the anterior chest wall musculature: abdominal wall, back and the stomach wall. The rate of worm recovery was 56%.

Cat #77 first showed ova positive stools on 13 June 1969 or about 227 days after skin infection (prepatent period). It died 28 days following the first ova positive stool, probably due to rupture of a gastric tumor caused by the G. spinigerum adults. On autopsy 46 organisms were found (rate of worm recovery was 54%) of which 30 were mature worms, 14 immatures and 2 advanced third-stage larvae infecting many organs as follows: 12 mature (8 females and 4 males) free in stomach cavity; 9 mature (6 females, 3 males) located in a gastric tumor measuring about 2.0 cm. in diameter; 7 mature (5 females, 2 males) found attached to the peritoneal fat near the outer surface of a stomach tumor; 2 mature females in the left diaphragm. Fourteen immature worms (12 females, 2 males) were located in the following organs: 9 females attached to peritoneal fat near the lower part of the stomach, 4 (2 females, 2 males) located in the stomach tumor, and 1 female in the intercostal muscles of the lower chest wall musculature and one in the left diaphragm.

Cat #83 showed the first ova positive stool on 2 September 1969, after a prepatent period of 310 days. Stool examination was first negative 89 days later (patent period). The animal is still being kept for further observation.

Cat #84 first showed ova positive stools on July 1969 (a prepatent period of 127 days). The first ova negative stool was found 100 days later (patent period). The animal was kept for further observation.

Cat #87 and #89 died during the period of ova negative stools probably as the result of vaccination against distemper on the same date of worm development and the infectivity rates in the animal. On necropsy there were a total of 41 worms recovered with the following details: 37 mature (22 females and 15 males) and 2 immature female G. spinigerum found

in a round gastirc tumor measuring 2.0 cm. in diameter. In addition there was 1 immature female located in the right lung and 1 immature female attached to the peritoneal tissue near the lower part of the stomach. The worm recovery rate was 63%.

Dog #2 showed the first G. spinigerum ova positive stools on 22 May 1969 (prepatent period of 247 days after the first skin infection); the first ova negative stool was on 3 February 1970 (patent period of 257 days). The animal was kept for further study.

Dog #9 showed the first G. spinigerum ova positive stools on 3 June 1969 (prepatent period of 22 days); the period of ova positive stools was 58 days (patent period). The animal was then sacrificed 57 days after the last ova positive stool; autopsy showed no stomach lesion, but there were 9 advanced third-stage larvae measuring 6.0-6.8 x 0.6-0.7 mm found in the muscles of the hind legs (4 larvae), in the intercostal muscles (3 larvae), and in the flesh of the anterior abdominal wall (2 larvae). The worm recovery rate is 4.7%.

Dog #12 the animal always showed ova negative stools on weekly stool examinations. It was sacrificed on 26 February 1970, 498 days after the skin infection and on autopsy revealed a total of 2 G. spinigerum larvae in the muscles of the back and fore-leg measuring 8.0 x 0.7 mm and 4.0 x 0.6 mm (worm recovery rate 2.3%).

Dog #13 first became ova positive 112 days after the first skin infection or 93 days after the last (prepatent period). The animal then continued showing ova positive stools for more than 329 days up to the date of termination of the study (17 October 1969).

Dog #14 was sacrificed on 23 July 1969 after 253 days (8 1/2 months) of patency. Autospy showed a gastric tumor of about 2.0 cm in diameter containing 19 mature worms (9 males and 10 females), and there were 12 mature worms (3 males, 9 females) found freely mixed with the stomach contents. In addition, there was 1 immature worm found in the right side of the diaphragm and another in the lower part of the esophageal wall. The total burden was 33 worms (rate of worm recovery about 49%).

Dog #18 was given on August 13, 1969, one transcutaneous infection with 64 G. spinigerum larvae in 1 1/2 hours (rate of successful penetration, 100%). The animal up to the end of 31 March (230 days after the skin infection) was still showing ova negative stools. It was kept for further observation.

The result of this experimental investigation on skin penetration of dogs by G. spinigerum larvae is summarized in Table 2.

SUMMARY: Observations of cats and dogs transcutaneously infected with third-stage larvae of G. spinigerum were continued. Prepatent periods in the cats ranged from 60 to 310 days, and the periods of positive stools (patent periods) varied from 89 to more than 197 days. Recovery of inocula as viable worms ranged from 7.0% to 94.4%.

In dogs, the prepatent period was from 96 to 247 days. One dog continued to be negative for 498 days, and at autopsy only 2 larvae were recovered. The period of patency (ova production) ranged from 58 days to 329 days. The worm recovery rate varied from 2.4% to 85%.

There appeared to be a continuous reduction of the number of worms harbored by both dogs and cats as the infections persisted. From rates as high as 94% in cats the recovery rate decreased to 7% from rates as high as 94% obtained from cats 22 days after exposure, the rates decreased to 7% 320 days after exposure. In dogs, 85% could be recovered the day after penetration of the larvae and only 4.7% of the inoculum was found 280 days after exposure.

Table 1. Skin penetration by Gnathostoma spinigerum advanced third-stage larvae in 10 adult domestic cats.

Animal No.	Source of larvae	Date and frequency of infection	No. of larvae penetrated thru the skin and %	Days from skin penetration of larvae to first ova positive stool (prepatent period)	Days from first ova positive stool to first ova negative (patent period)	Autopsy findings			Remarks
						No. of worms recovered and %	Stages of worms recovered	Organs infected by worms	
*Cat 38	white mice & snake-headed fish	13-22/8/68 3 times	53 (62%)	154 +	-	27 (51%) males, females and larvae	immature adult and larvae	stomach abdominal wall chest wall omentum diaphragm	Sacrificed on 14 Jan 69 (day 154 of prepatent period)
Cat 73	white mice dog	5-6/9/68 2 times	42 (93%)	176	144 +	3 males (7.0%)	mature adult	Gastric tumor and omentum	Died of unknown causes with ova positive stool (day 144, on 22 July 69)
Cat 74	snake-headed fish	17/9/68 1 time	46 (90%)	195 +	-	26 males, females and larvae (56%)	immature adult and larvae	stomach diaphragm, costal, abdominal and back muscles, abdominal fat.	Died perhaps of vaccination (on 1 April 69, day 195 of prepatent period)
Cat 77	white mice	29/10/68 1 time	85 (100%)	227	28 +	46 mature and immature and larvae (54%)	mature and immature adults and 1 larva	stomach diaphragm peritoneum muscles of abdomen and chest.	Died during ova positive stools (day 28 of patent period, on 11 July 69)
Cat 83	white mice	30/10/68 1 time	61 (100%)	310	89	-	-	-	Still alive, ova negative stool up to 31 March 70 (118 days after last positive stool)
Cat 84	white mice	25-28/2/69 2 times	44 (66.7%)	127	100	-	-	-	Still alive, ova negative stool up to 31 March 70 (172 days after last positive stool)
Cat 87	white mice	10/3/69 1 time	18 (100%)	22 +	-	17 larvae (94.4%)	larvae	liver, skin, diaphragm abdomenal fat	Died perhaps of vaccination on 1 April 69 (day 22 of prepatent period)
Cat 89	white mice	10/3/69 1 time	45 (75%)	22 +	-	38 larvae (84.4%)	larvae	liver abdomenal muscle abdomenal fat	Died perhaps of vaccination on 1 April 69 (day 22 of prepatent period)
Cat 91	white mice	17/7/69 1 time	59 (93%)	60	197 +	-	-	-	Still alive, ova positive stool up to 31 March 70 (day 197 of patent period)
Cat 97	white mice	7/8/69 1 time	10 (100%)	60 +	-	7 larvae (70%)	larvae	liver	Died on 6 Oct 69 (day 60 of prepatent period)

*Reported in 1969 Annual Report.

Table 2. Skin penetration by Gnathostoma spinigerum advanced third-stage larvae in 9 adult domestic dogs.

Animal No.	Source of larvae	Date and frequency of infection	No. of larvae penetrated thru the skin and %	Days from skin penetration of larvae to first ova positive stool (prepatent period)	Days from first ova positive stool to first ova negative (patent period)	Autopsy findings			Remarks
						No. of worms recovered and %	Stages of worms recovered	Organs infected by worms	
Dog 1	white mice	15/10/68 1 time	65 (79.3%)	231	31 +	41 mature males & females (63 %)	mature adult	stomach lung omentum	Sacrificed on 3 July 69 with ova positive stools (day 31 of patent period)
Dog 2	snake & snake-headed fish	17-23/9 /68 2 times	76 (100%)	247 (from first skin infection)	257	-	-	-	Still alive, ova negative stools up to 31 March 70 (56 days after last positive stool)
Dog 9	white mice	24/10-4/11/ 68 2 times	192 (81.4%)	222 (from first skin infection)	58	9 larvae (4.7%)	larvae	hind leg muscle costal muscle abdomenal muscle, abdomenal fat	Sacrificed with ova negative stools, 26 Sept69 (57 days after last positive stool)
Dog 10	white mice & snake	28/10 - 12/11/68 2 times	119 (69.6%)	234 (from first skin infection)	228	-	-	-	Still alive, ova negative stools up to 31 March 70 (57 days after last positive stool)
*Dog 11	white mice	4/9/68 1 time	33 (97.1%)	1 +	-	28 larvae (85.0%)	larvae	skin abdomenal flesh.	Died on 5 Sept 68 with ova negative stool (day 1 of prepatent period)
Dog 12	white mice	16/10/68 1 time	88 (96.7%)	498 +	-	2 larvae (2.3%)	larvae	dorsal muscle fore-leg muscle	Sacrificed on 26 Feb 70 with ova negative stool (day 498 of prepatent period)
Dog 13	white mice	2-21/8/ 68 3 times	64 (38.6%)	112 (from first skin infection)	329 +	-	-	-	Discharged on 17 Oct 69 from the study for being uncontrollable.
Dog 14	white mice	8-20/8/ 68 2 times	68 (46.2%)	96 (from first skin infection)	253 +	33 immature females, mature males & mature females (48.5%)	immature females, mature males mature females	stomach diaphragm esophageal wall.	Sacrificed on 23 July 69 during ova positive stools (day 253 of patent period)
Dog 18	white mice	13/8/69 1 time	64 (100%)	230 +	-	-	-	-	Still alive, ova negative stool up to 31 March 70 (day 230 of prepatent period)

*Reported in 1969 Annual Report.

Studies of New Experimental Hosts, Life Cycles and Modes of transmission of Gnathostomes

Principal Investigator: Professor Svasti Daengsvang, M

Assistant Investigators: Boonsiri Sermswatsri[1]
Pichit Youngyi, B.Sc.[1]
Dhawee Guname, B.Sc.[1]
Phaibul Sirichakwal, B.Sc.[2]
Paisal Yingyourd, B.Sc.[2]
Rapee Machimasatha, B.Sc.[2]

OBJECTIVE: During this reporting period continuation of the study to determine the seasonal variation in the prevalence of adult G. spinigerum infections in dogs in the Bangkok and Thonburi areas and of advanced third-stage larval infections in snake-headed fish (Ophicephalus striatus), bought at the Ayuthaya and Phetburi markets, was undertaken. Poisonous snakes provided by the Thai National Red Cross Snake Farm were also studied. Studies of adult G. spinigerum and other gnathostome species obtained from infected pig stomachs at the Bangkok Slaughter House were continued. Additional studies of the susceptility of some common fresh water animals and other vertebrates as intermediate and paratenic hosts of G. spinigerum, G. hispidum and G. doloresi were performed.

DESCRIPTION: Examination of the gastrointestinal tracts of stray dogs killed at The Bangkok-Thonburi Municipality Rabies Control Unit was performed in the month of April of this reporting year to complete a study on the seasonal prevalence of G. spinigerum in the definitive host (dogs) in the Bangkok and Thonburi areas. This study was initiated in 1965 for G. hispidum and G. doloresi and 1968 for G. vietnamicum. Gnathostome worms were obtained from infected pig stomachs for species identification. This was made possible with the full cooperation and assistance of the Bangkok Slaughter House authorities.

Weekly stool examinations for gnathostome ova by the formalin-ether concentration method were done on cats and dogs brought to the SMRL animal house for experimental purposes; all cats and dogs that died were autopsied and examined for the presence of gnathostome worms in the gastrointestinal tracts and musculature. Stool samples collected from 40 young domestic pigs at a private Nakhornpathom pig farm by

---

* Retroactive in the previous reports.
1 Worked for some months during the year before resignation for further education.
2 Replacement for 1.

Dr. Markapol Tingpalapong of the SMRL Veterinary Medicine Department were examined by the formalin-ether concentration method for gnathostome ova.

In the month of April, 1969, about 2 kg. of fresh water snake-headed fish (22 fish) purchased at the markets in Ayuthaya and Phetburi (endemic areas) and posionous snakes from the Thai National Red Cross Snake Farm in Bangkok, which died spontaneously, were autopsied for G. spinigerum and other gnathostome larvae for the completion of a study on the seasonal prevalence rate of infection with G. spinigerum advanced third-stage larvae.

To avoid the experimental use of naturally infected animals with gnathostome larvae, autopsies were done on samples of snake-headed fish (Ophicepahalus striatus), catfish (Clarias batrachus), top minnow fish (Gambusia hollbrooki), small fighting fish (Trichopsis vittatus), toad tadpoles (Bufo melanostictus), frogs (Rana rugulosa) and fresh water crabs (Paratelphusa sexpunctatum), of which some were collected in the Bangkok area from public and private fresh water ponds and ditches, and others were bought at the markets at Bangkok and Thonburi.

Experimental infection was also continued on many vertebrates to determine additional potential second intermediate and paratenic hosts G. spinigerum and of G. hispidum. Additional numbers of white mice and one monitor lizard were fed with fully developed larvae of G. doloresi in cyclops to determine their ability to act as the second intermediate host of the worm.

PROGRESS: Table 1 summarizes the April, 1969, findings on examination of dog stomachs from the Bangkok Thonburi Municipality Rabies Control Unit, snake-headed fish purchased at the fish markets in Ayuthaya and Phetburi, and snakes from the Thai National Red Cross Snake Farm in Bangkok.

All 1,289 dog stomachs examined were found negative. During April of 1967 and 1968, 408 and 356 dog stomachs respectively, obtained from the Rabies Control Unit were also negative. Advanced third-stage larvae of G. spinigerum were found in 2 (9%) of 22 fresh water snake-headed fish bought at Ayuthaya and Phetburi markets showing 1 and 2 encysted G. spinigerum larvae each (at Ayuthaya 1 large fish negative and 1 of 6 small fish positive with 2 encysted larvae; at Phetburi 1 of 5 small fish positive with 1 encysted larva). These findings may be compared with 60.0% positive on examination of 8 small fish and 2 large fish (5 small fish and 1 large fish were positive) during April 1968. All 37 poisonous snakes obtained from the Thai National Red Cross snake farm (26 Naja naja, 1 Naja hannah, 8 Vipera russelli, and 2 [illegible]nerus gramineus) were found negative as compared with 6% positive on examination of 17 poisonous snakes in April 1968 (1 of 17 snakes was positive with the larva). Also during this year 4 small and 1 large snake-headed fish obtained from Bangkok and Thonburi markets (2 small

fish bought at Thonburi market and 1 large and 2 small bought at Bangkok market) were found negative for gnathostome larvae. Of an

Table 1. Result of study in April 1969 on the incidence of G. spinigerum infection in Bangkok domestic dogs and infection of second intermediate or paratenic hosts (snake-headed fish and poisonous snakes) with advanced third-stage larvae in endemic areas near Bangkok (Ayuthaya - Phetburi)

	Total
Dog stomach	
(No. pos./No. exam.)	0/1289
Infection with adult G. spinigerum % Positive	0
Ophicephalus striatus (snake-headed fish)	
(No. pos./No. exam.) Large*	0/1
Small**	2/21
Total	2/22
Infection with advanced third-stage larvae (in Ayuthaya-Phetburi) % Positive	9
Poisonous snakes (Naja naja & hannah, Vipera russelli, Trimeresus gramineus)	
(No. pos./No. exam.)	0/37
Infection with advanced third-stage larvae % Positive	0

* Large Ophicephalus striatus = 500 grams and over
** Small Ophicephalus striatus = Less than 500 grams

additional 15 young snake-headed fish weighing not more than 5 grams each and measuring about 1.5 cm - 6.0 cm x 0.2 cm - 0.8 cm collected from a roadside fresh water ditch about 15.0 kilometers north of SMRL, 1 was found infected with a non-encapsulated G. spinigerum advanced third-stage larva in its liver measuring 2.2 x 0.2 mm.

2 small catfish (Clarias batrachus) bought from a Thonburi market were found on autopsy to be negative for gnathostome infection.

Stool examinations of 28 domestic cats (#97 to #124) newly obtained

from Rajburi Province and nearby areas for experimental infection with gnathostomes; 2 (#102, #103) were found positive with gnathostome ova (7.2%) but 11 of them died of unknown causes of which one only (#104) showed on autopsy one immature male measuring 13.3 x 0.3 mm in the stomach tumor of about 0.5 cm in diameter. This animal never showed gnathostome ova on frequent stool examinations.

Stool samples collected from 40 young domestic pigs at a private Nakhonpathom pig farm were found to be negative for gnathostome infection.

A total of 659 adult gnathostomes were recovered from infected pig stomachs from the Bangkok Slaughter House of which none were G. spinigerum; however, 488 (206 males and 282 females) were identified as G. doloresi and 171 (58 males, 113 females) as G. hispidum, as compared with a total of 979 worms (359 G. hispidum = 80 males and 279 females; 620 G. doloresi = 86 males and 534 females) for 1967-68, and 1773 worms (425 G. hispidum = 182 males and 243 females); 1348 G. doloresi = 414 males and 934 females) for 1968-69. There were 3411 gnathostomes found in 3 years from infected pig stomachs; none were G. spinigerum, but 955 and 2456 were identified as G. hispidum and G. doloresi respectively. It is then reasonable to conclude that pigs are not the definitive host of, or naturally infected with, adult G. spinigerum.

Experimental infection with G. spinigerum larvae.

(a) Further determination of new second intermediate host and paratenic host on some animals commonly found in fresh water ditches and ponds was experimentally undertaken as follows:

After finding no natural infection with gnathostome larvae on examination of 3 small top-minnow fish (Gambusia holbrooki), 10 toad tadpoles (Bufo melanosticuts) and 6 fresh water crabs (Paratelphusa sexpundtatum) caught from a fresh water pond near the animal house of SEATO Medical Research Laboratory, 3 other top-minnow fish of the same size and from the same pond were experimentally in a 1000 ml beaker containing about 600 ml of fresh water with 18 cyclops infected with 37 fully developed larvae of G. spinigerum. These 3 fish on autopsies 5-6 days later were found to be infected with 2, 3 and 4 advanced third-stage larvae respectively in the skeletal muscle of which three larvae, one from each fish, were found dead. Measurements of all larvae revealed a variation in length of from 0.3-1.66 mm and in width of from 0.03-0.13 mm.

Four toad tadpoles of about the same size from the same pond were experimentally infected in a 500 ml glass beaker containing about 300 ml of fresh water with 10 cyclops infected with 30 fully developed larvae of G. spinigerum 10 days old. Of the tadpoles autopsied on different days, 2 examined on days 11 and 28 of the experiment were

negative for the infection; however, the other 2 tadpoles examined on days 11 and 21 were found to be infected each with 2 and 4 live advanced third-stage larvae of the worm in the digestive tract measuring in the range of 0.4-1.0 mm x 0.03-0.12 mm.

The results of experimental investigation showed for the first time that top-minnow fish and toad tadpoles, commonly found in fresh water ditches and wells, also act as additional second intermediate hosts of G. spinigerum. Moreover it is reasonable to assume that snake-headed fish may be infected with advanced third-stage larvae of the worm by feeding on the infected top-minnow and toad tadpoles (secondary infection or paratenic host).

Fifteen adult fresh water crabs were experimentally fed with different numbers of advanced third-stage larvae (3 to 40) obtained from experimentally infected white mice on autopsies at various days (8-29) after infection; all were negative for the infection. This study will be continued until a total of 50 crabs have been examined or until some experimental crabs are found positive.

The life cycle of G. hispidum.

(a) Experimental study for determining second intermediate host by feeding vertebrates with G. hispidum fully developed larvae in cyclops.

Six adult snake-headed fish and 2 catfish were found negative after being fed with fully developed larvae in cyclops as reported in the SMRL Annual Report for 1969.

During the reporting year the following food animals of man were studied to determine their ability to act as second intermediate hosts of the worm with consequent possible transmission to man. The results are as follows:

Of 2 young snake-headed fish (Ophicephalus striatus) weighing about 3 grams (#5 and #6) and each fed with 15 fully developed 10 day old larvae in 4 and 8 cyclops respectively, one fish was found on autopsy 7 days after the experiment to harbor 1 living third-stage larva of G. hispidum measuring 345 microns x 66 microns located in the liver. The other fish autopsied 32 days after the experiment showed no infection. Two small snake-headed fish weighing 100 gram and 140 grams respectively were infected; one (#7) fed with 50 fully developed 16 day old larvae in 29 cyclops and the other (#8) with 40 larvae of the same age in 21 cyclops. On autopsy, one of the fish (#8) 5 days after the experiment showed 12 advanced third-stage larvae located in the stomach wall of which 10 were in the range of 662-800 microns x 85-100 microns. To determine further the infectivity, 8 larvae were then fed to one white mouse (#36); autopsy 21 days later showed no infection. The other experimental fish (#7) was sacrificed

41 days after the experiment. On necropsy it was also negative.

Four catfish (Clarias batrachus) fed with 50, 46, 30 and 30, 10-12 day old fully developed larvae in cyclops showed on autopsies 24, 28, 46 and 47 days after the experiment 21, 7, 3 and 4 living larvae in the stomach wall each of which was surrounded by a thin cyst wall measuring 266-400 x 71-194 microns. The dimensions of 7 larvae after being freed from the cyst wall were 1.2-1.4 x0.2-0.3 mm. To determine further the infectivity of these larvae in some warm-blooded vertebrates, 2 laboratory bred tree shrews (Tupaia glis) were fed with 9 and 10 larvae obtained from one of the infected fish. On autopsy 11 days later the tree shrews were negative for the infection.

Of 6 small fighting fish (Trichopsis vittatus) sacrificed 9 to 53 days after exposure to 68 cyclops infected with 89 G. hispidum fully developed larvae, 4 (66%) showed on autopsies 9 to 53 days after the experiment a total of 26 (30%) advanced third-stage larvae (25 in the stomach wall and 1 in the body flesh) of which 13 dead and 13 living. The dimensions of 19 larvae were 0.4-0.5 x0.05-0.08 mm. Eleven living larvae discovered in the stomach wall were then fed to a white mouse to determine their further infectivity; autopsy 14 days later revealed no infections.

Three frogs (Rana rugulosa) were previously reported negative with the infection after being fed with fully developed larvae in cyclops (Annual Progress Reports 1968 and 1969). During this reporting year each of 9 frogs was fed with 50 G. hispidum fully developed larvae 10-16 days old in cyclops; an additional frog was given 30 larvae. On autopsies of these frogs, 4-50 days after the experiment, 4 (40%) sacrificed 48-50 days after being fed with the larvae were found positive with 1, 1, 2 and 11 advanced third-stage larvae of the worm encysted in the flesh of the abdominal wall, leg, back and anterior chest wall of the animals.

Two tree shrews were fed with 30 fully developed 15 day old larvae each in cyclops. On autopsy 83 days later, one was found negative for the infection and another had 1 encysted larvae measuring 1.0 mm x 0.8 mm in the flesh of its right hind leg.

A monitor lizard (Varanus nebulosus) was found negative on autopsy after being fed with a total of 62 fully developed larvae in cyclops.

Snake-headed fish (2), catfish (4), and small fighting fish (4) were found to be experimentally susceptible to G. hispidum; advanced third-stage larvae could be found developing several days in the stomach wall; in addition, one larva was found in the flesh of an infected fish. Four frogs and 1 tree shrew were also found to be experimental hosts in which G. hispidum advanced third-stage larvae could develop after being fed with larvae in cyclops; survival in the flesh of the leg, abdominal wall and back for some days was noted.

These findings show that snake-headed fish, catfish, small fighting fish, frog and tree shrew can act as the second intermediate host of the parasite.

Further study to determine second intermediate hosts of G. hispidum is to be undertaken.

(b) Experimental study for determining paratenic hosts (secondary infection) by feeding vertebrates with G. hispidum advanced third-stage larvae obtained from an infected fish and two infected white mice; the results were as follows: one young fish showed on autopsy 3 days after being fed with the 6 larvae obtained from the mouse 4 living larvae of which 1 was found in the body flesh and 3 in the liver. A few hours later these 4 larvae were fed to another young fish which was sacrificed 33 days later and showed on examination 3 living larvae in its intestinal wall. The third fish, sacrificed 41 days after being fed with 5 living advanced third-stage larvae 179 days old obtained from an infected white mouse, was found to be negative for the infection on autopsy.

Two adult laboratory bred tree shrews (Tupaia glis) were fed with 9 and 10 G. hispidum 24 day old advanced third-stage larvae obtained from a catfish infected with fully developed larvae in cyclops. Autopsy 11 days after the experiment was negative for the infection.

It is clear that the advance third-stage larvae when obtained from infected mice could infect snake-headed fish and later removed from the fish could be transmitted to a second fish. On the other hand third-stage larvae from fish did not become well established when fed to a mouse or a tree shrew. The problem is to be further studied.

The study on the life cycle of G. doloresi.

This is an experimental study for determining the second intermediate host by feeding vertebrates with G. doloresi fully developed larvae in cyclops of which the results are as follows:
31 laboratory bred adult white mice (Mus musculus) which had been fed a total of 1407 G. doloresi fully developed larvae 10-12 days old in cyclops were autopsied 12-225 days after feeding 12 mice (39%) yielded 23 encysted G. doloresi third-stage larvae from the musculature. When considered with the 6 laboratory infections obtained in mice previously (1969 Annual Report) a total of 18 (40%) of 45 white mice have been experimentally infected with a total of 34 larvae.

One monitor lizard (Varanus nebulosus) was fed with 90 G. doloresi fully developed 10 day old larvae in cyclops. On autopsy 32 days later it was negative for the infection.

This study is continuing.

SUMMARY: 1289 stomachs from stray dogs killed at the Bangkok and

Thonburi Municipality Rabies Control Unit were examined during April 1969. All were negative. Thirty seven poisonous snakes obtained from the Thai National Red Cross Snake Farm were also found to be negative for gnathostome larvae. Two (9.0%) of 22 fresh water snake-headed fish bought at Ayuthaya and Phetburi markets were found positive with 1 and 2 encysted G. spinigerum larvae. Many fresh water fish, frogs, and toad tadpoles obtained from Bangkok and nearby areas were examined for the presence of natural infection with gnathostome larvae; only 1 young snake-headed fish was found positive wtih 1 non-encapsulated G. spinigerum larva.

On frequent examinations of 28 domestic cat stools (#97 to #124 obtained for experimental purposes during this period from Rajburi Province and nearby areas, 2 (7.2%) were found positive with G. spinigerum ova. Of the 28 cats, 11 which died in captivity, showed autopsies only 1 immature adult male G. spinigerum in a small gastric tumor. Of 659 gnathostome adults recovered from pig stomachs at the Bangkok Slaughter House, 488 were identified as G. doloresi and 171 as G. hispidum. Forty pig stool samples collected at Rajburi Province were found negative for gnathostome ova.

Experimental infection with G. spinigerum larvae for determination of additional second and paratenic hosts during this period showed for the first time 3 top minnow fish and 4 toad tadpoles acting as second intermediate host of the worm. Fifteen fresh water crabs were found negative after being fed with the advanced third-stage larvae obtained from white mice.

The study on the life cycle of G. hispidum has proved for the first time the development of advanced third-stage larvae in snake-headed fish, catfish, small fighting fish frogs and a tree shrew after being fed with many fully developed larvae in cyclops. However, the advanced third-stage larvae removed from infected fish could not be recovered after being fed to 2 white mice and 2 tree shrews for determining further infectivity of the larvae for mammals. Two snake-headed fish were found to be infected with advanced third-stage larvae first removed from white mice and from the fish after being first infected with the larvae from a white mouse.

Additional study on the life cycle of G. doloresi in laboratory bred white mice fed with many fully developed larvae of the worm in cyclops showed 12 (39.0%) of 31 experimental mice positive with 23 (1.6%) encysted advanced third-stage larvae of the worm.

Infectivity of Gnathostoma spinigerum Fully Developed Larvae and Gnathostoma hispidum Advanced Third-Stage Larvae in Primates.

Principal Investigator: Professor Svasti Daengsvang, Med.D.*

Assistant Investigators: Boonsiri Sermswatsri[1]
Pichit Youngyi, B.Sc.[1]
Dhawee Guname, B.Sc.[1]
Phaibul Sirichakwal, B.Sc.[2]
Paisal Yingyourd, B.Sc.[2]
Rapee Machimasatha, B.Sc.[2]

OBJECTIVE: This study was undertaken to determine whether or not direct infection of primates by Gnathostoma spinigerum larvae in cyclops is possible and whether or not infection of primates with G. hispidum advanced third-stage larvae can be established.

DESCRIPTION: A preliminary attempt was made to determine the development of G. spinigerum fully developed larvae in cyclops to a splenectomized adult gibbon (#1) provided by the SEATO Veterinary Medicine Department; to determine also the susceptibility of an adult monkey #17 (Macaca irus) with G. hispidum advanced third-stage larvae found in some cold blooded experimental food animals commonly consumed by people (catfish, frogs), living larvae freshly obtained from experimental catfish (10 larvae) and from frogs (14 larvae) were fed to the animal (Total 24 larvae). Concurrent weekly examinations of blood samples drawn from the femoral vein were made to determine the changes in total and differential white blood cell counts and for liver function tests. Intradermal tests with G. spinigerum advanced third-stage larvae unfractionated antigen in modified Coca's solution (kindly prepared by Dr. Savanat Taranvaniz, Acting Chief Department of Microbiology and Immunology, the Faculty of Tropical Medicine, Mahidol University) were done weekly to determine skin sensitivity. One control gibbon and one control monkey were also provided for the same tests. In addition, one adult monkey (#86) was fed with 22 G. hispidum advanced third-stage larvae removed from infected white mice to determine the susceptibility of the primate to larvae removed from a warm blooded animal, and to follow the course of migration of the worms after penetrating the stomach wall.

---

* Retroactive in previous Reports
1 Worked for some months during the year before resignation for further education.
2 Replacement for 1.

PROGRESS: (1) G. spinigerum in primates. Infections of primates (Macaca speciosa and Macaca irus) developed larvae obtained from cyclops (see Annual Progress Reports of 1967 and 1968). During this reporting period an adult female splenectomized gibbon was fed 510 G. spinigerum infective larvae from 238 cyclops. At autopsy, 118 days after exposure, 16 (3.0%) living G. spinigerum third-stage larvae were found in various organs. Ten were encysted in the musculature of the legs, 2 in the muscles of the back, 1 in abdominal fat, 1 in the omentum and one each in the diaphragm and/the liver. Three of these encysted larvae measured from 5.2-6.8 mm in length by 0.4 to 0.6 mm in width. The sizes of 11 cysts ranged from 1.3-2.0 mm x 1.7 mm. The larvae were almost double the size third-stage larvae found in the second intermediate host, the fresh water snake-headed fish. These results indicate that the gibbon is a comparatively good paratenic host for this parasite.

During the course of the infection in this gibbon, the differential white blood count was determined weekly without a significant change being observed. Weekly skin sensitivity tests using 1:50,000 and 1:16,000 dilutions of G. spinigerum larval antigen in modified Coca's solution showed positive skin reactions from 50 days after infection until sacrifice of the animal. The total serum protein was unchanged, but the globulin increased from 3.9 mg% to 4.9 mg% after the first 16 days of the infection and remained elevated until the experiment was terminated. The liver function tests showed a change in the zinc turbidity (1.9 units pre-infection to 6.0 units 37 days after the experimental feeding) and remained elevated throughout the balance of the experiment.

The experiment will be repeated. (2) G. hispidum in primates. This experiment was designed to determine whether or not G. hispidum third-stage larvae from cold blood food animals of man (fish and frogs) would further infect primates. Previously 2 adult monkeys (Macaca irus) were infected with G. hispidum larvae from experimentally infected white mice (see Annual Report, 1968). During the present reporting period the latter findings were confirmed, when 1 larva was found in a muscle of a monkey fed 22 G. hispidum larvae 13 days prior to autopsy.

An adult Macaca irus was fed 24 G. hispidum third-stage larvae obtained from a catfish (10 larvae) and from frogs (14 larvae). On autopsy, 92 days after the feeding, no worms were found. Throughout the course of the experiment weekly tests were performed for differential white blood count, liver function and skin sensitivity using G. spinigerum failure of larvae from cold blooded animals to infect a primate warrants further investigation.

SUMMARY: An adult splenectomized gibbon was fed infective larvae from cyclops and a 3.0% recovery of third-stage larvae from various sites was achieved. The larvae were larger than those recovered from fish, suggesting that the gibbon may be a good paratenic host for the parasite.

The gibbon became skin test positive 50 days after exposure to the larvae, showed a rise in globulin level and an increased value in the zinc turbidity test.

An attempt to infect a monkey with G. spinigerum obtained from cold blooded animals was unsuccessful. It was confirmed that the same species recovered from white mice, however, will infect primates.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 046, Parasitic infections of man and animals

Literature Cited:

References:

1. "Parasites of Man and Domestic Animals in Vietnam, Thailand, Laos, and Cambodia," D.B. Segal, J.M. Humphrey, S.J. Edwards, and M.D. Kirby. Experimental Parasitology 23, 1968, 412-464.

2. Diseases and Parasites of Buffaloes. Part III, Parasitic and Miscellaneous Diseases. R.N. Mohan. Veterinary Bulletin, 38, 1968, 735-756.

3. Parasites of Domesticated Animals in Thailand II - Worm Parasites of Cattle, Water Buffalo, Sheep and Goat Z. de Jesus, J. Waramontri. Report to South East Asia Treaty Organization, 1961.

4. Textbook of Veterinary Clinical Parasitology Vol 1. Helminths E.J.L. Soulsby. F.A. Davis Company, Philadelphia, Pa. 1965.

5. Nematode Parasites of Domestic Animals and Of Man. N.D. Levine, Burgess Publishing Co., Minneapolis, Minnesota, 1968.

6. Veterinary Pathology, H.A. Smith, T.C. Jones, Lea and Febiger, Philadelphia, 1968.

7. Briggs, N.T., Wellde, B.T., and Sadun, E.H.: Military Med., 131 (Suppl.): 1237, 1966.

8. Aisenberg, A.C.: J. Exptl. Med. 125: 833, 1967.

9. Siraganian, R.P., Secchi, A.G., and Osler, A.G.: J. Immunol. 101: 1130, 1968.

10. Kagan, I.G.: J. Parasit. 49: 773, 1963.

11. Duxbury, R.E. and Sadun, E.H.: Exp. Parasit. 20: 77, 1967.

12. Garcia, E.G. et al.: Jour P.M.A., 44: 149, 1968.

13. Levine, L., "Micro-complement Fixation" in Weir, D.M., Ed., "Handbook of Experimental Immunology" F.A. Davis, Philadelphia, 1967.

14. Yamaguti, S.: Systema Helminthum I. Digenetic Trematodes. 1958, p. 979.

15. Saoud, M.F.A.: On a New Trematode, Tremajoannes buckleyi gen. et sp. nov. (Lecithodendriidae) from Central American Bats with Some Notes on Phaneropsolus (Diesing, 1850) Braun, 1901. J. Helmin. 1964, 38: 97.

16. Lie Kian Joe: Some Human Flukes from Indonesia. Doc. Neerl. Ind. Morb. Trop. 1951, 3: 105.

17. Muench, H. 1959.: Catalytic models in epidemiology. Harvard University Press, Cambridge, 110 pp.

18. Sadun, E.H. and Maiphoom, C. 1953.: Studies on the epidemiology of the human intestinal fluke, Fasciolopsis buski (Lankester) in central Thailand. Amer. Jour. Trop. Med. and Hyg. 2: 1070-1084.

19. Hsieh, H.C. 1960.: Studies on the epidemiology of Fasciolopsis buski in south Taiwan. Formosan Sci. 14: 95-119.

20. Campbell, C.H.: J. Parasit. 41: 483, 1955.

21. Chipman, P.B.: J. Parasit. 43: 593, 1957.

22. Thorson, R.E.: J. Parasit. 42: 21, 1956.

23. Dusanic, D.G.: Expt. Parasit. 19: 310, 1966.

24. Miyazaki, Ichiro. 1954.: Studies on Gnathostoma occurring in Japan. (Nematoda: Gnathostomidae). II. Life history of Gnathostoma and morphological comparison of its larval forms. Kyushu Memoirs of Med. Sci. 5: 123-139.

25. Efimov, A.Z. 1948.: Occurrence of Gnathostoma spinigerum in Lutreola lutreola, collected papers on helminthology dedicated to K.I. Skryabin on his 40th. Anniversary. Moscow. pp. 109-114. (in Russian).

26. Anderson, R.C., 1964.: Gnathostoma miyazakii n. sp. from the otter (Lutra C. Canadensis) with comments on G. sociale (Leidy, 1858) of mink (Mustela vison). Canadian Jour. Zoology 42: 249-254.

27. Hoa, Le-Van 1965.: A new gnathostome G. vietnamicum n. sp. from an otter, Lutra elioti in Vietnam. Bull. Soc. Pathol 58: 228-235. (French text- English Summary).

28. Prommas, C., and Daengsvang, S. 1933.: Preliminary Report of a study on the life cycle of Gnathostoma spinigerum. Jour. Parasitol. 19: 287-292.

29. Golovin, O.V. 1956.: Biology of the nematode Gnathostoma hispidum, Doklado, Akad. Nauk. S.S.S.R., 1956. III (I), 242-244 (in Russian) and Helminthological Abstracts. 25: 265-266.

30. Miyazaki, I. 1960.: On the Genus Gnathostoma and human Gnathostomiasis with special reference to Japan. Exp. Parasitol. 9: 338-370.

31. Daengsvang, S., Thienprasitthi, P., and Chomcherngpat, P., 1966.: Further investigations on natural and experimental hosts of larvae of Gnathostoma spinigerum in Thailand. Am. J. Trop. Med. Hyg. 15, 727.

32. Epidemiologic Studies of 484 Typical Cases and the Etiologic Role of Angiostrongylus cantonensis, (In press)

33. Manning, G.S. and Ratanarat, C. 1970.: Fasciolopsis buski (Lankester, 1857) in Thailand. Amer. Jour. Trop. Med. and Hyg. In Press.

Publications:

1. Diggs, C.L. and Osler, A.G.: J. Immunol. 102: 298, 1969.

2. Manning, G.S., Diggs, C.L., Viyanant, V., Lertprasert, P. and K. Watanasirmkit, 1970.: Preliminary report on Phaneropsolus bonnei Lie Kian Joe, 1951, a newly discovered human intestinal fluke from northeastern Thailand. J. Med. Ass. Thailand 53 (2): 173-178.

3. Daengsvang, S., Sermswatsri, B., and Youngyi, P., 1969.: Spontaneous cure of natural and induced Gnathostoma spinigerum infection in cats. Ann. Trop. Med. and Parasitol. 63: No. 4.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6468	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	047
b. CONTRIBUTING				
XXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Metabolic Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine; 010100 Microbiology; 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	2.5	148
	CURRENT 71	2.5	148

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Altstatt, LTC L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Bourgeois, MAJ C. H.

NAME: Johnsen, MAJ D. O. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Metabolic Diseases; (U) Intermediary Metabolism; (U) Vitamin Deficiency; (U) Nutrition and Infection; (U) Clinical Research

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To identify metabolic diseases of men and animals of Southeast Asia which are of military importance because they may either complicate or mimic infectious disease.

24. (U) Metabolic diseases common to this area which may alter the course of infectious disease (e.g. malnutrition) or which may mimic infectious disease (e.g. Reye's syndrome) are characterized by clinical findings or laboratory examinations. Animal models are sought which allow identification of characteristic features of the disease and the impact of the disease on concurrent infection.

25. (U) 69 07 - 70 06 Experiments with rhesus monkeys given aflatoxins provided further evidence that aflatoxin is involved in the etiology of Udorn encephalopathy. A rapid test for the measurement of serum ammonia is being evaluated as a means for identifying this syndrome. No satisfactory explanation of the apparent black water fever syndrome occurring in Thai troops, with negative peripheral smears, has been obtained. For technical reports see SEATO Annual Progress Report, 1 Apr 69 - 31 Mar 70.

*Available to contractors upon originator's approval

BLANK PAGE

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 047, Metabolic diseases of man and animals

Investigators.
Principal: Curtis H. Bourgeois, LTC, MC
Associate: Dirck L. Brendling, MAJ, MC; Warren Y. Brockelman, CPT, MSC; Prachengchit Chandruang, M.D.[1]; Dhira S. Comer, M.D.; Robert B. Cotton, MAJ, MC; Charles R. Egelston, MAJ, MC; Hilary Evans, MAJ, MC; Allen M. Glasgow, CPT, MC; Richard A. Grossman, MAJ, MC; Supha Harikul, M.D.[1]; Robert L. Hickman, MAJ, VC; Dennis O. Johnsen, MAJ, VC; Chaiyan Kampanart-sanyakorn, M.D.[2]; Niyom Keschamras, M.D.[3]; Azorides R. Morales, M.D.[3]; Lloyd C. Olson, MAJ, MC; Pratoom Potitong, M.D.[4]; James D. Pulliam, MAJ, VC; Rattana Rattanawongsa, M.D.[5]; Ronald C. Shank, Ph.D[6]; Visith Sitprija, M.D., Ph.D.[4]; Thomas J. Smith, LTC, MC; Prayot Tanticharoenyos, DVM; Markpol Tingpalapong, DVM; Verachart Chaicumpa, DVM; Preecha Vichitbandha, M.D.[5]; William L. Wooding, MAJ, VC; Soontorn Aowchumpa, DVM; John C Bell, SFC, E7; Robert W. Davey, SFC; Kamnual Dhiensiri, M.D.; Damri Chawalitrujiwong; Kwanyuen Lawhaswasdi, DVM; Ronald E. Marshall, SP5, E5; Aysom Siriwangchai; Jerm Pomsdhit; James P. Slowey, SFC, E7; Curtis A. Stewart, SP6, E6; Suriyont Trapukdi; Edward Williams, SFC

1. Dept of Pediatrics, Udorn Provincial Hospital, Udorn, Thailand
2. School of Public Health, Rajavithi Road, Bangkok, Thailand
3. Director, Udorn Provincial Hospital, Udorn, Thailand
4. Korat Hospital, Korat, Thailand
5. Children's Hospital, Bangkok, Thailand
6. Dept of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Encephalopathy and Fatty Degeneration of the Viscera in Thai Children

Principal Investigators:

Curtis H. Bourgeois, LTC, MC
Dhira S. Comer, M.D.
Robert B. Cotton, MAJ, MC
Hilary Evans, MAJ, MC
Richard A. Grossman, MAJ, MC
Supha Harikul, M.D.[1]
Dennis O. Johnsen, MAJ, VC
Chaiyan Kampanart-sanyakorn, M.D.[2]
Niyom Keschamras, M.D.[3]
Lloyd C. Olson, MAJ, MC
Pratoom Potitong, M.D.[4]
Rattana Rattanawongsa, M.D.[5]
Ronald C. Shank, Ph.D.[6]
Thomas J. Smith, LTC, MC
Prayot Tanticharoenyos, DVM
William L. Wooding, MAJ, VC

Assistant Investigators:

Robert W. Dewey, SFC
Damri Chawalitrujiwong
Arporn Siriwangchai
Suriyont Trapukdi
Edward Williams, SFC

OBJECTIVE: A systematic study of the epidemiology, etiology, pathology and clinical features of Encephalopathy and Fatty Degeneration of the Viscera in Thai Children, (EFDV).

DESCRIPTION: From 1 January 1969 until 31 December 1969, epidemiologic, clinical and pathologic data were collected on all patients admitted to the Udorn Provincial Hospital with diagnoses of

---

1. Dept of Pediatrics, Udorn Provincial Hospital, Udorn, Thailand
2. School of Public Health, Rajavithi Road, Bangkok, Thailand
3. Director, Udorn Provincial Hospital, Udorn, Thailand
4. Korat Hospital, Korat, Thailand
5. Children's Hospital, Bangkok, Thailand
6. Dept of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

encephalopathy, encephalitis or convulsions.

PROGRESS: A total of 109 patients were included in the study.

The diagnosis of "probable" EFDV was made if the following clinical features were present:

1. Sudden onset of convulsion or coma;
2. Serum glucose of less than 50 mg% (Folin-Wu) and/or cerebrospinal fluid glucose less than 40 mg% and/or SGOT greater than 80 sigma units;
3. Absence of neutrophils and no more than 10 lymphocytes in the spinal fluid.

A case was considered "definite" EFDV, if:

1. The above clinical criteria were present and a liver biopsy showed characteristically small vacuole fatty metamorphosis;
2. An autopsy revealed cerebral edema without cellular infiltration, and a characteristic pattern of fatty metamorphosis in the liver, kidney, and heart.

Using the above criteria, 40 cases were diagnosed as "definite" EFDV (12 biopsies and 28 autopsies), and 27 were diagnosed as probable EFDV. Except for hypoglycemia sixteen additional patients met all criteria for a diagnosis of probable EFDV; all of these patients had had IV glucose before the blood sugar determination.

Epidemiologic aspects

Of the 67 definite or probable cases, 38 were girls and 29 boys. All but six patients were between 18 months and 6 years of age. Forty-six (70%) of the patients died.

All but four of the patients came from rural areas. Since 10% of the population of Udorn province lives in the city of Udorn, and the rural population has much less ready access to the hospital than those in the city, the predominately rural distribution of this disease is emphasized. Two patients were siblings and immediate neighbors of a third child admitted two weeks earlier. In two other instances two children were from the same village. These were the only instances of clusters in the geographic origin of cases. However, 33 of all 162 siblings (20%) of patients, and 26 of the 58 between 18 months and six years of age, were ill within two weeks of their siblings' illness. In

most instances this consisted of non-specific symptoms such as fever, URI's and/or headache.

Clinical syndrome

In nine of the 67 patients there was no history of symptoms prior to the onset of convulsions and coma. In the other 58 such prodromal symptoms tended to be mild and in most were less than 24 hours in duration. These included symptoms of URI, fever, headache, diarrhea, abdominal pain and vomiting.

In most patients, then, the onset of symptoms indicating serious disturbances of central nervous system function were unexpected and acute. Each of the 67 patients was brought to the hospital because of convulsions and coma. One patient died a few minutes after admission. Of the other 66, 54% were febrile at the time of admission, 46% had abnormal respiratory patterns (tachypnea, hyperpnea, Cheyne-Stokes, etc), and 23% showed hepatomegaly. Only three patients were jaundiced, and three others had gross irregularities in cardiac rhythm. All patients appeared well-nourished and in no patient was a skin rash present.

All but one patient was semi-comatose or comatose on admission, and 43 patients exhibited seizures. Thirty-one of these were in status epilepticus. Twenty-three of the patients displayed intermittent decerebrate posturing. Only one patient showed signs of meningeal irritation.

In all patients symptoms evolved acutely despite therapeutic attempts to correct hypoglycemia and electrolyte abnormalities and control cerebral edema. Thus 25% of the 46 fatal cases died within twelve hours of the onset of CNS symptoms, and 61% within 24 hours. No patient died later than three days after onset. Conversely, survivors generally had improved markedly within 48 hours of hospitalization. Only two patients still showed significant residual effects upon discharge, and both of these continued to improve thereafter. The clinical syndrome presented by these patients was remarkably constant. In most instances the diagnosis could be correctly made on the basis of history and physical examination, and in all cases where autopsy subsequently established a tissue diagnosis, the clinician had correctly diagnosed whether the patient had EFDV syndrome or not.

A summary of laboratory data is shown in Table 1.

LABORATORY RESULTS

A = Autopsy - Proven
B = Biopsy - Proven
C = Clinically Diagnosed

TEST	NO.	RANGE	MEDIAN	MEAN	STD. DEV.	COMMENTS
Glucose						
A	23	12-90	30	32.8	18.7	
B	8	13-65	31.5	27.1	16.2	
C	27	0-214	26.9	39.3	37.5	
SGOT						
A	23	50-1900	112	-	-	74%> 75
B	9	78-1250	250	-	-	100%> 75
C	26	120-156	133.5	133.2	-	100%> 75
SGPT						
A	23	16-875	69	-	-	70%> 40
B	9	30-1000	75	-	-	78%> 40
C	26	32-75	48.3	49.3	-	92%> 40
Total Bilirubin						
A	21	0.0-9.2	0.5	-	-	19%≥ 2.0
B	9	0.0-3.6	0.8	-	-	22%≥ 2.0
C	25	0.0-8.9	0.3	-	-	16%≥ 2.0
Prothrombin Time						
A	7	24-40	33	32.0	-	86%≥ 25
B	10	17-83	20.5	28.8	-	20%≥ 25
C	11	15-35	22	21.9	-	18%≥ 25
CO_2 Content						
A	18	7.4-20.9	16.9	15.6	-	83%< 19
B	12	10.8-26.5	13.8	15.9	-	75%< 19
C	24	8.9-26.0	18.0	16.2	-	75%< 19
Chloride						
A	26	80.5-122	101.5	100.7	-	38%< 98
B	12	91.5-108	101.5	101.0	-	17%< 98
C	26	75-105.8	96.1	97.6	-	50%< 98

TEST	NO.	RANGE	MEDIAN	MEAN	STD.DEV.	COMMENT
Potassium						
A	24	4.1-7.4	5.8	5.8	-	58%> 5.6
B	10	3.2-7.4	4.7	4.8	-	10%> 5.6
C	26	3.2-7.5	4.8	4.9	-	12%> 5.6
Sodium						
A	24	116-158	131.5	132.8	-	50%< 132
B	10	120-160	137.0	138.9	-	20%< 132
C	26	120-156	132.5	133.3	-	42%< 132
Hematocrit (%)						
A	20	28-44	36	35.6	-	20%< 32
B	12	19-43	36	34.2	-	17%< 32
C	23	26-42	34	34.8	-	22%< 32
PTT						
A	7	57-166	78	89.9	-	3/7 > 80
B	10	40-106	59	61.3	-	1/10> 80
C	11	44-489	72	115.7	-	4/11> 80
WBC						
A	19	900-23,100	12,[illegible]0	12,100	-	53%> 11,000
B	12	4,000-33,300	13,00[illegible]	15,600	-	58%> 11,000
C	23	5,200-48,500	15,100	16,300	-	70%> 11,000
Polys (%)						
A	16	13-87	69.5	-	-	
B	12	27-92	69	-	-	
C	23	37-90	73	-	-	
Lymphs (%)						
A	16	15-87	28.5	-	-	
B	12	7-64	28.5	-	-	
C	23	10-61	27	-	-	
Total Protein						
A	13	5.7-7.5	6.3	6.6	-	
B	5	6.1-7.5	6.4	6.6	-	
C	15	6.0-7.7	6.8	6.8	-	

Pathology

A detailed report of the pathologic findings is now in preparation. The availability of more case material and fresh biopsy specimens have provided the following new observations:

1. There is ten to fifteen per cent enlargement of hepatocytes and hepatocyte nuclei;
2. Markedly enlarged irregular hepatocyte nucleoli are present in most cases;
3. Diffuse lymphocytolysis is a constant finding;
4. Neuronal degeneration is commonly found.

Lipid analyses

Specimens of liver and kidney were homogenized in a Waring blender, lyophilized, weighed and extracted three times with chloroform:methanol according to the method of Folch. The tissue was relyophilized, re-weighed and the extractable lipid expressed as milligrams per gram of lyophilized tissue. The chloroform: methanol extracts were evaporated to dryness and then brought up to a volume of twenty-five cc. in chloroform. For chromatographic studies, and aliquot was adjusted with chloroform so that ten cc. of sample represented one gram of lyophilized tissue, thin layer chromatography was performed using commercially available sheets (Eastman Chromagram System) according to the manufacturer's instructions. Identification of the various lipid fractions was accomplished by using known standards, published Rf values and spot tests.

Chemical analyses for total lipid, cholesterol, triglycerides, free fatty acids and phospholipids were performed on each extract. Two samples were taken from each tissue specimen and 2 replicate analyses were performed on each extract.

Lipid analyses were performed on liver and kidney specimens from 7 "controls" (patients dying with disease other than EFDV) and on 17 patients dying with EFDV. The average weight of lipid extractable from one gram of lyophilized liver and kidney tissue from "control" patients were 232 mg. and 199 mg. respectively; for patients with EFDV, the values were 558 and 349 respectively. Chromatographic analyses of the lipid revealed a marked increase in triglycerides, diglycerides and free fatty acids in the liver, with only triglycerides being increased in the kidney. Chemical analyses of the hepatic lipids revealed:

Result (Per. Gm. lyophilized tissue)	"Control"	Patient
Free Fatty Acids (mEq.)	0.138	0.276
Phospholipid (mg.)	52.0	39.0
Triglycerides (mg.)	10.7	109.5
Cholesterol (mg.)	11.5	9.0
Total Lipid (mg.)	212	550

Results of the renal lipid analyses are not yet completed

Viral studies

Specimens of brain, liver, heart, kidney, and lung were collected under aseptic conditions and stored at -60°C in individual, sterile, plastic bags.

After thawing rapidly, a 20% suspension was made by grinding with sterile sand in Hanks balanced salt solution; the suspension was then clarified by centrifugation, and inoculated into suckling mice and primary monolayer cultures of rhesus monkey kidney, Hela and WI-38 cells. Some specimens were also inoculated into primary human embryonic kidney cells. Thereafter all systems were observed for evidence of virus, i.e., illness in suckling mice, cytopathic effects, hemadsorption, or challenge virus resistance (using echo virus type 11 in monkey and human kidney cells.)

Serological tests were limited to hemagglutination-inhibition against arbovirus group A and B antigens. Paired sera (on admission and at least two weeks later) collected from survivors with documented disease were extracted with acetone and tested against dengue types 1-4, Japanese B encephalitis, and Chikungunya virus.

A total of 89 specimens from 26 autopsies were cultured for virus. These included tissues from 26 brain specimens, 20 lungs, 11 livers,

10 kidneys, 8 spleens, 7 hearts, 5 nodes, one thymus and one acute serum. All specimens were negative for a transmissible agent. A total of eleven paired sera obtained during the acute illness and at least two weeks later were tested for hemagglutination-inhibiting antibodies to dengue types 1-4, Japanese B encephalitis and Chikungunya viruses. None showed evidence of recent infection with these agents.

Acute toxicity of aflatoxin B_1 in the monkey

Further analyses of the mycotoxin obtained from the left over food samples revealed the toxin to Aflatoxin B_1 (see 1969 Annual Report). This was confirmed by ultraviolet, infrared, mass spectroscopic and nuclear magnetic resonance analyses (courtesy of Dr. George Buchi, Mass. Inst.).

In order to establish the LD_{50} of this toxin in monkeys, and to observe the biochemical and pathologic responses to acute toxicity, the following experiment was carried out.

Five groups of four female macaques were given a oral single dose of 0.5, 1.5, 4,5, 13.5 or 40.5 mg/kg. of chromatographically pure, crystalline aflatoxin B_1. The toxin was prepared from the Aspergillus flavus grown from the B. Kota food sample. A control group of 5 animals received no toxin. All animals receiving 40.5 and 13.5 mg/kg and one animal receiving 4.5 mg/kg. died.

All 9 of the animals that died became drowsy 2-3 days after toxin administration; some experienced vomiting and/or convulsion; all these animals showed grossly fatty livers and kidneys with little other macroscopic changes. Hepatic cell necrosis was pronounced in the animals receiving the higher doses. Fatty degeneration of the hepatocytes, renal tubular epithelium and myocardium were constant findings. Similar, but less severe, changes were seen in tissues from the surviving monkeys (sacrificed at the end of 7 days).

The LD_{50} for aflatoxin B_1 in the monkey was calculated for the test period of 6 days according to the method of Litchfield and Wilcoxon. The LD_{50} is 7.8 mg/kg body wt. with 95% confidence limits of 3.5 - 17.6 mg/kg body wt.; the slope function of the LD_{50} plot is 2.25.

Serum chemical analyses revealed that by day 3 after ingestion of toxin all the gross changes in test values had occurred in those that either went on to die or recovered. By day 1 some of the animals that later showed disease had early changes.

The following tests showed no evidence of significant alterations (compared to controls or their respective dose groups) over these 3 days:

Cholesterol
Total Protein
Electrolytes (Na, K, Cl)
Gamma Globulin
A/G Ratio

The following tests showed changes at higher dose levels but were difficult to analyze because of the wide variability of values for control and test animals and the absence of day 0 values:

Total Lipids (depressed)
Triglycerides (increased)
SGOT, SGPT (increased)
Alpha 2 and Beta Globulin (depressed)

Serum glucose and nonesterified fatty acids were the most interesting tests. Both showed changes (glucose decreasing, NEFA rising) that appear to be dose-related. But, whereas NEFA increases (as well as phospholipid decreases) were noted on day 1, values for several animals in the 3 high dose groups the glucose levels were still stable (see Table 1):

Table I. Mean values on days 1 and 3 for serum glucose, NEFA and phospholipid determinations.

Dose Mg/Kg BW (4 monkeys/group)	Glucose (Mg%)		NEFA (Mg/L)		Phospholipid (Mg%)	
	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3
Controls	93.0	67.0	.602	.910	161.2	158.1
0.5	97.2	76.0	.657	1.090	183.8	198.2
1.5	72.5	64.8	.530	.825	190.0	181.9
4.5	64.8	50.0	1.080	1.665	140.6	146.9
13.5	83.5	35.0	.810	2.268	103.1	121.9
40.5	87.8	15.5	.740	2.535	100.8	126.2

The fairly large variation between days for these glucose and NEFA levels makes this observation harder to substantiate. But analysis of the paired glucose-NEFA values by animal revealed a nonsignificant correlation coefficient ($r = -.257$) for day 1 but a highly significant value ($r = -.906$) for day 3 values. This substantiates the definite changes on day 3, whereas any day 1 changes were not yet concurrently present. A closer look at the paired values on day 3 reveals 2 populations of results, both fairly normal or both very abnormal, with no intermediate values seen. Thus, even though a graded dose-response relationship is present for each test, values of both tests hardly overlap. This is also true for the survivors versus dead groups regardless of dose (Table II).

Table II. Comparison of Day 3 NEFA and GLUCOSE values in survivors and those that later died.

Test		Died n=9	Survived n=11
NEFA	RANGE	1.40-3.19	0.62-1.57
GLUCOSE		8.5-60	60-91
NEFA	MEAN	2.458	1.037
GLUCOSE		23.60	68.64

Day 3 values were further analyzed by log dose-response regression procedures. The NEFA values were uncorrected; for the glucose values the variable used was day 3 value as a proportion of day 1 value to try to stabilize the sizeable mean differences within each dose group. Both curves had highly significant slopes, .903 for NEFA and -0.332 for glucose, but a good linear fit was found only for the NEFA values. Phospholipids may likewise be changing early (Table I) but further analysis is not possible due to missing values thereafter.

The significance of these findings lies not only in being able to document the biochemical changes leading to the observed histologic damage but also in demonstrating graded responses by dose and early, though definitely abnormal, changes. The possibility of diagnosing less severe and non-fatal disease (? pathogenesis dose-dependent)

opens up many optimistic areas for investigation.

Aflatoxin assay

Quantitative chemical analysis for aflatoxins were performed on freshly frozen autopsy tissues, using a slightly modified version of the method of Eppley. Confirmation of identity was made by the chemical derivatives method of Andrellous and Reid. Results of analyses of tissue specimen are given below. Analyses were also performed on 7 non EFDV patients.

AFLATOXIN ANALYSES
Non EFDV Patients

Diagnosis	Age	Sex	Brain	Liver	Kidney	Stool	Stomach Contents	Intestinal Contents
			2		2			
Auto Accident	17	M	T	-	T	-	-	-
Auto Accident	20	M	ND	-	ND	-	-	-
Diphtheria	11/2	F	T	ND	-	ND	-	T
Diphtheria	1	F	3	3	-	-	-	-
Pneumonia	2	M	3	3	T	-	-	-
Miliary T.B.	4	M	-	T	ND	-	-	-
Nasopharyngeal abscess	2	M	-	T	2	-	-	-

AFLATOXIN ANALYSES
EFDV Patients

Case Number	Age	Sex	Brain	Liver	Kidney	Stool	Stomach Contents	Intestinal Contents
UA-5 (009)	1 1/2	F	T	T	-	-	T	-
UA-9 (013)	3	M	-	T	-	T	T	-
UA-18 (028)	2	F	-	T	T	T	ND	-
UA-20 (032)	4	F	T	-	T	ND	T	-
UA-24 (035)	6	F	3	3	3	123/15	T	-
UA-25 (038)	2	M	2	3	-	ND	T	-
UA-26 (039)	12	M	ND	ND	-	ND	ND	-
UA-27 (040)	4	F	2	2	4	ND	T	-

The fairly large variation between days for these glucose and NEFA levels makes this observation harder to substantiate. But analysis of the paired glucose-NEFA values by animal revealed a nonsignificant correlation coefficient ($r = -.257$) for day 1 but a highly significant value ($r = -.906$) for day 3 values. This substantiates the definite changes on day 3, whereas any day 1 changes were not yet concurrently present. A closer look at the paired values on day 3 reveals 2 populations of results, both fairly normal or both very abnormal, with no intermediate values seen. Thus, even though a graded dose-response relationship is present for each test, values of both tests hardly overlap. This is also true for the survivors versus dead groups regardless of dose (Table II).

Table II. Comparison of Day 3 NEFA and GLUCOSE values in survivors and those that later died.

Test		Died n=9	Survived n=11
NEFA	RANGE	1.40-3.19	0.62-1.57
GLUCOSE		8.5-60	60-91
NEFA	MEAN	2.458	1.037
GLUCOSE		23.60	68.64

Day 3 values were further analyzed by log dose-response regression procedures. The NEFA values were uncorrected; for the glucose values the variable used was day 3 value as a proportion of day 1 value to try to stabilize the sizeable mean differences within each dose group. Both curves had highly significant slopes, .903 for NEFA and -0.332 for glucose, but a good linear fit was found only for the NEFA values. Phospholipids may likewise be changing early (Table I) but further analysis is not possible due to missing values thereafter.

The significance of these findings lies not only in being able to document the biochemical changes leading to the observed histologic damage but also in demonstrating graded responses by dose and early, though definitely abnormal, changes. The possibility of diagnosing less severe and non-fatal disease (? pathogenesis dose-dependent)

opens up many optimistic areas for investigation.

Aflatoxin assay

Quantitative chemical analysis for aflatoxins were performed on freshly frozen autopsy tissues, using a slightly modified version of the method of Eppley. Confirmation of identity was made by the chemical derivatives method of Andrellous and Reid. Results of analyses of tissue specimen are given below. Analyses were also performed on 7 non EFDV patients.

AFLATOXIN ANALYSES
Non EFDV Patients

Diagnosis	Age	Sex	Brain	Liver	Kidney	Stool	Stomach Contents	Intestinal Contents
			2/T		2/T			
Auto Accident	17	M		-		-	-	-
Auto Accident	20	M	ND	-	ND	-	-	-
Diphtheria	11/2	F	T	ND	-	ND	-	T
Diphtheria	1	F	3	3	-	-	-	-
Pneumonia	2	M	3	3	T	-	-	-
Miliary T.B.	4	M	-	T	ND	-	-	-
Nasopharyngeal abscess	2	M	-	T	2	-	-	-

AFLATOXIN ANALYSES
EFDV Patients

Case Number	Age	Sex	Brain	Liver	Kidney	Stool	Stomach Contents	Intestinal Contents
UA-5 (009)	1 1/2	F	T	T	-	-	T	-
UA-9 (013)	3	M	-	T	-	T	T	-
UA-18 (028)	2	F	-	T	T	T	ND	-
UA-20 (032)	4	F	T	-	T	ND	T	-
UA-24 (035)	6	F	3	3	3	123/15	T	-
UA-25 (038)	2	M	2	3	-	ND	T	-
UA-26 (039)	12	M	ND	ND	-	ND	ND	-
UA-27 (040)	4	F	2	2	4	ND	T	-

Case Number	Age	Sex	Brain	Liver	Kidney	Stool	Stomach Contents	Intestinal Contents
UA-28 (041)	7	M	3	3	-	108/19	T	-
UA-29 (042)	3	F	3	3	ND	ND	5/2	-
UA-31 (045)	2	M	-	-	4	-	-	-
UA-32 (049)	12	M	ND	3	-	-	127/15	81/10
UA-34 (053)	10	F	2	2	1/T	-	-	-
UA-35 (056)	6	F	2/T	2	-	ND	116/19	-
UA-36 (057)	5	F	ND	ND	ND	13/4	ND	-
UA-38 (061)	10	F	ND	T	ND	ND	ND	-
UA-39 (074)	13	M	ND	47/6	3	T	11	-
UA-41 (082)	2	M	-	-	-	ND	2	-
UA-44 (101)	3	M	3	2	7	ND	ND	ND
UA-46 (103)	2	F	T	T	T	-	-	-
UA-47 (105)	18/12	F	-	-	-	ND	ND	T

SUMMARY: Between 1 January and 31 December 109 cases of possible encephalopathy and fatty degeneration of the viscera (EFDV) were studied at the Udorn Provincial Hospital. A diagnosis of either "definite" or "probable" EFDV was made in 67 cases. Clinical, laboratory and pathologic data was collected and analyzed. Viral studies on 89 specimens from 26 cases showed no evidence of viral infection. Tissue lipid analyses revealed the increased hepatic lipids to be predominately triglycerides and free fatty acids. Aflatoxin assays on autopsy specimens were positive in over 90% of cases. The LD_{50} for aflatoxin B_1 in the macaques was established as 7.8 mg/kg body wt. Animals receiving a lethal dose of the toxin developed clinical and pathologic findings similar to patients with EFDV.

Ammonia Metabolism: Reye's Syndrome as a Model for Elevated Ammonia

Principal Investigator: Allen M. Glasgow, CPT , MC

Associate Investigators: Robert B. Cotton, MAJ, MC
Kamnual Dhiensiri, M.D.

OBJECTIVES:

1. Determine if ammonia is elevated in Reye's syndrome in Thailand;
2. Develop ion exchange resins as treatment for elevated ammonia;
3. See if treatment of elevated ammonia will increase survival in Reye's syndrome.

BACKGROUND: Elevated ammonia has been reported to be associated with encephalopathy in several disease states. Ammonia has been reported elevated in Reye's syndrome in the United States.

Blood ammonia will be measured in patients with encephalopathy and fatty degeneration of the viscera (EFDV) in order to determine: (1) if it could have etiologic significance in the encephalopathy; (2) if EFDV is similiar to Reye's syndrome in this respect. Blood ammonia will also be measured in patients with Japanese B encephalitis to see if blood ammonia can be used to differentiate the two conditions.

It is anticipated that if the encephalopathy of EFDV is related to elevated ammonia that this would prove a useful model for study of treatments of elevated ammonia. EFDV would be a good model for the following reasons: (1) lack of other medical problems as in chronic hepatic failure; (2) probable reversible nature of lesion (vs acute fulminant hepatitis); (3) well defined end point (death); (4) large number of cases available over relatively short period.

Another phase of this study will look at the possibility of using an ionic exchange resin in the treatment of elevated ammonia. Ionic exchange resins have been shown to lower blood ammonia in laboratory animals. Decreasing stool pH has also been shown to lower blood ammonia. It is hoped to combine these effects by using an acidic ionic exchange resin enema that would work according to the following equation.

BLOOD | BOWEL

$$NH_4^+ \leftrightarrow H^+ + NH_3 \quad \vdots \quad NH_3 + H^+ \leftrightarrow NH_4^+ + RESIN \leftrightarrow RESIN\ NH_4^+$$

It is hoped that this might prove to be a simple, effective therapy for conditions characterized by elevated ammonia.

PROGRESS: (1) Ammonia Assay. The ammonia assay, although a modification of a previously reported principle[1], is reported here is some detail because it offers certain advantages in the volume of blood required, speed and ease of analysis and accuracy of results:

Blood is drawn and immediately transfered to tubes (containing 50 u dried heparin) on ice and mixed. Blood, 3.0 ml is added to 3.0 M ice cold perchloric acid, mixed and centrifuged in the cold. To 3.0 ml ice supernatant is added 1.5 ml 3.0 M KOH need not be exactly 3.0 M but should be adjusted so that neutralized extract is neutral to slightly acidic.) 0.01 ml 0.2% methyl red is added and the solution titrated to an orange or "just" yellow color with the above solutions of perchloric acid and KOH (with practice it is possible to easily perform this titration). This involves a small dilution but it perchloric acid and KOH are properly prepared this is less than 1%). The neutralized solution is centrifuged in the cold and the supernatant removed and allowed to come to room temperature. To cuvettes add 2.0 ml supernatant, 1.0 ml PO_4 buffer-NADH (prepared immediately before use by disolving NADH in 0.5 M PO_4 buffer pH=7.5 and adjusting to read .600 to .700 at 340 mu in spectrophotometer) and 0.1 ml 0.25 M∞ Ketoglutarate. Mix and read initial OD_{340}. Add .05 ml glutamate dehydrogenase, mix, and let stand 30' at room temperature, read final OD_{340}.

NH_4^+ in um/ml = $OD_S - OD_B \times .76$

OD = Initial-final OD_{340}

S = Sample

B = Water Blank

Figure 1. Shows A Representative Standard Curve.

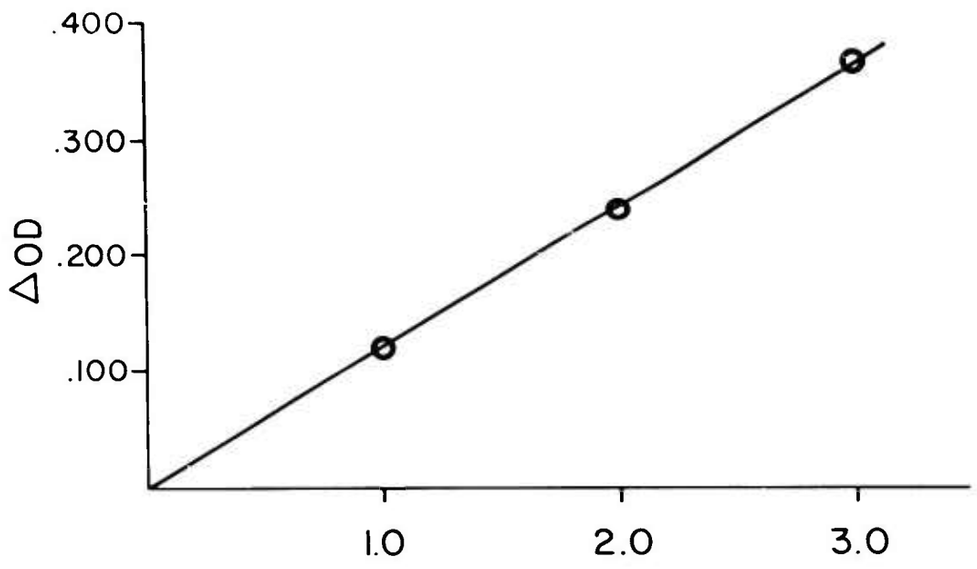
Other experiments showed reproducibility is good (duplicates within 5-10%) and that recovery from blood of added ammonia is completed. The neutralized extract is stable up to 2 weeks following addition of 0.5 ml 0.5 M PO_4 buffer pH=7.5 to 2.0 ml extract and freezing and storing at -60°C.

Total time until extract is stable is about 30 min. total time for assay about 90-120 min. 10-15 samples can be run by one operator simultaneously.

2. Removal of NH_4^+ by ion exchange resin. Kayexalate is used as the resin. It is converted from its Na^+ form to a $Na^+ - K^+$ form by repeated washing with a solution containing 3,777 mg Na^+ (as NaCl) and 200 mg

FIGURE 1

SHOWS A REPRESENTIVE STANDARD CURVE



K^+(as KCl)/100 ml and then repeated distilled H_2O washing. (Washing is most easily performed using suction on a large funnel). The completeness of equilibration is then tested by mixing a quantity of resin with a solution containing 377 mg Na^+ and 20 mg K^+/100 ml (i.e. approximate plasma concentration) and measuring the change in the concentration of Na^+ and K^+.

The resin thus prepared will remove NH_4^+ in vitro but the removal is dependent on salt concentration (Table 1) as well as the amount of resin (Table 2).

Two attempts have been made to lower blood ammonia in monkeys using the resin by stomach tube and enema. These attempts were unsuccessful. However, several technical difficulties were encountered including low control blood pH (probably secondary to anesthesia), only mildly elevated ammonia, and poor distribution of resin (resin remained in stomach and rectum). Further attempts will be made to overcome these difficulties.

3. Blood ammonia in EFDV in Thailand. Measurement of levels of blood ammonia in patients with EFDV will be done in the near future.

Table 1. 68 mg resin added to 10 ml above solutions containing .001 mg N/ml as NH_4^+Cl

Na mg/100 ml	K mg/100 ml	% NH_4^+ REMOVED
377	20	24
188	10	36
94	5	39
37.7	2	57
0	0	100

Table 2. Resin added to 10 ml solution containing 377 mg Na, 20 mg K/100 ml and .001 mg N/ml as NH_4^+Cl

RESIN	% NH_4^+ REMOVED
34	4.4
68	17.1
136	28.2
272	42.7

Pathology of Diseases Common in Thailand

Principal Investigators: Curtis H. Bourgeois, LTC, MC
Prachenchit Chandruang, M.D.[1]
Dhira S. Comer, M.D.
Hilary Evans, MAJ, MC
Niyom Keschamras, M.D.[2]
Azorides R. Morales, M.D.[3]
Visith Sitprija, M.D., Ph.D.[4]
Preecha Vichitbandha, M.D.[5]

Assistant Investigators: Damri Chawalitrujiwong
Suriyonta Trapukdi

1. Pathology of the Cardiac Conduction Disturbances in Diphtheric Myocarditis.

OBJECTIVE: A clinical-pathologic analysis of 2 cases of diphtheric myocarditis.

DESCRIPTION: The pathologic changes in the hearts of two patients with fatal diphtheritic myocarditis were studied, particular attention being focused on the involvement of the conduction system, correlated with cardiac conduction disturbances. Except for the absence of fat in sinus and atrioventricular node fibers, the changes seen in the specialized tissue of the heart were similar to those affecting the working musculature.

It is suggested that the extensive accumulation of fat in myocardial fibers, including bundle of His and bundle branches, may play a significant role in the genesis of the transitory conduction disturbances so commonly associated with the disease.

PROGRESS: The project has been completed. A manuscript has been accepted by the Archives of Pathology.

---

1 & 5 Departments of Pediatrics and Pathology, Siriraj Hospital, Mahidol University, Bangkok, Thailand
2 Director, Udorn Provincial Hospital, Udorn, Thailand
3 Dept. of Pathology, Henry Ford Hospital, Detroit, Michigan 48202
4 Dept. of Medicine , Chulalongkorn Hospital, Bangkok, Thailand

II. The Liver in Opisthorchis viverrini Infection.

OBJECTIVE: To describe the pathologic findings in the liver and biliary tract of patients infected with the fluke O. viverrini.

DESCRIPTION: Livers and extrahepatic biliary tracts obtained at autopsy in Udorn Provincial Hospital were studied by postmortem cholangiography, dissection, and histologic examination.

PROGRESS: Specimens from ten patients were examined. The project has been completed. A manuscript is in preparation.

III. Renal Lesions in Human Leptospirosis.

OBJECTIVE: To describe the histology and ultrastructure of renal tissue obtained from patients with leptospirosis and to correlate morphologic findings with renal function.

DESCRIPTION: Percutaneous renal biopsy and renal function tests were carried out in patients with leptospirosis admitted to Chulalongkorn University Medical Center.

PROGRESS: Nine patients were studied. The project has been completed.

IV. Nephrotic Syndrome in Thailand.

OBJECTIVE: To describe the histology and ultrastructure of kidney biopsy specimens from patients with the nephrotic syndrome and to correlate morphologic findings with clinical picture, renal function, and response to therapy.

DESCRIPTION: Percutaneous renal biopsies and renal function studies were carried out in patients with nephrotic syndrome admitted to the renal unit of Chulalongkorn University Medical Center.

PROGRESS: The project has been completed. Twenty-seven patients were studied.

This work was presented at the Third Annual Meeting, Far East Chapter, Association of Military Surgeons, Tachikawa, Japan, 4-7 November 1969.

A manuscript is in preparation.

Nutritional and Health Requirements for Development and Maintenance of Conventional Animal Colonies

Principal Investigators: Dennis O. Johnsen, MAJ, VC
Robert L. Hickman, MAJ, VC
William L. Wooding, MAJ, VC
James D. Pulliam, MAJ, VC
Paul C. Smith, MAJ, VC
Prayot Tanticharoenyos, D.V.M.
Verachart Chaicumpa, D.V.M.

Assistant Investigators: Kwanyuen Lawhaswasdi, D.V.M.
James P. Slowey, SFC, E-7
John C. Bell, SFC, E-7
Curtis A. Stewart, SP6, E-6
Ronald E. Marshall, SP5, E-5

OBJECTIVE: The objective of this study is to characterize and improve the quality of conventionally produced and maintained laboratory animals in Thailand and to investigate those problems that may be of significance to medical research.

DESCRIPTION: Monthly disease surveillance programs have been instituted in all the rodent production colonies to determine the incidence and extent of parasitic infestations, latent virus infections, bacterial infections, and presence of other pathological conditions which might affect the outcome of investigations utilizing these animals as biological models. The information obtained from this disease screening program is used to continually evaluate the quality of animals bred in the laboratory and to assure their uniformity. Locally purchased animals, such as primates, are also subjected to a variety of diagnostic examinations during their extensive quarantine period and at intervals during their stay in the laboratory. Every animal that dies spontaneously or shows signs of non-experimentally induced disease, is thoroughly necropsied and the cause of illness is determined if possible. The resulting body of information is analyzed to determine what problems exist in the colony and what improvements should be made in the standards of husbandry, management, and medical care to bring about their solution.

PROGRESS: Information concerning the deaths and significant illnesses of all those animals in the colony except rodents and poultry are present in Table 1. With the death of 24 gibbons during the report period, mortality in this species was especially high. The most

important cause of death in the gibbons were respiratory tract infections. Following an experiment in which a small number of gibbons were inoculated with two strains of influenza virus, an epizootic began which resulted in significant illness in at least 30% of all the animals in the gibbon colony and the deaths of four animals. A manuscript describing this epizootic, which is one of the new instances where human influenza virus has caused a spontaneous clinical infection in animals, has been submitted for clearance and subsequent publication. Three months following this outbreak there was another outbreak of respiratory disease which claimed the lives of another four animals. Although attempts were made to determine the etiologic agent involved, serologic studies and attempts at virus isolation were unsuccessful. Both the clinical course of the second epizootic and the pathological lesions in the dead animals resembled the first outbreak caused by influenza virus. Another problem in the gibbon, which also manifests itself frequently in the lungs, is the continuing infections caused by migrating Strongyloides larvae. Routine stool examinations show that this parasite is widespread through the colony and additional pathological evidence confirms its importance as reported in last year's annual report. Preventive measures being taken to reduce infections consist of steam cleaning cages at least once a week if possible and worming the entire colony at the time blood samples are collected each quarter. It is too early at this time to assess the effectiveness of these measures. No deaths occurred during the year from malignant lymphoma in contrast to the four deaths reported last year. In screening blood samples taken from each of the gibbons every quarter no further developing cases of lymphoma have been discovered. Two deaths in gibbons were of special interest. One occurred in a gibbon that had been splenectomized and used in studies with malaria. It suddenly developed signs of an acute hemolytic crisis and died within 8 hours. Necropsy lesions and examination of stained blood smears showed that malaria parasites of a type found only in gibbons were responsible for its death. The other case involved a gibbon that had been given water seeded with Pseudomonas pseudomallei as part of a study concerning the infectivity of this organism. Following an illness of several days accompanied by a high fever, the animal died. At necropsy the lungs were discolored and contained many small abscesses which resembled the lesions of melioidosis described in humans. Nevertheless, Chromobacterium janthinum was isolated from these lesions. The finding of this bacteria in gibbons supports findings reported by Groves, et.al. in Kaula Lumpur in which similar deaths were caused in several gibbons by the same organism. Macaques, in contrast to the gibbons, appear to be much hardier animals. Only one death

occurred in a rhesus monkey; the death apparently resulted from blockage of the urethra by calculi. There continue to be many quarantined animals that are infected with Shigella or Plasmodium inui. Both conditions respond favorably to treatment and have not caused problems later. None of the primates in the colony reacted to the intrapalpebral inoculation of mammalian tuberculin during routine testing.

Disease problems in the rodent breeding colonies were negligible. There has been no reappearance in newly established colonies of the Salmonella infections that necessitated the destruction of both the hamster and guinea pig colonies. It is likely that better security and sanitation procedures have reduced the likelihood of such infections being introduced into the colony. The bacteriologic findings obtained from the monthly disease screening program are summarized for the year in Table II. The bacterial fauna of these animals is unremarkable. Of the 220 mice examined, eleven had gross lesions of the lungs or respiratory lesions identical with those described last year in the study on murine pneumonia. There were no other gross lesions observed except in one animal with a fatty liver. A small percentage of rats, approximately 5%, also have lesions resembling chronic murine pneumonia. It is possible that the incidence of this disease will be further reduced as the Sprague Dawley rats are replaced with the Fischer strain which is less susceptible to this disease. The rats were also infected to a small degree with the tapeworm cyst, Cysticercus fasciolaris, which most likely results from contact with bedding contaminated by dogs or cats. In hamsters there was an approximately equal incidence, less than 2%, of pulmonary and hepatic lesions whose cause was undetermined. Pneumonic lesions were present in less than 2% of the animals necropsied and were the only lesions of significance found. Rats and guinea pigs were free of endoparasites, but mice were found to be infected with pinworms, Aspicularis tetraptera and Syphacia obvelata, and the dwarf tapeworm, Hymenolepis nana. Unidentified helminth ova were found in the feces of two of the hamsters examined. The control of these endoparasites is virtually impossible without having even hot water in the areas where cages are washed. The only ectoparasites found in the rodents were mild louse infestations on rats and guinea pigs.

Table 1. Summary of Deaths and Significant Illnesses of Animals in the SMRL Animal Colony from April 1969—March 1970

Species	Code #	Nature of Death or Clinical symtoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Gibbon White—handed	S—1	Constipation, inappetence, dehydration, weakness.	Blood cult: Chromo- Heart: bacterium Lung: janthinum Kidney: E. coli S. intest, GPO 119:B14 Stool:	1. Pneumonia 2. Hepatic 3. Cysticercosis, heart 4. Strongyloidosis	Stool exam: Strongyloides larvae	
Gibbon White—handed	S—12	Depressed, loss of appetite, vomit & dehydrated.	Stool E. coli 0127: B8 and 0112: B11	Not Completed.	Blood smear: (—ve) Stool exam: (—ve)	
Gibbon White—handed	S—30	Found dead in cage	Mediastinal lymph- node: Enterobacter Lung: aerogenes Stomach: Heart: Providencia and E. coli Intestine: E. coli Liver: No bacterial growth after 48 hrs. incubation.	1. Pneumonitis, interstitial, modarate, with hemorrhage, lung. 2. Congestion, moderate, kidney, brain. 3. Nephritis, interstitial, chronic, focal, kidney. 4. Congestion, severe, liver. 5. Autolysis, stomach, liver, brain.	Virus isolation: (—ve)	
Gibbon White—handed	S—62	Found dead in cage	Small intestine: Shigella sonnei form I Kidney: Liver: Proteus Lung: mirabilis Nodule from diaphragm:	not completed	None	

Table 1 (Continued)

Species	Code#	Nature of Death or Clinical symptoms	Becteriology findings	Pathology findings	Other findings	Remarks
Gibbon, White-handed	S-62	Found dead in cage	Small intestine: Shigella sonnei form I Kidney: Liver: Proteus Lung: mirabilis Nodule from diaphragm:	Not Completed.	None	
Gibbon, White-handed	S-64	Found dead in cage	Pleural effusion: Staphylococcus epidermidis and alpha Streptococcus Kidney: alpha Streptococcus Heart bld: alpha Streptococcus Liver: E. coli Enterobacter cloacae alpha Streptococcus	1. Pneumonia, exudative, subacute, lung. 2. Congestion moderate lung, adrenal, kidney, uterus, liver, tonsil. 3. Lymphadenitis, suppera-tive, with hemorrhage, lymph node. 4. Autolysis, intestine, kidney, liver. 5. Basal cell tunor, skin.	None	
Gibbon, White-handed	S-79	Constipation, inappetence, dehydration, weakness	Lung: Proteus spp. Liver: E. coli, Aerobacter aerogenes Pseudomonas spp. and Proteus spp. Small Int: No enteric bacterial patho-gen detected. Cecum:	1. Pulmonary strongyloidosis, 2. Pneumonia 3. Pulmonary hemorrhage 4. Multiple abscesses	Stool exam: Numerous strongyloides larvae	

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Gibbon, White–handed	S–80	Found dead in cage	Liver: No bacterial growth after 24 and 48 hrs. incubation	1. Pneumonia, supperative, marked, lung. 2. Reticular hyperplasia, with hemorrhage, lymph node 3. Enteritis, subacute, with Balantidium coli, large intestine 4. Hepatitis, periportal, mild, liver. 5. Congestion, moderate, liver, kidney, adrenal, cerebrum, pars 6. Nephritis, interstitial, chronic. kidney 7. Extramedullary hemato-poiesis, lymph node.	None	
Gibbon, White–handed	S–84	Found dead in cage	Trachea: Proteus spp. Enterobacter spp. Lung: Proteus spp. Liver: alpha Streptococcus	1. Pneumonia supperative, diffuse, with hemorrhage, lung. 2. Splenitis, severe, with necrosis, spleen. 3. Congestion, moderate. liver. heart.	None	
Gibbon, White - handed	S–87	Found dead in cage	Dura-mater: Enterobacter aerogenes Pleural fluid: Peritoneal fluid:	1. Pneumonitis interstitial, subacute, moderate, lung 2. Congestion, moderate, with autolysis, liver, brain, kidney.	Virus isolation: (–ve)	

Table · (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Gibbon, White handed	B—50	None	Lung: Paracolobactrum intermedium, E. coli Liver: Paracolobactrum intermedium, E. coli, alpha Streptococcus Heart bld: No bacterial growth after 48 hrs. incubaction.	1. Pulmonary strongyloidiasis 2. Pulmonary hemorrhage 3. Pneumonia	None	
Gibbon, White—handed	B—57	Found dead in cage	Pericardial fluid: Enterobacter cloacae, E. coli, Proteus mirabilis. Vagina: Enterobacter cloacae, E. coli, Proteus mirabilis Liver: Enterobacter cloacae Lung: No bacterial Heart bld: growth after 48 hrs. incubation.	1. Pleuritis, chronic, moderate, lung 2. Pneumonia, acute, moderate with diffuse hemorrhage, lung. 3. Fatty degeneration, moderate, kidney 4. Nephritis, interstitial, chronic, focal, kidney. 5. Congestion, moderate, liver. 6. Lymphoid hypoplasia, spleen. 7. Autolysis, liver, large intestine pancreas, adrenal.	Stool exam: Strongyloides ova and larvae	
Gibbon, White—handed	B—30—S	Diarrhea occurred about 2 wks. with occasional vomiting. Anorexia & dehydration 2 days before death.	Stool: No enteric bacterial pathogen detected.	1. Pneumonia, interstitial, with congestion and parasites, lung. 2. Nephritis, interstitial, chronic, focal, with autolysis, kidney. 3. Autolysis, marked, with nematodes, intestine. 4. Autolysis, with multiple cysticercosis liver.	Stool: No ove and parasites seen.	

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Gibbon, White-handed	B--31--S	Acute parasitic anemia	Blood: No bacterial growth after 24 and 48 hrs. incubation.	1. Malaria 2. Interstitial nephritis, mild. 3. Alveolar emphysema, mild. 4. Hepatitis, focal.	Blood smear: Malaria parasites.	
Gibbon, White-handed	P--10	Found dead in cage	Stool: No enteric bacterial pathogen detected.	1. Nephritis interstitial, chronic, kidney. 2. Congestion, mederate, heart, liver, meninges, cerebrum cerebellum. 3. Cysticercus, cyst, cerebrum. 4. Hemorrhage massive, lung.	Stool exam.: Giardia lamblia cyst, E. coli cyst, Strongyloides larvae.	
Gibbon, White-handed	B--57			8. Necrosis, hemorrhage, fallopian tube. 9. Endometritis, with hemorrhage, uterus. 10. Enteritis, subacute, with nematode larvae, small intestine.		
Gibbon, White-handed	B--79	Presumed dead	None	None	None	Kled Kaeo Island

Table 1. (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other finding	Remarks
Gibbon, White–handed	B–80	Respiratory failure due to acute pulmonary hemorrhage.	Trachea: No result Lung: back.	1. Hemorrhage, severe, lung. 2. Nephritis, interstitial, mild, kidney. 3. Congestion, moderate, kidney, liver, intestine. 4. Autolysis, liver, intestine.	None	
Gibbon, White–handed	B–87	Found dead in cage	Liver: No bacterial Heart bld: growth after 48 hrs. incubation Large intestine content: No enteric bacterial pathogen detected.	1. Pneumonia, acute, severe with edema and hemorrhage, lung 2. Congestion, moderate, with fatty degeneration, kidney. 3. Splenitis, moderate, spleen. 4. Lymphadenitis, with necrosis, lsmph node. 5. Necrosis, severe, liver. 6. Enteritis, subacute, severe, with paritonits, large intestine 7. Metritis, necrotic, acute, uterus.	Stool exam: E. coli cyst Virus isolation: (–ve)	
Gibbon, White–handed	S–66	Complete fracture of the listal end of left femur. Amputated due to infaction.	None	None	None	Treat with Cortisone acetate and chloramphe icol, intra-muscularly.

Table 1. (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Gibbon, White-handed	S-73	Watery diarrhea	None	None	None	Kaopectate and Terramycin per oral.
Gibbon, White-handed	VM-64	Endoparasites	None	None	Stool exam.: Whip worm ova	Treat with thiabendazole orally
Gibbon, White-handed	VM-65	Endoparasites	None	None	Stool exam.: Whip worm ova.	Treat with thiabendazole orally
Gibbon, White-handed	VM-66	Endoparasites	None	None	Stool exam.: Hook worm ova.	Treat with thiabendazole per oral.
Monkey, Rhesus	KL-9	Found dead in cage.	Urine: Bacillus spp. Lung: Pseudomonas spp. Proteus spp.	1. Peribronchitis, chronic, with edema and lung. 2. Congestion with autolysis, liver. 3. Urethritis, purulent, with necrosis penis. 4. Nephritis, focal subacute, with autolysis, kidney. 5. Necrosis, focal heart.	Calculi analysis not completed	

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Gibbon, White handed	PC 2	Subnormal emp., distended be . dilated pupils, loss of appetite, weakness, dehydration, watery diarrhea, and prostration. About 10 ml of clear ascitic fluid was present with enlargement of mesenteric lymph nodes and gall bladder.	Rectal swab: No enteric bacterial pathogen detected.	1. Enteritis, mucoid, severe, with nematodes, large intestine. 2. Pneumonia, moderate, with hemorrhage lung. 3. Autolysis and congestion, liver, kidney, brain.	None	
Gibbon, White–handed	PC -3	Anesthetic accident	None	1. Pneumonia, interstitial, acute, severe, lung. 2. Congestion, modarate, liver. 3. Congestion, severe, kidney, brain.	Virus isolation: (–ve)	
Gibbon, Capped	VM–17	None	None	None	None	Suffocation during handling
Gibbon, White–handed	PC–1	Watery diarrhea, Strongyloidosis	None	None	Stool exam.: Strongyloides larvae.	Treat with thiabendazole per oral.

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
White-handed Gibbon,	B-46	Diarrhea	Stool: No enteric bacterial pathogen detected.	None	None	Symptomatic treatment.
Monkey, Rhesus	KL-14	None	Spleen: Enterobacter spp.	1. Degeneration, vacuolar, moderate, liver. 2. Pigment lung, spleen.	Blood smear: (P. inui + ve)	
Monkey, Cynomylogus	MS-91	Endoparasites	None	None	Stool exam.: Hook worm ova. Strongyloides larvae.	Treat with thiabendazole orally
Monkey, Cynomologus	MS-92	Endoparasites	None	None	Stool exam.: Hook worm ova, Strongyloides larvae, Giardia lamblia cyst.	Treat with thiabendazole orally
Monkey, Cynomologus	MS-93	Endoparasites	None	None	Stool exam.: Hook worm ova, Strongyloides larva, I. butschlii cyst	Treat with thiabendazole orally
Monkey, Cynomologus	MS 94	Endoparasites	None	None	Stool exam.: Hook worm ova, Strongyloides larvae.	Treat with thiabendazole orally

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Monkey, Cynomologus	MS 95	Endoparasites	None	None	Stool exam.: Hook worm ova, E. coli cyst, Giardia lamblia cyst.	Treat with thiabendazole per oral.
Monkey, Cynomologus	MS 96	Endoparasites	None	None	Stool exam.: Hook worm ova, Strongyloides larvae, E. coli cyst.	Treat with thiabendazole per oral.
Monkey, Cynomologus	MS--97	Endoparasites	None	None	Stool exam.: Hook worm ova.	Treat with thiabendazole per oral.
Monkey, Cynomologus	MS--98	Primate malaria	None	None	Blood smear: (P. inui + ve)	Treat with Primaquine PQ_4 and Resochin, intramuscularly.
Monkey, Cynomologus	MS--100	Endoparasites	None	None	Stool exam.: Hook worm and Whip worm ova.	Treat with thiabendazole per oral.

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Monkey, Cynomologus	MS–101	Endoparasite	None	None	Stoal exam.: Strongyloides larvae.	Treat with thiabendazole per oral.
		Shigellosis	Stool: Shigella sonnei form I	None	None	Treat with Chloromycetin, intramuscularly.
		Primate malaria	None	None	Blood smear: (P. inui + ve)	Treat with Primaquine PQ_4 and Resoch in, intramuscularly.
Monkey, Cynomologus	MS–102	Endoparasite	None	None	Stool exam.: Strongyloides larvae.	Treat with thiabendazole per oral.
Monkey, Cynomologus	MS - 103	Endoparasite	None	None	Stool exam.: Hook worm ova Whip worm ova, and Strongyloides, larvae.	Treat with thiabendazole per oral.
Monkey, Cynomologus	VM–55	Endoparasite	None	None	Stool exam.: Hook worm ova, Strongyloides larvae, E. coli cyst, I butschlii cyst.	Treat with thiabendazole per oral.
		Shigellosis	Stool: Shigella sonnei form I	None	None	Antibiotic treatment.
Monkey, Cynomologus	VM–56	Endoparasites	None	None	Stool exam.: Hook worm ova, Strongyloides larvae, Giardia lamblia cyst.	Treat with thiabendazole per oral.

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Monkey, Cynomologus	VM–58	Shigellosis Endoparasites	Stool: Shigella sonnei form I None	None None	None Stool exam.: Hook worm ova, Whip worm ova.	Antibiotic treatment. Treat with thiabenda-zole orally
Monkey, Cynomologus	VM–59	Endoparasites	None	None	Stool exam.: Hook worm ova, Whip worm ova, E. coli cyst.	Treat with thiabenda-zole orally
Monkey, Cynomologus	VM–60	Shigellosis Primate malaria Endoparasites	Stool: Shigella sonnei from 1 None None	None None None	None Blood smear: (P. inui + ve) Stool exam.: Hook worm ova, Whip worm ova.	Antibiotic treatment. Anti-malaria treatment Treat with thiabenda-zole orally
Monkey, Cynomologus	VM–67	Endoparasites	None	None	Stool exam.: Strongyloides larvae.	Treat with thiabenda-zole orally
Monkey, Cynomologus	VM–68	Endoparasites	None	None	Stool exam.: Strongyloides larvae.	Treat with thiabenda-zole orally

6001
1009

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology findings	Other findings	Remarks
Monkey, Cynomologus	VM—69	Endoparasites	None	None	Stool exam.: Whip worm ova and Strongloides larvae.	Treat with thiabenda-zole orally
Monkey, Cynomologus	VM—70	Endoparasites	None	None	Stool exam.: Hook worm ova.	Treat with thiabenda-zole orally
Monkey, Cynomologus	VM—72	Shigellosis Endoparasites	Stool : Shigella flexneri 2 None	None None	None Stool exam.: Strongyloides larvae.	Antibiotic treatment. Treat with thiabenda-zole orally
Monkey, Cynomologus	VM—73	Primate mararia Endoparasites	None None	None None	Blood smear: (P. inui + ve) Stool exam.: Strongyloides larvae.	No treat-ment. Treat with thiabenda-zole orally
Monkey, Cynomologus	VM—74	Endoparasite	None	None	Stool exam.: Strongyloides larvae.	Treat with thiabenda-zole orally

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology fineings	Other findings	Remarks
Monkey, Cynomologus	VM--75	Endoparasite	None	None	Stool exam.: Strongyloides larvae.	Treat with thiabenda-zole orally
Monkey, Cynomologus	VM--76	Endoparasite	None	None	Stool exam.: Hook worm ova, Strongy-loides larvae.	Treat with thiabenda-zole orally
Monkey, Cynomologus	VM--77	Endoparasite	None	None	Stool exam.: Strongyloides larvae.	Treat white thiabenda-zole orally
Monkey, Cynomologus	VM--78	Primate malaria Endoparasite	—	—	Blood smear: (P. inui + ve) Stool exam.: Hook worm ova.	No treat-ment Treat with thiabenda-zole orally
Monkey, Stumptailed.	VM--79	Endoparasite	None	None	Stool exam.: Hook worm ova.	Treat with thiabenda-zole orally

Table i (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology finding	Other findings	Remarks
Canine	Pup #1	Died due to air passage obstruction during anesthesia.	None	None	None	Dirofilariasis Study.
Canine	Pup #2	Demodectic mange	None	None	Skin scrapping: Demodex canis.	Treat with "Ectoral" and B-complex.
Canine	Pup #4	Canine Distemper	None	None	None	Antibiotic and supporting treatment.
Feline	#103	Gangrene, wet, hind quarter. Found dead in cage.	None	None	None	Treat with "Ancylol", subcutaneously.
Feline	#106	Endoparasites	None	None	Stool exam.: Hook worm ova.	Treat with "Ancylol", subcutaneously.
Feline	#109	Endoparasites	None	None	Stool exam.: Hook worm ova.	Treat with "Ancylol", subcutaneously.

Table I (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology findings	Pathology finding	Other findings	Remarks
Feline	#111	Endoparasites	None	None	Stool exam: Hook worm ova	Treat with "Ancylol", subcutaneously.
Feline	#112	Endoparasitse	None	None	Stool exam: Hook worm ova	Treat with "Ancylol", subcutaneously.
Feline	#113	Endoparasites	None	None	Stool exam: Hook worm ova.	Treat with "Ancylol", subcutaneously
Feline	#114	Endoparasites	None	None	Stool exam.: Hook worm ova.	Treat with "Ancylol", subcutaneously
Bovine	—	Unknown	None	1. Mesenchymonc	Stool exam.: Hook worm ova.	Treat with "Ancylol" subcutaneously
Bovine	-	Unknown	None	1. Pneumonia, lobar, acute	None	

Table 1. (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacterioiogy finding	Pathology findings	Other findings	Remarks
Bovine	D1--23	Depress, abdominal distension but normal appetite.	Lung: Proteus spp. Intestine: Proteus spp. Heart bldg: No bacterial growth after 72 hrs. incubation.	1. Congestion, moderate, with antoloysis, lung. 2. Congestion and hemorrhage, lymph node. 3. Edteritis, acute, diffuse, with autolysis, small intestine. 4. Serositis, purulent, serosa, bladder. 5. Congestien, mild, liver. 6. Informalion, purulent, spermatic card.	None	
Ovine	#1	Neoplasia in the posterior nares, coughing and sneering with nose bleeding, labored breathing and loosing weight.	Post. neres: Staphylococcus epidermidis, alpha Streptococcus, Diphtheroids.	1. Granuloma, peribronchiol, lung. 2. Fat infiltration, periportal, moderate, liver. 3. Adenocarcinoma, turbinates.	None	Sacrificed
Ovine	#28	None	Abd. fluid: Proteus mirabilis, Sarcina spp. Streptococcus fecalis, Staphylococcus aureus, Becillus spp. not B. anthracis and Enterobacter aerogenes Thoracicfluid: Staphylococcus aureus, Proteus mirabilis, Enterobacter aerogenes.	1. Periportal hepatitis 2. Toxis hepatitis. 3. Renal hemosiderosis. 4. Cortical necrosis, kidney.	None	
Ovine	#29	Dystocia	None	None	None	
Ovine	#35	None	None	None	None	Decayed
Ovine	#45	Shedding soft stool and stiff gait, and separate from the flock.	Heart bld: Alpha Streptococcus	1. Autolysis, with bacteria, liver, spleen, intestine. 2. Congestion, moderate, lung. 3. Necrosis, moderate, kidney.	None	

Table 1 (Continued)

Species	Code #	Nature of Death or Clinical symptoms	Bacteriology finding	Pathology findings	Other findings	Remarks
Ovine	#79	Stiffness of the extremities, persistant tremor, grind and salivation, temp. 108°F.	Swab from spleen: Bacillus spp. (not B. anthracis), Aerobacter cloacae, Pseudomonas spp.	1. Congestion and edema, moderate, lung. 2. Congestion, moderate, liver. 3. Funiculitis, acute and chronic, with necrosis, spermatic cotd.		
Rabbit New Zealand White		Stomach rupture	Lung: No bacterial growth after 72 hrs. incubation. Liver: Staphylococcus epidermidis Enterobacter spp.	1. Hemorrhage and edema, lung. 2. Congestion, moderafe, liver.		
Rabbit New Zealand White	--	Progressive weight loss, extremely emaciated and rear quarters soiled.	Lung: E. coli, Alpha streptococcus Liver: E. coli	1. Pneumonitis, interstitial, mild, with edema and congestion, lung. 2. Congestion, moderata, kidney. 3. Necrosis, midzonal, with coccidia, live·	None	
Rabbit New Zealand White	—	Found dead in cage.	Lung: Proteus spp. E. coli Uterus: Proteus mirabilis Heart bld: No bacterial growth after 24 and 48 hrs. incubation.	Not completed	None	

Table II. Bacteriologic findings-Breeding Colony Disease Screening Program

Animal	Organs Cultured	Proteus Spp	Bacillus Spp	Pseudomonas	A. Strep.	Micro-coccus	S. epidemidas	Diptheroids	Paracolon	Providencia	Enterobacter aerogenes	Proteus mirabilis	E. Coli	Paragenoides	P. coliforme	S. aureus	Shigella flexeri II	Average # Organisms per # Animal Screened
MICE	Lung	2	3		63	42	33	1		28			1					25/220
	Stool	140		12					6	16	4		139				1	53/220
RATS	Lung		7		55	8	29				1		98		10			25/200
	Stool	72		53														47/200
HAMSTERS	Lung	16			37		16			5								19/165
	Stool	59		8						31			12			5		23/165
GUINEA-PIG	Lung	6	2		7		13											9/110
	Stool	24	41	26						6	2		37	3				16/110

Gibbon Menstrual Cycle and Breeding Study

Principal Investigators: Dennis O. Johnsen, MAJ, VC
Markpol Tingpalapong, D.V.M.
Verachart Chaicumpa, D.V.M.

Associate Investigarors: Robert L. Hickman, MAJ, VC
Prayot Tanticharoenyos, D.V.M.
William L. Wooding, MAJ, VC

PURPOSE: In this study the reproductive cycle of the female gibbon and semen of the male gibbon is characterized and related to other physical parameters of breeding performance with the ultimate goal of obtaining reproduction of the gibbon in a laboratory environment.

DESCRIPTION: During this report period continuing emphasis has been placed on determining the length and characteristics of the menstrual period, the physical characteristics of gibbon semen, and in the effect of combinations of hormones on inducing ovulation in the female. Also during this report period male and female pairs of gibbons were reestablished in eight large outdoor cages that were moved to Bangkok for the purpose of allowing natural mating.

PROGRESS: Data characterizing the female menstrual cycle was obtained by vaginal swabs taken three times a week; in those females that exhibited some degree of regularity swabs were taken seven days a week. At the same time the transverse width of the everted vulva was measured to determine if changes that occurred, if any, were associated with menstrual bleeding. Data from the twenty-nine gibbons that were examined in this manner in shown in Table 1. Of interest is the variability in the duration of the menstrual flow and the intervals between menstruation. The variability in bleeding is probably largely artefactual since swabbing only three times a week does not yield accurate results. However, information obtained from swabs taken seven days a week indicates that bleeding ordinarily lasts from three to four days. The variability in the length of the menstrual cycle is consistent with observations made in the last annual report. These intervals are graphically portrayed in figure 1 and show an average interval of approximately 18 days, a period of time considerably less than what would be expected from information collected on other primates. When compared to various times of the year, there seems to be no relationship between the occurrence of menstruation and the season. Measurements made on the transverse diameter of the vulva show that in non-pregnant animals the degree of eversion can vary from none at all to three centimeters. Eversion

seems to be most pronounced two to three days before bleeding; during bleeding eversion decreases. However, the degree of variability in this finding is so great that it cannot be considered a reliable indicator for determining the stage of the menstrual cycle.

Examination of gibbon semen was continued using the same techniques described in the last annual report. The semen samples were collected from sedated gibbons by electroejaculation using 5 to 15 volts A.C. 50 cycle current and a stimulus of 1 to 2 seconds applied at intervals of 2 to 4 seconds. The results of the semen examinations are presented in table 2 and do not differ significantly from those reported last year. In an effort to develop the capability for performing artificial insemination an experiment was designed to determine what type of semen expander was the most suitable for diluting and preserving the small volumes of semen collected from the gibbons. Four expanders, egg yolk and dextrose, egg yolk and saline, heated milk, and human seminal plasma, were used in the experiment. The 0.2 to 0.3 ml. to collected semen was diluted with 1 ml. of the expander being examined and kept for varying lengths of time in a 37°C. waterbath. At 30 minute intervals slides were prepared from the diluted specimens and examined for specific activity, motility, and viability. The results shown in figure 2 show that the most suitable expander of those examined is the egg yolk-normal saline solution.

A necessary prerequisite to successful artificial insemination is determining when ovulation in the female occurs. Because of the extreme irregularity in the menstrual cycles of the females studied in the colony the use of exogenous hormones to induce ovulation appeared to be a practical alternative to using biological indicators. As reported in the last annual report it is possible to regulate stages of the menstrual cycle in a gibbon by giving a course of treatment with progesterone or related compounds. However, as laparotomies and direct observation of the ovaries have shown, it is not possible to predict the time that ovulation would occur with this method. A more complex method used to induce ovulation in macaque species was evaluated in four gibbons that had been cycling at regular intervals. For five days each animal was given 5 mg. of progesterone intramuscularly, then 1 mg. follicle stimulating hormone (Anteron, Schering) intramuscularly for the next four days, and finally 500 international units of human chorionic gonadotrophin (Pregnyl NV. Organon) intramuscularly. A laparotomy was performed on each gibbon in succession at 10 hours, 14 hours, 18 hours and 22 hours following the last injection; times chosen to bracket the ovulation time induced in macaques. The ovaries of these animals all showed multiple follicle development, and although the follicles were mature enough to rupture with even the most delicate manipulation, there was no evidence that any had ruptured

spontaneously.

Eight pairs of gibbons have been mated for approximately six months in the large 20 foot by 20 foot cages moved to Bangkok from Prabuddhabat. The females at first were examined rectally for pregnancy at monthly intervals. In addition, vaginal swabs were taken from the females for the menstrual cycle study and weekly semen samples were being collected from the males. Because none of the animals became pregnant over a period of approximately five months, a decision was made to limit the handling of them to only once each quarter and largely eliminate the excitement and trauma that is unavoidable when these animals are restrained. Since that time rectal exams have been performed each quarter in addition to the collection of blood samples for the leukemia screening program, physical examination, and administration of anthelmintics and other necessary treatment. There have been no pregnancies diagnosed.

Growth and Development of the Gibbon

Principal Investigators:	Dennis O. Johnsen, MAJ, VC Prayot Tanticharoenyos, D.V.M. Dirck L. Brendlinger, MAJ, MC*
Assistant Investigators:	Markpol Tingpalapong, D.V.M. Jerm Pomsdhit

PURPOSE: The production of gibbons from the gibbon breeding program has offered a unique opportunity to measure certain parameters of growth and development in animals where birthdates are known. The purpose of this study is to relate distinctive developmental features to the age of these young gibbons so that the age of animals with unknown birthdates may be accurately determined.

DESCRIPTION: Growth and development is measured in the following ways in young gibbons:

a) Body weights are taken regularly on each of the gibbons in the study;

b) The time of eruption and notable characteristics of wear on the teeth are observed and recorded quarterly;

---

* Assistant Chief, Diagnostic Section, Fitzsimons General Hospital, Denver, Colorado.

TABLE 1 RESULTS OF VAGINAL SWAB EXAMINATIONS

Gibbon#	Bld_1	Int	Bld_2	Int	Bld_3	Int	Bld_4	Int	Bld_5	Int	Bld_6	Int	Bld_7	Int	Bld_8	Int	Bld_9	Int	Bld_{10}	Int	Bld_{11}	Int	Bld_{12}	Int	Bld_{13}	Int	Bld_{14}	Int	Bld_{15}	Int	Bld_{16}	Int	Bld_{17}
B-66-S	3	14	1	47	1	23	1	15	1	15	1	16	1	15	1	21	1	17	3	15	1	15	1	17	1	14	3	18	11	13	3	42	3
B-85	1	44	3	44	2	69	3	47	3	28	1	29	1	21	1	15	1	18	1	29	1	24	3	6	3	13	3	17	3	35	1	14	
P-7	1	32	1	13	1	143	1	18	1	17	3	27	1	27	1	17	3	31	1	37	1	16	1										
S-2	3	35	3	11	1	76	1	15	3	16	6	14	3	13	3	16	3	14	3	11	3												
B-86	1	22	3	99	1	79	1	25	1	20	1	35	1	15	1	29	3	18	1	23	1												
B-57	1	29	3	16	6	36	1	3	9	33	1	3	1	87	3	38	1	23	3														
B-30-S	1	69	3	8	1	20	1	19	1	52	1	23	1	10	6	17	3																
B-11	1	20	1	75	1	19	1	22	1	18	1	27	1	13	1																		
B-88	2	21	1	71	1	79	1	18	1	79	3	11	3	57	3																		
B-7	1	68	1	107	3	46	1	24	1	21	3	25	8	40	1																		
B-87	1	79	1	81	2	42	1	31	1	22	1	63	1	116	1	33	D																
P-10	1	21	3	17	3	16	1	26	1	37	1	11	1	D																			
B-70	1	25	1	48	3	3	1	366	3	6	1																						
S-86	1	28	1	25	1	32	1	8	1	81	1	38																					
B-9	1	41	1	177	1	17	1	117	1																								
B-50	1	35	1	18	1	21	1	33	1	D																							
B-25	3	38	1	9	1	38	1																										
P-2	1	33	1	11	1	4	1																										
B-14-S	1	228	3	18	1																												
B-4	1	64	1	110	1																												
DZ-1	1	122	1	44	1																												
S-90	1	165	2	10	1																												
B-51	1	96	1																														
B-37	1	99	1																														
S-95	1	177	3																														
P-12	1	17	1																														
S-84	1	107	1	D																													
S-10	-																																
S-62	N	-	D																														

Bld_1, Bld_2, Bld_3, ... = The first, second, third, time of menstrual bleeding. (The first day of bleeding to the last day of bleeding.

Int = Time interval (from last day of bleeding to the next first day of bleeding)

D = Dead

Table 2. Average Gibbon Semen Values

Animal Number	Volume ml.	Density	Mass Activity	Motility %	pH	Concentration per mm^2	Nigrosin Staining		Cell Staining
							Prox. %	Dist. %	
DZ-2	0.3	Thick	++++	86	7.0	1,682,740	2	-	Neg.
S-98	0.24	Slightly thick	++	51	7.1	1,082,058	2	-	Neg.
B-12	0.13	Slightly thick	+++	59	7.1	1,426,870	1	-	Neg.
B-8	0.2	Slightly thick	+++	64	7.2	1,004,286	2	1	Neg.
B-21	0.12	Thick	++	46	7.1	1,421,631	1	1	Neg.
B-46	0.19	Thick	+++	73	7.0	2,472,727	1	1	Neg.
S-72	0.24	Thick	++	Fair	7	524,000	0.2	0.2	-
S-76	0.1	Thick	++	Good	7.2	247,000	Present	-	-
S-53	0.1	Thick	++	Bad ab.	7.4	116,300	Present	Absent	-
S-74	0.1	Thick	++	Fair	7.1	566,000	Present	Absent	-
B-83	0.1	Thin	+	Bad ab.	7	no spermatozoa	-	-	-
B-18-S	0.1	Thin	++	Bad ab.	7.1	36,000	Absent	Absent	-

FIG. 1 FREQUENCY OF MENSTRUAL BLEEDING RELATED TO THE INTERVAL OF TIME BETWEEN MENSTRUAL PERIODS.

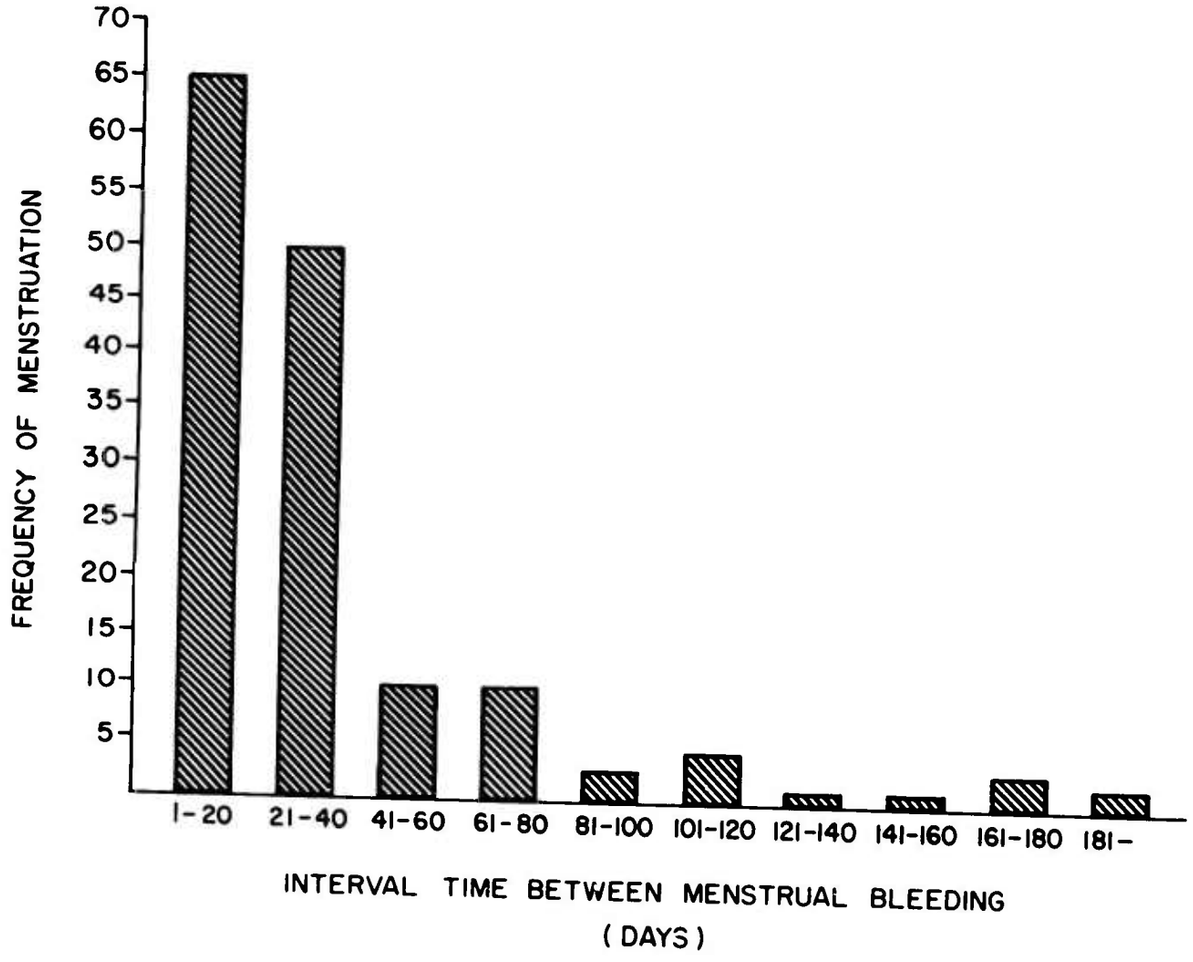


Figure 2. Gibbon Semen Survivability in various expanders (% surviving)

Egg yolk/dextrose	50	40	20	10	<10	0
Egg yolk/saline	60	40	37	33	30	0
Milk	60	20	0			
Human seminal plasma	0					
	5 min.	30 min.	60 min.	90 min.	120 min.	overnight

c) Skeletal development is evaluated radiographically at quarterly intervals following birth. In measuring skeletal development, emphasis is placed on the development of the hands, wrists, ankles, and feet;

d) The time that obvious signs of sexual development occur, such as descension of the testicles in the male and the onset of menstruation in the female, are observed and recorded.

PROGRESS: Information that has been accumulated during the course of this study is presented in Tables 1 and 2. The following developments characterize the growth and development of the gibbon at the ages indicated:

6 months. The proximal radial epiphysis and styloid process of the ulna become visible radiographically. Two juvenile incisors are present in both the upper and lower jaw.

9 months. Eight carpal bones are present and the distal epiphysis of the third metacarpal becomes equal in width to its diaphysis.

12 months. The proximal epiphysis of the proximal first phalanx and the first metacarpal appears. The proximal epiphysis of the proximal third phalanx, proximal radial epiphysis, and distal radial epiphysis become equal in width to their diaphyses. The upper central permanent incisors are present.

15 months. The radial styloid process appears and the distal ulnar epiphysis becomes equal in width to the ulnar diaphysis. Several changes in bone shape occur during this time that are distinctive. The carpal bones become modeled and tend to conform more to the shape of the bones they articulate with as in the adult carpus. The metacarpals as well become funnelized, or assume a more triangular shape. The proximal epiphysis of the proximal third phalanx of the hand is in transition between its earlier distal convex shape to a more flattened appearance.

18 months. The distal epiphysis of the third metacarpal of the hand becomes nearly square shaped instead of round. The proximal radial epiphysis is "capping" and becoming wider than the diaphysis. At least one patella ossifying from two centers, appears during this period.

21 months. The proximal epiphysis of the first metacarpal reaches a width equal to that of its diaphysis. Changes in shape include the proximal epiphysis of the proximal third phalanx of the foot becoming concave in shape, and the distal radial epiphysis becoming "capped"; the distal radial epiphyseal plate begins to assume a sigmoid shape. The second lower incisor has appeared by this time.

24 months. The distal epiphysis of the third metacarpal is square.

Due to the influenza epizootic that passed through the gibbon colony in August that is described elsewhere in this report, two of the three infant

gibbons died. It is hoped that further pregnancies in the breeding colony will result in the additional young gibbons that are necessary to complete this study. As of the present time, the remaining female gibbon has shown no signs of sexual development.

Table 1. Growth in Infant Gibbons
(in grams)

Age	PC 1	PC 2	PC 3
3 mos.			447
6 mos.		838	1,150
9 mos.	1,190	980	1,610
12 mos.	1,210	1,200	2,050
15 mos.	1,350	1,240	
18 mos.	1,800	1,560	
21 mos.	2,120		
24 mos.	2,400		

Table 2. Gibbon Skeletal and Dental Development

Age (Mos)	Proximal Epiphysis Proximal 1st Phalanx, hand $\frac{1}{3}$	Proximal Epiphysis Proximal 3rd Phalanx, hand $\frac{2}{3}$	Ossification of Seasamoid Process, metacarpo-phalangeal joint	Distal Epiphysis 3rd Metacarpal, hand $\frac{3}{4}$	Proximal Epiphysis 1st Metacarpal	Number of Carpal bones	Proximal Epiphysis Proximal 3rd phalanx, foot	Fusion, of Distal Radial Epiphysis $\frac{2}{3}$	Proximal Radial Epiphysis	Ulnar Epiphysis: Fusion of Distal Ulnar Epiphysis $\frac{1}{3}$	Ulnar Epiphysis: Styloid Process Present	Remarks	Dental Formula $\frac{ic\ o\ m}{ic\ o\ m}$
3 (1)*	Abs.	Unk	Abs.	Unk	Abs.	7	Unk.	Unfused present	Abs.	Unfused present	Abs.		
6 (2)	Abs.	Unfused present	Abs.	Unfused present round	Abs.	7	Unk.	Unfused	Unfused	Unfused	pres.		$\frac{ii\ c\ p\ m}{ii\ c\ p\ m}$
				$\frac{3}{4}$				$\frac{3}{4}$	$\frac{3}{4}$	$\frac{1}{2}$	indis-tinct		
9 (3)	Abs.	Unfused $\frac{3}{4}$	Abs.	Unfused $\frac{1}{1}$	Abs	7-8	Unk.	Unfused $\frac{3}{4}$	Unfused $\frac{3}{4}$	Unfused $\frac{1}{2}$	pres. dis-tinct $\frac{1}{2}$		$\frac{ii\ c\ p\ m}{ii\ c\ p\ m}$
12 (3)	Unfused present $\frac{1}{3}$	Unfused Convex distally $\frac{1}{1}$		Unfused not round $\frac{1}{1}$	Unfused present $\frac{1}{2}$	8	Unfused present $\frac{1}{1}$	Unfused $\frac{3}{4}$	Unfused $\frac{1}{1}$	Unfused $\frac{3}{4}$	pres.		$\frac{1i\ c\ p\ m}{1i\ c\ p\ m}$

Table 2. (Continued)

Age (Mos)	Proximal Epiphysis Proximal 1st Phalanx, hand	Proximal Epiphysis Proximal 3rd Phalanx, hand	Ossification of Seasamoid Process, metacarpo-phalangeal joint	Distal Epiphysis 3rd Metacarpal, hand	Proximal Epiphysis 1st Metacarpal	Number of Carpal bones	Proximal Epiphysis Proximal 3rd phalanx foot	Fusion, of Distal Radial Epiphysis	Proximal Radial Epiphysis	Ulnar Epiphysis: Fusion of Distal Ulnar Epiphysis	Ulnar Epiphysis: Styloid Process Present	Remarks	Dental Formula
15 (3)	Unfused $\frac{1}{2}$	Unfused tends to flatten $\frac{1}{1}$		Unfused $\frac{1}{1}$	Unfused $\frac{1}{2}$	8	Unfused flatter $\frac{1}{1}$	Unfused Radial Styloid present $\frac{1}{1}$	Unfused $\frac{1}{1}$	Unfused $\frac{1}{1}$	pres.	Metacarpal funnelization begins Carpals Modeled	$\frac{Ii\ c\ p\ m}{Ii\ c\ p\ m}$
18 (1)	Unfused $\frac{1}{2}$	Unfused $\frac{1}{1}$	Abs.	Unfused Nearly Square $\frac{1}{1}$	Unfused $\frac{3}{4}$	8	Unfused $\frac{1}{1}$	Unfused $\frac{.}{1}$	Unfused Capped $\frac{7}{6}$	Unfused $\frac{1}{1}$	Patella present	Patella present	$\frac{Ii\ c\ p\ m}{Ii\ c\ p\ m}$
21 (1)	Unfused $\frac{1}{2}$	Unfused flat $\frac{1}{1}$	Abs.	Unfused $\frac{1}{1}$	Unfused $\frac{1}{1}$	8	Unfused concave $\frac{1}{1}$	Unfused sigmoid capped $\frac{1}{1}$	Unfused $\frac{8}{7}$	Unfused $\frac{1}{1}$		Metacarp well funnelized	$\frac{Ii\ i\ c\ p\ m}{I\ I\ c\ p\ m}$

Table 2. (Continued)

Age (Mos)	Proximal Epiphysis Proximal 1st Phalanx, hand	Proximal Epiphysis Proximal 3rd Phalanx, hand	Ossification of Seasamoid Process, metacarpo-phalangeal joint	Distal Epiphysis 3rd Metacarpal, hand	Proximal Epiphysis 1st Metacarpal	Number of Carpal bones	Proximal Epiphysis Proximal 3rd phalanx foot	Fusion, of Distal Radial Epiphysis	Proximal Radial Epiphysis	Ulnar Epiphysis		Remarks	Dental Formula
										Fusion of Distal Ulnar Epiphysis	Styloid Process Present		
24 (1)	Unfused $\frac{2}{3}$	Unfused $\frac{1}{1}$	Abs.	Unfused Square $\frac{1}{1}$	Unfused $\frac{1}{1}$	8	Unfused Cancave $\frac{1}{1}$	Unfused $\frac{1}{1}$	Unfused $\frac{8}{7}$	Unfused $\frac{1}{1}$	pres.		$\frac{I}{I}\ \frac{i}{I}\ \frac{c}{c}\ \frac{p}{p}\ \frac{m}{m}$

* Indicates number of observations
**Indicates epiphysis to diaphysis width ratio

Behavior and Ecology of Gibbons on Kled Kaeo Island

Principal Investigator:	Warren Y. Brockelman, CPT, MSC
Associate Investigators:	Dennis O. Johnsen, MAJ, VC Robert L. Hickman, MAJ, VC William L. Wooding, MAJ, VC
Assistant Investigators:	James P. Slowey, SFC, E-7 Ronald E. Marshall, SP5, E-5

OBJECTIVE: The primary objective of placing gibbons on Koh Kled Kaeo was to establish a self sustaining colony under semi-natural conditions and to study those aspects of behavior related to reproduction in a free ranging environment.

DESCRIPTION: Field observations are made periodically on the social behavior and ecology of the free ranging gibbons. Special attention is paid to factors that may influence reproductive activity among the animals.

PROGRESS: Twenty adolescent and adult gibbons were introduced onto the island in early 1967. At the present time seven animals remain. There are three heterosexual pairs and a single unpaired male. Deaths from undetermined causes have been responsible for the deaths of six of the original animals, two simply disappeared, and five have been returned to the laboratory because of antisocial behaviour or medical reasons. The heterosexual pairs are composed of female 6 and male 1, female 18 and male 11, and female 14 and male 19. Male 5 is the single unpaired male.

During the report period infants were born and the third female on the island was found to be in late gestation. Following the death of female 8, the birth of whose infant was reported in the last annual report, her baby was cared for by her mate, male 19. When male 19 subsequently remated, his new mate, female 14, assumed the task of caring for the infant. Although approximately two years passed before the birth of the first offspring, the fact that all the pairs remaining are reproducing suggests that some degree of normalcy and stability has been achieved on the island. However, the practical problems associated with maintaining these animals are indicated by their high mortality rate and the difficulty we have experienced in detecting and treating their illnesses and determining the causes of their deaths. The elusiveness of free ranging gibbons and the densely overgrown and rugged terrain on the island make it impossible to know the whereabouts of every animal each day and to capture all of them at will. There are probably ecological factors affecting the gibbons in this semi-natural environment that are not common to their normal habitat in

tall forests. For example there is a much greater opportunity for increased endoparasite exposure because of their greater contact with the ground. The question of whether or not a two to three year adaptive period is required for a colony such as this one to become established is one that can be answered by how well the remaining animals are able to survive.

Because of the departure of the principal investigator behavioral data for this report is not available. Such observations are therefore limited. The paired gibbons defend a general territory whose boundaries are fluid rather than fixed and which are frequently challenged by members of other pairs or single animals. It is not uncommon for several animals to follow persons from one end of the island to the other. The presence of four new infants offers a unique opportunity to observe their behavioral and social development under circumstances that are much more favorable than feral gibbons in their normal arboreal habitat.

Bovine Infertility Study

Principal Investigators:	Charles R. Egelston, MAJ, VC Dennis O. Johnsen, MAJ, VC William L. Wooding, MAJ, VC Robert L. Hickman, MAJ, VC
Assistant Investigators:	Ronald E. Marshall, SP5, E-5 Soontorn Aowchumpa, D.V.M.

PURPOSE: The development of four regional breeding centers for beef cattle as part of the Thai counterinsurgency program has indicated that bovine infertility is a widespread and costly problem in Thailand. Although infertility can result from such different factors as nutritional deficiencies, poor husbandry practices, and adverse climatic effects, the role that infectious disease might be playing in infertility was a problem that could be more easily studied with available laboratory resources. From disease surveys that have been made, it was apparent that both brucellosis and leptospirosis were widespread among the cattle population but their effect on the newly established herds and breeds in the regional breeding centers was unknown. Because infertility in cattle is militarily important as a probable significant economic problem in Thailand, a comprehensive study was begun to determine the extent of the problem and the effect infectious disease might have on it.

DESCRIPTION: The study was conducted at the western regional breeding center of the Thai Ministry of Defense's Mobile Development Unit near Saiyok in Kanchanaburi Province. The area consists of approximately 200 acres of newly developed irrigated pasture with excellent facilities

for handling and caring for the cattle that are kept there. In addition to having a resident veterinarian and several trained technicians who assist him in inseminating cows and performing other veterinary activities, there is a hospital barn, and small laboratory and pharmacy where specimens may be collected, cultured, and preserved for future study. Visits were made to the breeding center at monthly intervals by the principal investigators for the purpose of performing rectal examinations on each cow for pregnancy diagnosis, assessing the health of the reproductive tract, and determining if established pregnancies were progressing satisfactorily. Cows that failed to conceive after three services, or which aborted, received a complete genital examination which included culturing for bacteria. In addition, sera for both Brucella and Leptospirosis titer determinations were collected quarterly or at those times when they were indicated for diagnosis, such as following an abortion. In support of these activities a detailed system of keeping records of the breeding history of cows and the diagnostic tests performed was instituted. Efforts were made to facilitate the collection of diagnostic specimens at any time by stocking media and necessary instruments at the breeding center and writing and demonstrating to resident personnel those procedures to be followed when performing such a technique as a necropsy.

PROGRESS: The records accumulated in this study confirm the impression that breeding performance should be improved. The information presented in Table 1 partially explains one reason for the observed infertility. Examination of the teeth of the cows showed that many animals were present in the herd that were probably too young to be productive. Exclusive of this group there were only twenty two animals, or approximately 20%, that were infertile. However, the conception rate, as calculated by determining the number of pregnancies by the number of breeding as diagrammed in figure 2, shows that there is a great amount of rebreeding occurring and that conception is well below 50% and probably averages approximately 30%. Initially the investigators have felt that this low rate of conception was due to the failure of responsible personnel to properly detect heat and inseminate correctly. In a number of cases cows were bred several times following the time pregnancy diagnosis later showed that conception had taken place. Accordingly, responsible personnel received a month of supervised intensive training in the application of breeding principles and artificial insemination techniques in late 1969. However, in spite of this, no improvement occurred in the conception rate. Such performance often characterizes herds that are affected by cattle infertility diseases. In support of this possibility is the data presented in Table 3. The serum titers to various strains of Leptospira suggest an exposure to this disease in well over half the animals. Present also, but of apparently less significance, is the number of animals that have positive serum titers for

brucellosis. Unfortunately, the laboratory findings for follow-up serum samples in reactor animals could not be processed by the laboratory so it is not possible to determine what changes occurred in titers or how they were related to an individual animal's breeding performance. From the large number of pregnancy examinations that were performed during the course of the year, all pregnancies, once diagnosed, have preceeded to term without abortion. It was not possible to conduct an adequate laboratory follow-up of the two cows that died spontaneously during the year, but the causes of death were apparently not related to the herd problems that were experienced. Bacteriologic findings from cervical cultures of animals that have been repeatedly bred are not conclusive.

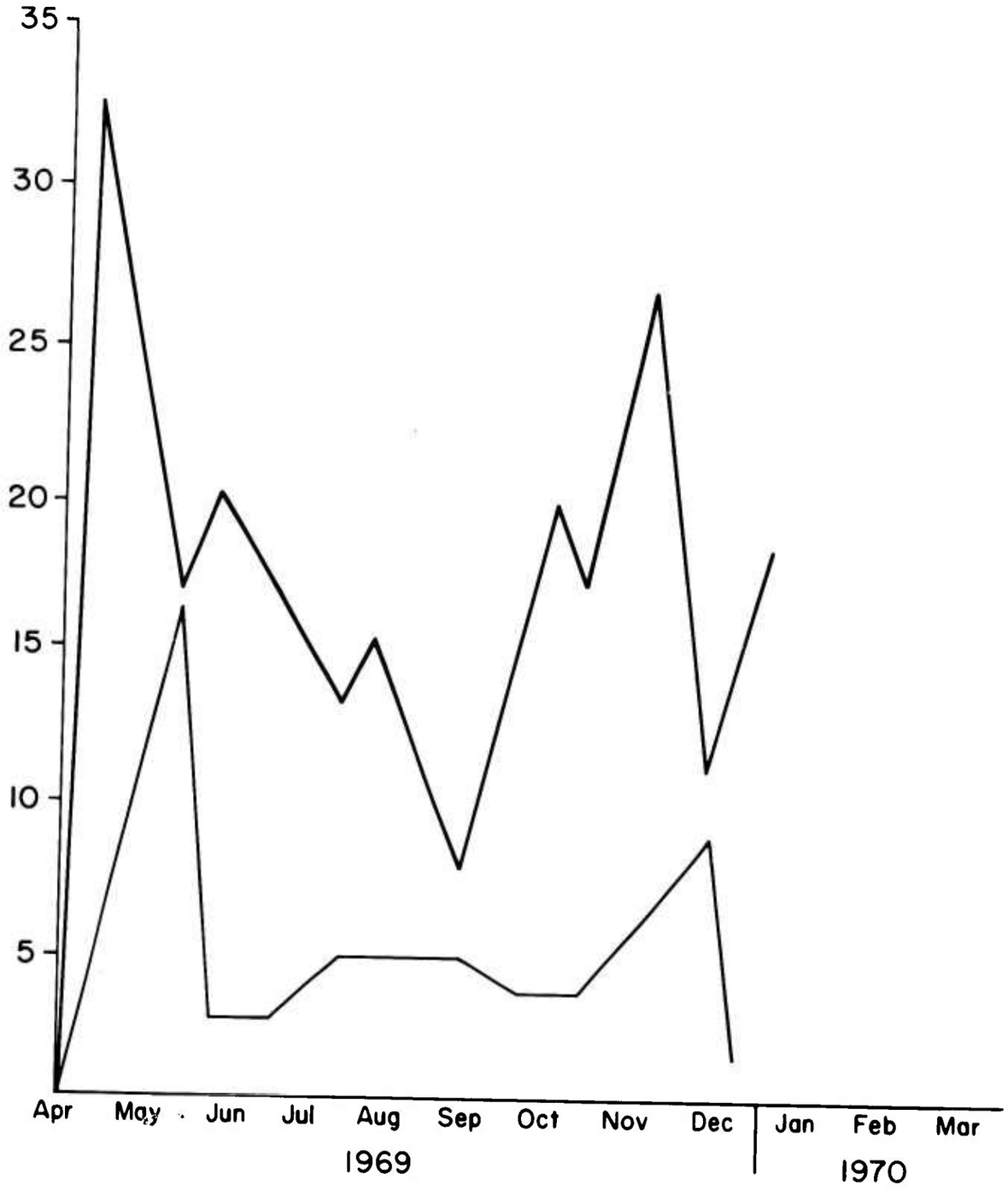
Table 1. Pregnancy Status of Study Cows

Pregnant cows	
Calved	30
Not yet calved	59
Non pregnant cows	
Bred	21
Anestrus	1
Adolescent (less than 2 yrs.)	29

Table 2. Leptospirosis And Brucellosis Titters

Number of Animals in Herd		140
Leptospirosis:	1/50 1/100 1/400 1/1600	11 33 26 14
Number of Positive Leptospirosis Titers/ Number Animals in Herd		84/140
Brucellosis: Positive titers:	1/25 1/50 1/100	26 9 1
Number of Positive Brucellosis Titers/ Number Animals in Herd		36/140

FIG. 2 BOVINE INFERTILITY PROJECT



NUMBER OF NEW PREGNANCIES DIAGNOSED PER MONTH
NUMBER OF ANIMALS BRED PER MONTH

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E.ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 047, Metabolic diseases of man and animals

Literature Cited.

References:

1. Olson, L., Bourgeois, C.H., Keschamras, N., Harikul, S., Grossman, R., and Smith, T. Udorn Encephalopathy: cerebral edema and fatty degeneration of the viscera in Thai children. Am. J. Dis. Child. In Press.

2. Evans, H., Bourgeois, C.H., Comer, D.S., and Keschamras, N. Neuropsthology and encephalopathy and fatty degeneration of the viscera. Arch. Path. In Press.

3. Morales, A.R., Bourgeois, C.H., and Chulacharit, E. Encephalopathy and fatty degeneration of the viscera: pathology of the cardiac conduction system and myocardium. Am. J. Cardiology. In Press.

4. Shank, R.C., Johnsen, D.O., Tanticharoenyos, P., Wooding W., and Bourgeois, C.H. Acute toxicity of aflatoxin B_1 in the monkey. Nature. In Press.

5. Clin. Chem. Acta. October 1969, p. 185.

6. Siiprija, V.; and Evans, H.: The Kidney in human leptospirosis. Am. J. Med. (in press, 1970).

Publications:

1. Bourgeois, C. H., Keschamras, N., Comer, D.S., Harikul, S., Olson, L., Smith, T., and Beck, M. Udorn Encephalopathy: fatal cerebral edema and fatty degeneration of the viscera in Thai children. J. Med. Ass. Thailand 52:553-565, 1969.

2. Morales, A.R., Bourgeois, C.H., Trapukdi, S., and Chulacharit, E. Encephalopathy and fatty degeneration of the viscera. An electron microscopic study. Amer. J. Clin. Path. 52:755, 1969. (Abstr.)

3. Sitprija, V.; and Evans, H.: Renal Pathophysiologic Correlation in Human Leptospirosis. Abstract, IVth International Congress of Nephrology, Stockholm, June 22-27, 1969. Abstracts p. 186.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6469	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	048
b. CONTRIBUTING				
XXXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Rickettsial Diseases of Man and Animals (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 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

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	0.5	30
FISCAL YEAR CURRENT 71	0.5	30

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO
ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Grossman, MAJ R. A.
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Jatinandana, RADM S., RTN
NAME: Sankasuwan, MAJ V., RTA DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Rickettsial Diseases; (U) Scrub Typhus; (U) Murine Typhus; (U) Leptothrombidium arenicola

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 ecology of rickettsial diseases of military importance in Thailand and to determine the risk of acquired rickettsial diseases in Thai soldiers serving in Vietnam.

24. (U) Disease occurrence in Thailand is determined by case detection and laboratory methods. The disciplines of clinical medicine, medical entomology, epidemiology and rickettsiology are used to identify the various components of the ecosystem (e. g. vectors, hosts, reservoirs). Serologic methods form the basis of diagnosis in Thai soldiers serving in Vietnam.

25. (U) 69 07 - 70 06 Serologic studies of Thai soldiers returning after one year in Vietnam indicates no significant experience with scrub typhus. For technical reports see SEATO Annual Progress Report, 1 Apr 69 - 31 Mar 70.

*Available to contractors upon originator's approval

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

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 048, Rickettsial diseases of man and animals

Investigators.
Principal: Vichai Sankasuwan, Major MC, RTA*
Premthavi Bodhidatta, Flight Lieutenant MC, RTAF**

Associate: Richard A. Grossman, Major, MC
Bospan Prakoppanichakij, B.Sc. [Pharm]
Cheodchai Chuenchitra, B.Sc. [Med Tech]
Krasaere Nabnean, Second Lieutenant, RTA***

Survey of Rickettsial Diseases in Thailand

Principal Investigators: Vichai Sankasuwan, Major MC, RTA*
Premthavi Bodhidatta, Flight Lieutenant MC, RTAF**

Associate Investigator: Richard A. Grossman, Major, MC

Assistant Investigators: Bospan Prakoppanichakij, B.Sc. [Pharm]
Cheodchai Chuenchitra, B.Sc. [Med Tech]
Krasaere Nabnean, Second Lieutenant, RTA***

OBJECTIVE: To determine the distribution of rickettsial diseases in Thailand; identify arthropod vectors and mammal reservoirs and alternate hosts, and serve as required as consultative laboratory, and to determine the types and frequency of rickettsial infections of Thai soldiers serving in Vietnam.

DESCRIPTION: Search is being continued for evidence of infection of man and other animals by rickettsiae of scrub typhus, murine typhus, Q-fever and the spotted fever group in Thailand. Methods used are small animal trapping; collection, identification and pooling of their ectoparasites; inoculation of tissue specimens and ectoparasite pools in white mice or guinea pigs; and serologic testing of human and animal sera.

---

* Chief Investigator [Thai Component]
** Investigator, Division of Research [Thai Component]
*** Medical Research Technologist [Thai Component]

A new project was initiated this period to determine the frequency of rickettsial [and other] infections occurring in Royal Thai Army troops serving a one year tour in Vietnam. Blood is collected just before departing and on the day of return from Vietnam. Each soldier also fills out a questionnaire requesting information on past disease history as well as history of illness while in Vietnam.

PROGRESS: A field trip was made to Mae Sariang district, Mae Hongsorn province, in April 1969. Fifty [50] rodents were trapped - 9 Rattus rattus from which 25 fleas and 27 mites were collected, 40 R. exulans infested with 51 fleas, and one Menetes berdmorei. The 8 pools of exulans and the berdmorei were negative while 1 of 2 rattus pools was positive for Rickettsia tsutsugamushi. The 8 pools of fleas [Xenopsylla cheopis] were negative for rickettsial agents. The human serologic data from Mae Sariang collected in June 1969 are reported elsewhere in this volume.

In September 1969 a field trip was made to Kaeng Koi district, Saraburi province. From the 15 R. rattus trapped 1,530 chiggers, 73 mites and 34 fleas were collected. Other animals trapped were 5 R. exulans, 1 M. berdmorei and 1 Bandicota indica. The 6 pools made from these animals were negative for rickettsiae. Trips to Phuket and Satoon, on the Southern Thai peninsula, were made during November 1969 searching for the beach chigger, Leptothrombidium arenicola, using the black plate technique and animal trapping. None of these chiggers were recovered. Two strains of R. tsutsugamushi were isolated from R. rattus trapped at Phuket.

Over 1,000 Thai soldiers were bled before leaving for Vietnam and 465 second specimens were so far collected on returnees. Complement fixation tests showed positive [≥4-fold] titer rises in 2 to Q-fever and in 6 to murine typhus antigens. Immunofluorescent testing for scrub typhus, as well as analysis of the questionnaires, are pending.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 048, Rickettsial diseases of man and animals

Literature Cited.

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6470	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	049
b. CONTRIBUTING				
[redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Psychiatry and Behavioral Studies (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology; 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	2	118
FISCAL YEAR CURRENT 71	2	118

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Altstatt, LTC L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Firestone, MAJ M. H.

NAME: Russ, MAJ J. J. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Psychiatry; (U) Human Behavior; (U) Stress; (U) Drug Abuse; (U) Performance

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To clarify certain aspects of human behavior that are of particular military importance, namely, methods of minimizing duty time lost to psychiatric problems, sources of difficulty in transcultural endeavors and the management of drug abuse.

24. (U) All projects employ American trained psychiatrists with Thai trained advisers. Information is gained by systematic observation of human behavior and mental-status testing adapted to circumvent cultural differences.

25. (U) 69 07 - 70 06 A study of American adviser-Thai counterpart relationships has been completed. A comparison of cultural influence of psychiatric diagnosis has been completed. A preliminary survey of service facilities has indicated that the problem of drug abuse offers a fertile field for investigation. For technical reports see SEATO Annual Progress Report, 1 Apr 69 - 31 Mar 70.

* Available to contractors upon originator's approval

DD FORM 1 MAR 68 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 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S.E.ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 049, Psychiatry and behavioral studies

Investigators.
Principal: Harry C. Holloway, LTC, MC
Associate: William H. Biggers, M.D.; Curtis H. Bourgeois, Jr., MAJ, MC; Sanchai Bunlungpoh; Vivat Chavengchaiyong; Robert W. Dewey, SFC; Carter L. Diggs, MAJ, MC; Chiraphun Duangmani, M.D.; Marvin H. Firestone, M.D., MAJ, MC; Jimmie M. George, SSG, USAMEDS; Douglas J. Gould, Ph.D.; Richard A. Grossman, MAJ, MC; Harry C. Holloway, LTC, MC; Tien Jaiboonma; Vacharee Jan-Aiem; Winaisuk Kattipattanapongse; Supoch Khwanmitra, MAJ GEN, RTAH; Peter Kunstadter, Ph.D.*; Apakorn Kuntatun; Surarak Laosuwan; George S. Manning, CPT, MSC; Howard E. Noyes, Ph.D.; Utaiwan Pongsukree; Boonarb Punpanya, PHN; Kriangsak Rittaporn; Jonathan J. Russ, M.D., MAJ, MC; Phon Sangsingkeo, M.D.; Pricha Singharaj, M.D.; Chira Sitasuwan, M.D.; Cherdchalong Sivasomboon, R.N.; Thomas J. Smith, LTC, MC; Siriwat Sothornwit; Umneuy Sriroongroj; Chantana Sukavajana, M.D.; Aporn Surintramont, B.A.; Charas Suwanwella, M.D.; Gerald S. Suzuki, SSG, USAMEDS; Sukree Tumrongrachaniti, R.N.; Chinda Witayarut, P.H.N.; William L. Wooding, MAJ, VC

---

* Dept of Anthropology, University of Washington, Seattle

Prevalence Survey of Disease in Mae Sariang

Principal Investigators:
Richard A. Grossman, MAJ, MC
Harry C. Holloway, LTC, MC
Peter Kunstadter, Ph.D.*
Phon Sangsingkeo, M.D.
Pricha Singharaj, M.D.

Associate Investigators:
Curtis H. Bourgeois, Jr., MAJ, MC
Carter L. Diggs, MAJ, MC
Chiraphun Duangmani, M.D.
Douglas J. Gould, Ph.D.
Howard E. Noyes, Ph.D.
Thomas J. Smith, LTC, MC
William L. Wooding, MAJ, VC
Robert W. Dewey, SFC

OBJECTIVE: To make a preliminary survey of the point prevalence of diseases in an ethnically mixed neighborhood in Amphur Mae Sariang, Mae Hongsorn province.

DESCRIPTION: This report presents the results of biomedical data, collected in June and July, 1969, as part of a larger multidisciplinary survey attempting to assess the functional relationship between migration, ethnic membership and ecologic experience and these biomedical variables. A total census was performed [by P.K.] beforehand of one area, Ban Phae Chom Chaeng, just east of Mae Sariang town [about 500 kilometers northwest of Bangkok]. A cluster random sample [RS] of 50 of the 188 households present was selected for study. In addition all 14 households designated as being occupied by New Lua were similarly selected [but not included in random sample analyses]. These were ethnically Lua who were of special interest in that they had migrated into Mae Sariang from the surrounding hills within the previous year. Three of these New Lua households were drawn in the RS. The major ethnic groups identified were Lua, North Thai and Karen. Each person received an identifying number and all data collected were transferred to punch cards for analysis. The following specimens were collected from the sampled population, on a voluntary basis - peripheral and venous blood, urine, and stool. Also performed were rectal and nasopharyngeal swabbing, chest X-ray, and measurement of height, weight and head size [up to age 6].

In addition, animal feces and blood were collected, rats were trapped, mosquito fauna was studied, and water and soil samples were analyzed. These non-human samplings were performed in the same area and during

---

* Department of Anthropology, University of Washington, Seattle.

the same general time period as the human survey. A mass of behavioral and anthropologic data was also concurrently accumulated and there are plans to appropriately integrate such data with those presented here.

RESULTS: The main advantage of having had a total and accurate census on hand before drawing the sample was the opportunity thereby afforded to check the representativeness of the random sample. If the sample is truly representative of the target population, then obviously more credence is lent to the various prevalence determinations made on the sample collections as being precise estimates of population prevalences. Table 1 displays various characteristics of the target population and the cluster random sample, which includes 26.6% of the households present. The 95% confidence intervals bracket the true population value in each instance - total persons, number and proportion of males, and number and proportion of children. Adequate representativeness is further shown in the age-sex distributions of the population and sample [Tables II, III]. Figure 1 shows the age distribution for the target population plotted on normal probability graph paper. The fairly close straight-line relationship [after age 10] indicates that the cumulative age distribution is approximately normally distributed and is a major reason why such a relatively small sample of households [and not of people] is so highly representative over these ages.

The proportion of males is about 50% in the sample population. It is of interest to know whether this is true for larger as well as smaller households. Table IV shows that the proportion of males is clearly independent of household size [$p > 0.50$].

Participation in the survey was generally very good and over 95% of the sample population was interviewed, had height and weight measured and gave a urine specimen. Although over 90% came to the examining area during the 3 days [4-6 June] of concentrated testing, participation in giving specimens ranged from 35% for stool to 70% for peripheral blood specimens [Table V]. Over 80% of persons over age 3 received chest X-rays. Analysis of the missing and present distributions for hemoglobins failed to reveal any consistent pattern to seriously bias the data, although hidden errors may surely be masked and some caution must be used before extrapolating these data.

Tables VI and VII give the mean heights and weights for the random sample as well as for the 2 major ethnic groups present which account for 86.6% of the total persons in the RS. No marked differences are noted in these sex and age distributions, between the RS and ethnic group populations. The average adult in this area is approximately 5' 2" tall and weighs about 105 pounds. All the urine specimens were negative for glucose and phenylketones while 2% had significant proteinuria. Four persons [2.1%] were found to have active tuberculosis

on chest X-ray. Two were male, and the ages were 35,50,55 and 60 years. One sputum was positive for acid-fast bacilli. The X-rays were not interpreted for presence of other abnormalities. Malaria slides were made on all finger-stick collections and all were negative. A somewhat surprising result was that there were no positive VDRL serologic tests of the 182 performed [150 in RS]. This was checked by repeating.

Bacteriological evaluation of the water wells in the study area demonstrated high coliform counts, a finding consistent with the fact that all wells are uncovered and that privies are reportedly used by less than 50% of the households interviewed. During the 3 days in June, 21 persons complained of diarrhea, and of the 168 persons [64.4% of the RS] who gave swab or cup fecal samples, 8 had Shigella flexneri type 2, 2 had Sh. sonnei form 2, and 2 had Sh. dysenteriae. Tetracycline therapy [only Sh. sonnei was resistant] was made available to these and other symptomatic people thereafter.

In addition, 4 persons had Salmonella welteverden and 5 persons had enteropathogenic Escherichia coli [3 different types] isolated from their stools. There was no clustering of these isolates by age, sex or household. Soil, water and human sera specimens were tested for prevalence of melioidosis. None of 9 soil samples were positive; 3 out of 23 [13%] water samples were positive. Only 6 persons in the RS out of 168 sera tested [3.6%] had a positive melioidosis titer and 3 of these were from the same household [ages 14, 18 and 40]. Titers were 1:80, 1:40 and 1:16 respectively. Table VIII shows that 4/102 Old Lua sera were positive while no New Lua and North Thai sera were positive.

Hemoglobins were determined on 183 RS persons [70.1%]. The distributions for males age 0-14 and 15+are very similar [and approximately normal] and have similar means around 12 gm% [Figure 2]. Using WHO criteria about 40% of the male and 35% of the female populations of this study area are anemic [Table IX]. Closer inspection of the table reveals relatively less frequent anemia in young females but the numbers tested are small. There are no significant differences between the mean hemoglobins of the North Thai and Lua populations [Table VIII]. These 3 distributions should probably be age-and sex-adjusted before stating the foregoing with good assurance. It should be mentioned that evidence of gross malnutrition and deficiency diseases was not observed in this area.

The presence of certain stool parasites is often incriminated as contributing to levels of moderate anemia such as seen in this area. Of 91 RS stool exams only 14 [15.4%] were negative while 15 had one, 25 had 2, 24 had 3, and 13 had 4 or more parasites present. By far the most prevalent parasites were nematode ova. Similar prevalences were found for hookworm [47.3%], Ascaris [52.7%] and Trichuris [44.1%] eggs.

These 3 eggs were likewise similarly distributed by sex and age. However, they were differently distributed by ethnic group [Table VIII] where the New Lua group apparently has a much higher prevalence than the average. Three people [3.3%] had Taenia species eggs in their stool and adult worms were purged from 2 persons, one of which was identified as T. saginata. In addition Strongyloides stercoralis larvae, and Entamoeba coli and Giardia lamblia cysts were recovered. No E. histolytica were found. Considering the drawbacks in collection and handling methods of the stools, and that only one specimen was examined, it is reasonable to presume that these prevalences represent the minimum. It is likewise not possible to estimate the worm load of these parasites.

It is still of interest to attempt to correlate the presence of a known red blood cell ingestor, hookworm, with hemoglobin levels and presence of eosiniphilia. Significant eosinophilia [15% of differential count] was present in 30% of the 192 RS specimens examined. Figure 3 shows that the age prevalence of eosiniphilia is fairly constant except for a high prevalence early in life which would be expected as the response to the initial acquisitions of intestinal parasites and others. In contrast, the hookworm age prevalence curve fluctuates widely, although the large dip between ages 20-29 may reflect the small numbers examined [non-response rate was highest for this group]. Table X shows the mean hemoglobins by age for those persons positive and negative for hookworm or Ascaris. Although the numbers are too small for statistical evaluation and the male/female ratios are not necessarily similar, there does seem to be a trend [especially in the younger age groups] for lower hemoglobin values in the presence of hookworm infestation, with much smaller differences noted with the presence of Ascaris.

The remaining serological tests performed revealed only minor recent experience with most of the antigens tested. Overall prevalences in the RS were: trichinosis [SAFA test] 7.9%, scrub typhus [IF test] 1.6%, murine typhus [CF test] 0.8%, and Q-fever [CF test] 0.0%. Scrub typhus exposure probably occurred in upland areas since, of 9 positive sera, 8 were in New Lua persons. Of particular interest was the experience with group B arboviruses. HI antibody titers $\geq$ 1:40 to dengue and Japanese encephalitis were detected in 14.0 and 14.5% respectively [179 sera tested]. There was no sex difference and age prevalence revealed no consistent pattern for dengue, but JE antibody was absent until age 10 and then generally rose to a high of 58.3% for ages 50-59. Since the 2 viruses freely cross-react in the HI test, further inferences cannot be made other than being able to confidently state that no recent major exposure had occurred to these 2 agents in the study area. Two years earlier similar testing of sera of an upland village of New Lua revealed an absence of dengue antibody. The New Lua in the present study had migrated mostly from this same upland area and it is noteworthy that only one of 46 such

sera tested reacted to dengue and JE whereas about 16% of the Old Lua and North Thai were positive [Table VIII]. Only one serum [0.6%] was positive for Chikungunya, a 23 year-old male of Burmese and North Thai extraction.

Animal collections were made in the study area during this time period, in nonrandom fashion. Three trap-nights produced 15 rats [5 male], 3 Rattus rattus, and 12 R. exulans. Only one was flea-infested and none of the 9 sera tested was positive for plague HA antibodies. Twenty [20] dogs were investigated. One dog had a 26% eosinophil count and one had a 1:40 melioidosis titer. Only 2 out of 19 dogs had negative stools; 8 had hookworm ova, 4 had Ascaris ova, 2 had Spirocerca ova and one each had Dirofilaria mansoni and Trichuris ova. Twelve [12] cows were bled; all were negative for melioidosis, dengue 1 and Chikungunya but 2 had 1:1280 and 6 others had 1:40 titers to JE. Of the 24 cow stools examined 6 were negative and 16 of the remaining 18 had Fasciola ova exclusively. Six [6] out of 15 water buffalo stools were negative and 7 out of the remaining 9 had Fasciola ova.

Twelve [12] pigs were bled; one had a 1:40 melioidosis titer and 9 had significant JE titers [some were 1:1280 and 1:2560]. Dengue 1 reactivity paralleled the JE, but to a high titer of only 1:80. Only 5 of 54 pig stools were negative. Parasites present were hookworm, Ascaris, Trichuris, Spirocerca and Fasciolopsis buski ova, Strongyloides larvae, E. coli cysts, Balantidium coli and Coccidia.

Adult mosquito and larval collections were made in the study area [Ban Phae] at the start of the rainy season [May], during the peak of the rainy season [end of July], and 9 months later [April, 1970] during the hot, dry season. Similar collections were made at these times in an area just north of town [Ban Pong] as well as in Mae Sariang town proper. In May, 1969, both Culex gelidus and C. tritaeniorhynchus, proven vectors of Japanese encephalitis, were collected biting man. The most common domestic mosquito was Culex quinquefasciatus, which was often found breeding in domestic water containers inside houses.

Inspection was made of artificial containers in 30-60 houses in both Ban Phae and Ban Pong each survey. No Aedes aegypti larvae were found. In contrast, about 5% of the houses in the town center had A. aegypti larvae in the 2 dry-weather periods and 59/100 houses [59%] were positive in the rainy season collection. Four houses were also positive for A. albopictus larvae. At that time [28-30 July] houses in town had an average of 9.9 water storage containers while there were 7.6 per house in Ban Pong and only 4.2 per house in Ban Phae. The water containers used in Ban Phae are generally smaller [5-10 gallons] than those used in the town [30-60 gallons], and the people in Ban Phae usually refill the jars from nearby wells daily while the larger jars in town are usually used to store rain water.

The availability of fewer breeding sites in Ban Pong and Ban Phae, plus the fact that aegypti larvae are unlikely to reach maturity in jars that are emptied every 24-72 hours may be part of the explanation for their absence in these 2 areas. This finding may also explain the relatively low prevalence of dengue antibodies in the Ban Phae sera although these peoplefrequently visit the nearby town center and marketplace in the daytime. Although previous information is lacking, transmission of dengue virus was documented during July [when the aegypti larvae index had risen dramatically in the town] by serologic diagnosis of several town residents admitted to the local Mission Hospital with the clinical diagnosis of hemorrhagic fever. No patients from Ban Phae were similarly encountered.

Table 1. Population and Sample Characteristics and Sample Representativeness.

Variable of Interest	Sample Value	Population Value	Population Total Estimated from Sample Value	95% Confidence Interval of Population Value	Standard Error
Number of people	261	908	981	879 -1,083	50.81
Number of households [clusters]*	50	188	-	-	-
Average number of people per household	5.2	4.8	-	-	-
Number of males	130	439	489	420 -558	34.50
Proportion of males [%]	49.8	48.3	-	44.7- 54.9	.0257
Number less than age 15 years	125	410	470	395 -545	37.41
Proportion less than age 15 years [%]	47.9	45.2	-	44.0- 51.8	.0193

* Sampling fraction [f] = 50/188 = 26.6% of households.

Table II. Age-Sex Distribution of Target Population and Comparison with Random Sample [RS] Cumulative* Percentage Distribution.

Age [Yrs]	MALE				FEMALE				TOTAL			
	No.	%	Cumul. %	Cumul. % in RS	No.	%	Cumul. %	Cumul. % in RS	No.	%	Cumul. %	Cumul. % in RS
0-	79	18.0	-	-	77	16.4	-	-	156	17.2	-	-
5-	66	15.0	18.0	16.9	70	15.0	16.4	16.0	136	15.0	17.2	16.5
10-	57	13.0	33.0	31.5	65	13.9	31.4	30.5	122	13.4	32.2	31.0
15-	40	9.1	46.0	46.2	33	7.0	45.3	49.6	73	8.0	45.6	47.9
20-	26	5.9	55.1	57.7	32	6.8	52.3	57.2	58	6.4	53.6	57.5
25-	25	5.7	61.0	62.3	37	7.9	59.1	61.1	62	6.8	60.0	61.7
30-	65	14.8	66.7	66.9	65	13.9	67.0	69.5	130	14.3	66.8	68.2
40-	37	8.4	81.5	81.6	41	8.7	80.9	82.4	78	8.6	81.1	82.0
50-	27	6.1	89.9	90.0	28	6.0	89.6	93.1	55	6.0	89.7	91.6
60-	17	3.9	96.0	96.9	21	4.5	95.6	96.2	38	4.2	95.7	96.6
Total	439	99.9	99.9	100.0	469	100.1	100.1	100.0	908	99.9	99.9	100.0

* Less than [<] cumulative distribution.

Table III. Comparison of Target Population and Random Sample [RS] Age Distributions.

Age [Years]	Number		%		Cumulative %	
	Population	RS	Population	RS	Population	RS
0-	292	81	32.2	31.0	—	—
10-	195	69	21.5	26.4	32.2	31.0
20-	120	28	13.2	10.7	53.7	57.4
30-	130	36	14.3	13.8	66.9	68.1
40-	78	25	8.6	9.6	81.2	81.9
50-	55	13	6.1	5.0	89.8	91.5
60-	26	6	2.9	2.3	95.9	96.5
70-	12	3	1.3	1.2	98.8	98.8
Total	908	261	100.1	100.1	100.1	100.0

	Median Age [Yrs.]	
	Population	RS
Male	17.7	17.4
Female	18.9	17.3
Total	18.3	17.2

Table IV. Proportion of males in larger vs. smaller households.

Household Size	Proportion of Males*		Total
	> 50%	< 50%	
2-5	11	14	25
> 5	7	5	12
Total	18	19	37

*Excluding the 13 households containing 50% males.
X^2 (1) = 0.22 (with Yates correction).

Table V. Participation by the sample population for specified test procedures, by age.

Age (Years)	No. in RS	Height, Weight		Blood, finger		Blood, venous		Stool		Chest X-ray	
		No.	%	No.	%	No.	%	No.	%	No.	%
0-	43	43	100.0	24	55.8	19	44.2	15	34.9	21	48.8
5-	38	38	100.0	24	63.2	24	63.2	16	42.1	33	86.8
10-	44	42	95.4	32	76.2	32	76.2	14	31.8	35	79.5
15-	25	22	88.5	16	64.0	16	64.0	8	32.0	16	64.0
20-	11	10	90.9	10	90.9	10	90.9	3	27.3	10	90.9
25-	17	17	100	14	82.4	14	82.4	4	23.5	14	82.4
30-	36	32	88.9	26	72.2	26	72.2	12	33.3	26	72.2
40-	25	23	92.0	20	80.0	21	84.0	9	36.0	20	80
50-	13	13	100	11	84.6	12	92.3	8	61.5	11	84.6
60+	9	9	100	6	66.7	5	55.6	4	44.4	7	77.8
Total	261	249	95.4	183	70.1	179	68.6	93	55.6	193	73.9

Table VI. Average heights (cm) for random sample (RS) and ethnic categories, by age and sex.

Age (Years)	RS		North Thai		Old Lua		New Lua	
	M n=118	F n=131	M m=34	F n=31	M n=58	F n=79	M n=34	F n=41
0-4	83.9	79.2	82.2	76.7	89.4	80.9	78.0	76.1
5-9	106.1	106.3	106.0	107.9	100.5	107.1	100.5	110.6
10-14	134.8	129.8	135.7	132.5	134.2	128.4	*144.7	133.0
15-19	154.7	147.4	*153.3	*146.9	154.3	146.8	*136.5	136.2
20+	158.1	149.1	161.4	150.8	157.3	148.5	157.9	145.4

*Includes less than 4 persons.

Table VII. Average weights (kg) for random sample (RS) and ethnic categories, by age and sex.

Age (Years)	RS		North Thai		Old Lua		New Lua	
	M n=117	F n=131	M n=34	F n=31	M n=58	F n=79	M n=34	F n=41
0-4	11.4	10.0	10.1	9.1	12.8	10.7	10.5	9.1
5-9	16.8	16.8	16.3	16.2*	17.0	17.4	16.4	18.8
10-14	25.2	27.7	36.8	29.2	28.6	26.9	*39.0	30.3
15-19	48.5	44.4	44.7*	44.5*	49.4	44.3	*33.4	40.5
20+	48.9	45.0	42.9	45.4	48.0	45.3	48.9	45.6

*Includes less than 4 persons.

Table VIII. Prevalence of various test results in the Lua and North Thai populations*

Ethnic Group	Arbovirus Serology			Melioidosis		Eosinophils		Hookworm Ova		Hemoglobin (Gm%)			
										Male		Female	
	No. Tested	%HI≥1:40		No. Tested	%HA ≥1:40	No. Tested	% ≥15%	No. Tested	% (+)	No. Tested	Mean	No. Tested	Mean
		Dengue	JE										
rth Thai	40	15.0	17.5	40	0.0	43	27.9	18	27.8	17	12.4	22	12.3
ɔld Lua	113	15.9	15.9	102	3.9	113	28.3	58	55.2	52	12.1	61	12.3
New Lua**	46	2.2	2.2	40	0.0	53	25.4	29	72.2	26	11.8	20	11.4
TOTAL	199	12.6	13.1	182	2.2	209	28.2	105	60.0	95	12.1	103	12.1

* Data not adjusted for differences present between the age and sex distributions of the 3 groups.

**New Lua represents Lua having moved into study area ≤1 year. Includes all 14 New Lua households, 3 of which were drawn in the RS.

Table IX. Frequency of anemia* in random sample, Mae Sariang, June 1969.

Age (Years)	Critical Hemoglobin Value* (Gm%)	Hemoglobins less than critical values					
		Male		Female		Total	
		No.	%	No.	%	No.	%
0-4	10.8	3/11	27.3	0/13	0.0	3/24	12.5
5-9	11.5	3/11	27.3	2/13	15.4	5/24	20.8
10-14	12.5	4/14	28.6	8/18	44.4	12/32	37.5
Adults	Male14.0 Female12.0	25/50	50.0	24/53	45.3	49/103	46.7
Total	-	35/86	40.7	34/97	35.1	69/183	37.7

*WHO criteria, in WHO Technical Report Series 182, Geneva, 1959.

Table X. Relation of stool parasites to mean hemoglobin values in RS.

Age* (Years)		Hookworm			Ascaris		
		No. (Males)	Mean Hgb Gm%	Mean Hgb Diff.	No. (Males)	Mean Hgb Gm%	Mean Hgb Diff.
0-9	Pos	6 (3)	11.6	1.3	15 (7)	12.1	0.5
	Neg	15 (7)	12.9		6 (3)	12.6	
10-14	Pos	5 (3)	10.4	1.7	9 (6)	12.1	0.8
	Neg	6 (5)	12.1		2 (2)	12.9	
15-44	Pos	18 (9)	12.4	0.6	15 (6)	12.6	0.2
	Neg	15 (3)	13.0		18 (5)	12.8	
45+	Pos	9 (3)	11.9	0.3	5 (2)	10.6	2.3
	Neg	4 (2)	12.2		8 (3)	12.9	
Total		78 (35)			78 (34)		

* Not adjusted for differences in distribution of males and females in both the positive and negative groups.

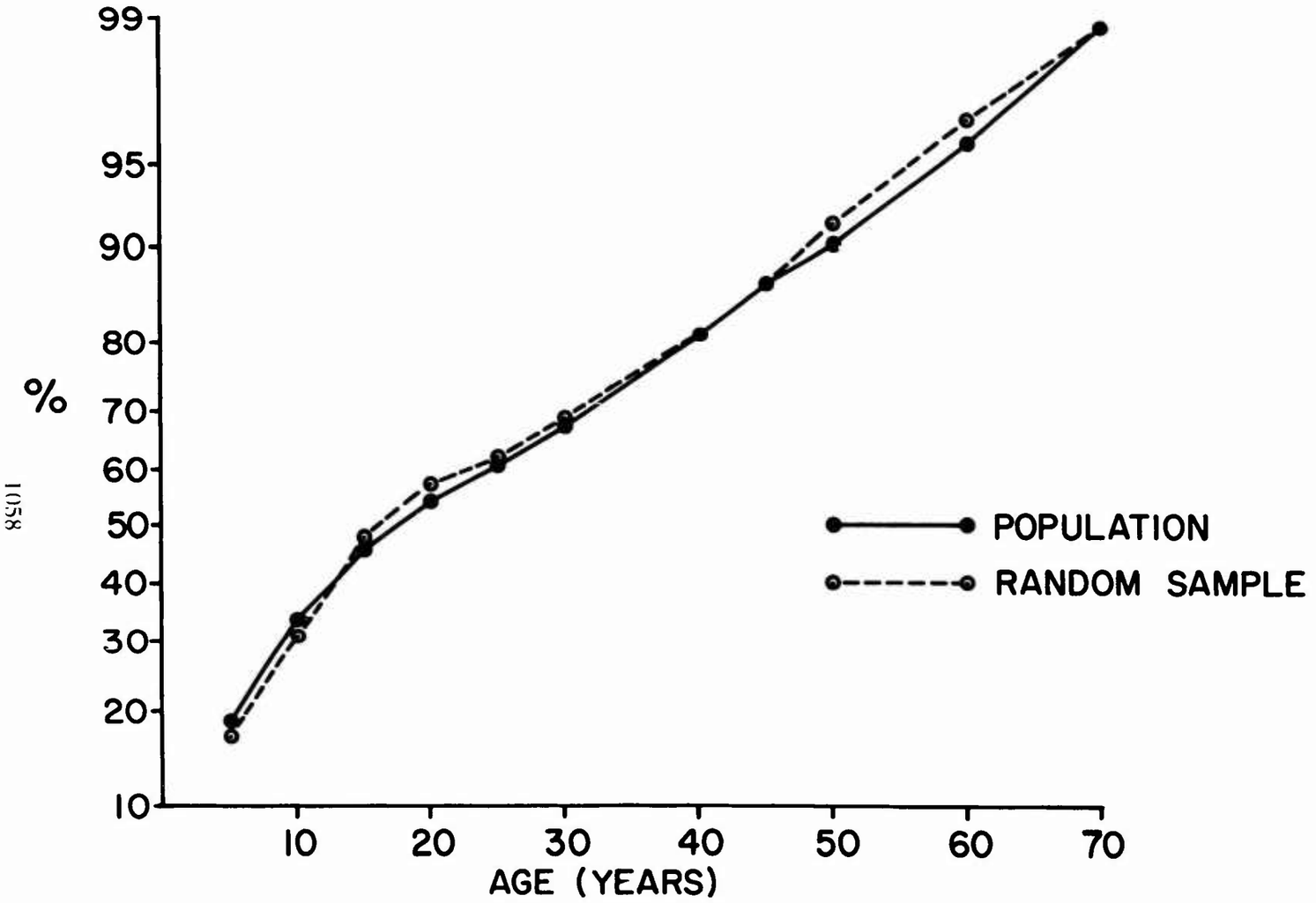


FIGURE 1. POPULATION AND SAMPLE CUMULATIVE PERCENTAGE AGE DISTRIBUTION, BAN PHAE CHOM CHAENG, MAE SARIANG, JUNE, 1969.

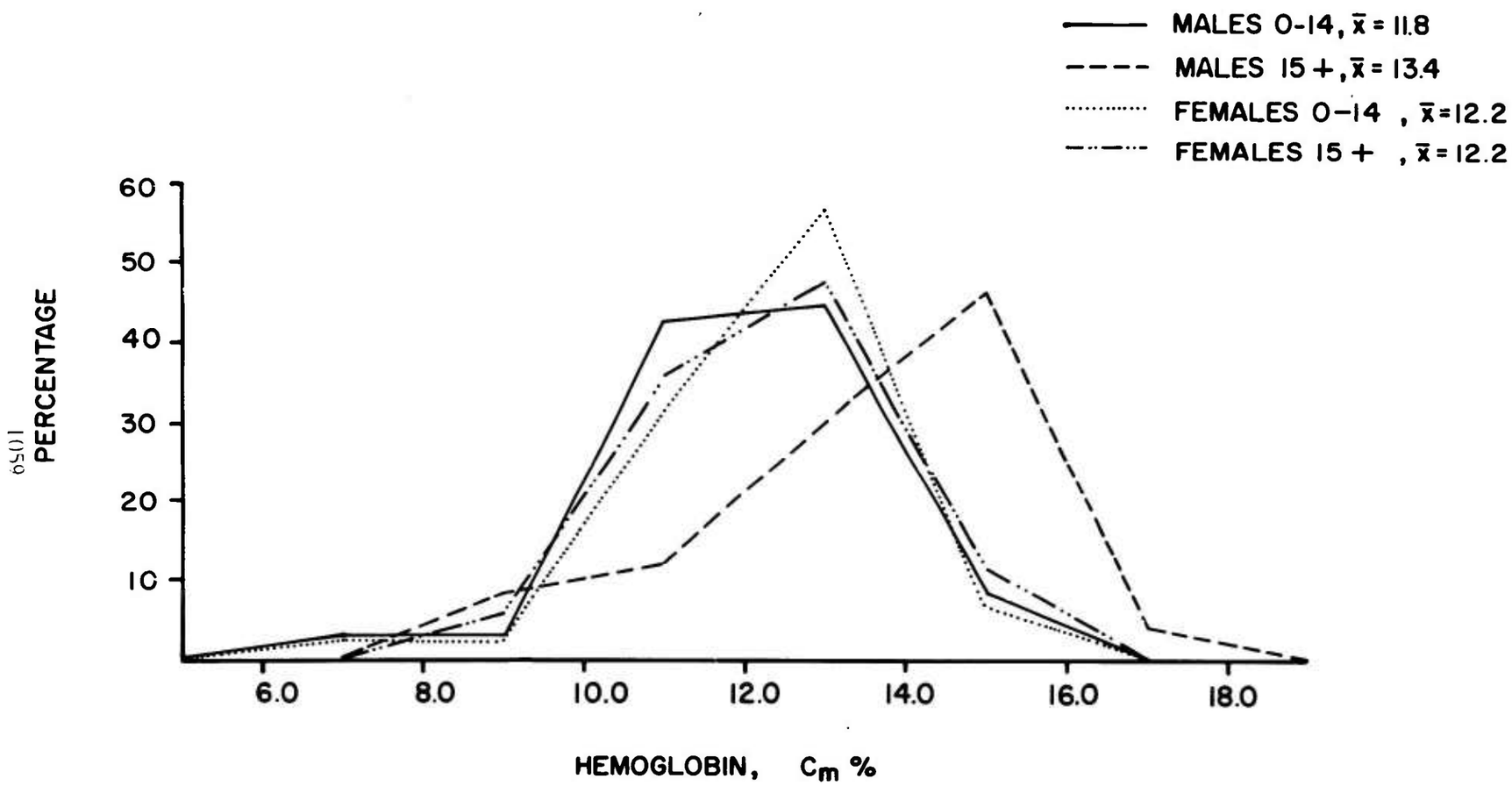


FIGURE 2. FREQUENCY OF HEMOGLOBIN VALUES

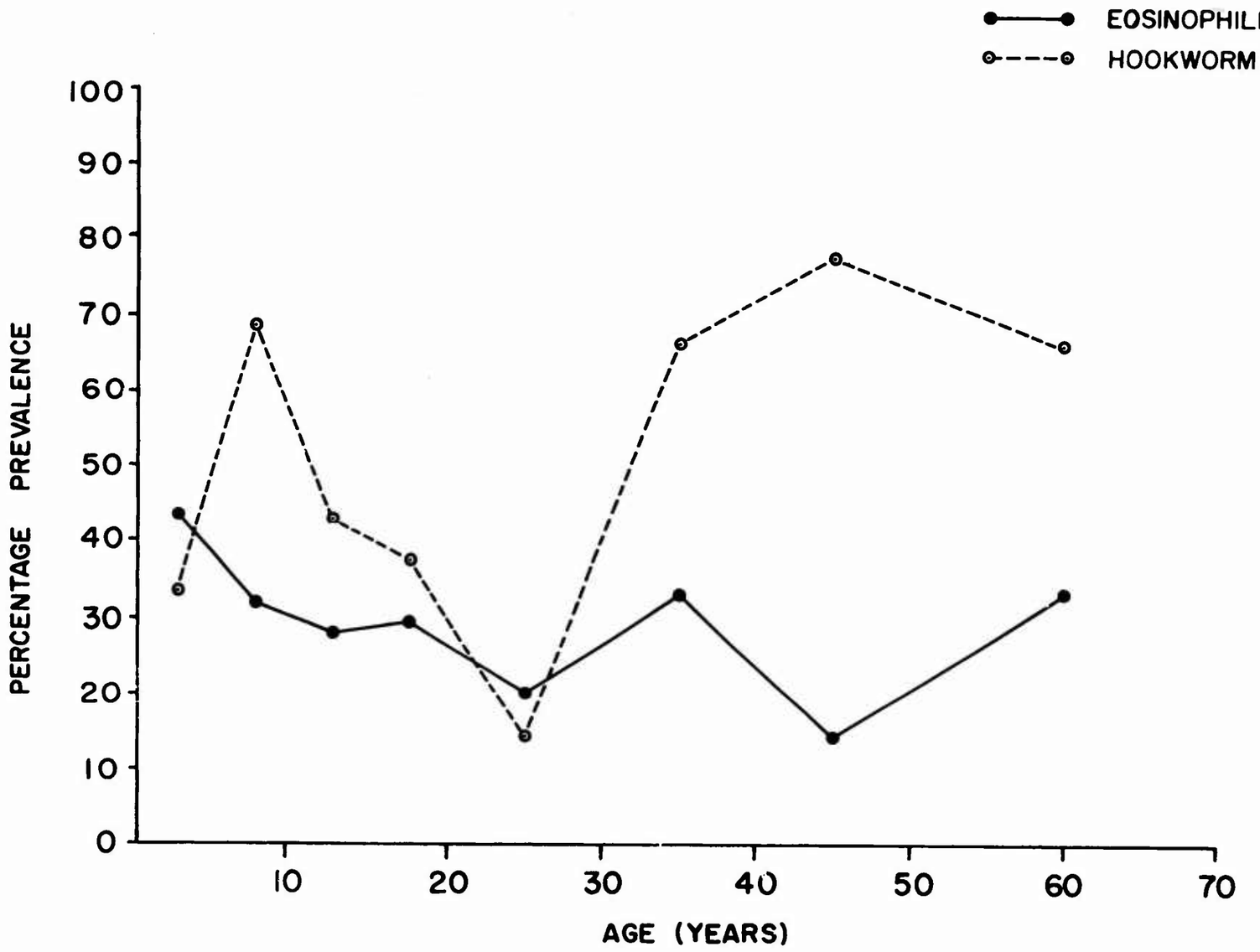


FIGURE 3. AGE-PREVALENCE OF HOOKWORM OVA AND EOSINOPHILIA *

* DISTRIBUTION OF ASCARIS AND TRICHURIS OVA PREVALENCES ARE SIMILAR.

An Investigation of Clinical and Psychometric Techniques in the Study of Cerebral Dysfunction

Principal Investigators: William H. Biggers, M.D.
Charas Suwanwella, M.D.
Harry C. Holloway, LTC, MC

Assistant Investigators: Boonarb Panpanya, P.H.N.
Vacharee Jan-Aiem

OBJECTIVE:

1. Development of a clinically valid, brief Thai mental status questionnaire for measuring mental changes in conscious patients secondary to disruption of brain function by a defined neurosurgical lesion;

2. To describe the shifts in symbolic formulations concerning illness made by the patient and patient's family during the course of the patient's illness.

DESCRIPTION: See previous Annual Reports.

PROGRESS: No new work was done on this project during the last year. The data was returned to WRAIR in September 1969 for final write-up.

Disease Morbidity and Culture in an Ethnically Mixed Neighborhood

Principal Investigators: Phon Sangsingkeo, M.D.
Richard A. Grossman, MAJ, MC
Pricha Singharaj, M.D.
Harry C. Holloway, LTC, MC
Peter Kunstadter, Ph.D.

Associate Investigators: Douglas J. Gould, Ph.D.
Howard E. Noyes, Ph.D.
Thomas J. Smith, LTC, MC
Chiraphun Duangmani, M.D.
Curtis H. Bourgeois, Jr., MAJ, MC
Carter L. Diggs, MAJ, MC

George S. Manning, CPT, MSC
Robert W. Dewey, SFC
Gerald S. Suzuki, SSG, USAMEDS
Jimmie M. George, SSG, USAMEDS

Project Coordinator: Harry C. Holloway, LTC, MC

OBJECTIVES:

1. To make a preliminary survey of the point prevalence of disease in a predominantly Lua' and North Thai lowland urban neighborhood in Amphoe Mae Sariang, Changwat Mae Hong Son;

2. To assess the functional relationship between migration, ethnic membership and ecologic experience and selected biomedical parameters. This portion of the study will be exploratory and will evaluate the practicality of using biomedical, social scientific, and psychological tools for collecting information about the interaction of social behavior and disease frequency. We will examine the possibility of using biologic measures to independently validate statements derived from observation and analysis of human behavior in a changing social and ecological setting where long term residents and migrants reside together.

BACKGROUND: See previous Annual Report.

PROGRESS: Collected data during the reporting period can be found in the study reports by the Department of Epidemiology and Special Studies. Complete analysis of the data will be done by Dr. Peter Kunstadter and LTC Harry C. Holloway who have returned to CONUS.

A Study of the Normal American Family in Thailand

Principal Investigator: Harry C. Holloway, LTC, MC

Assistant Investigators: Aporn Surintramont, B.A.
Boonarb Panpanya, P.H.N.

PURPOSE: The purposes of this study are:

1. to describe the adaptive responses of normal American families living in Chiang Mai, Thailand;

2. to add to the information concerning the medical system of Chiang Mai town and examine its functional significance for American households;

3. to compare and contrast the history of the adaptation of normal families with historical material already collected from families with a clinically disturbed member.

METHOD: Semi-structured interviews using a series of six protocols with all of the residents within a household group who volunteered their time for research.

PROGRESS: Additional material useful for general comparative purposes was collected from a number of families in Bangkok. This consists of transcribed tapes of a group of families discussing family problems. Considering the differences in method of data collection employed, the information can be used in only limited comparisons.

The data collected from all sources has been reviewed and shipped to WRAIR, where analysis is being completed by the principal investigator.

Advisor/Counterpart Relationships: An Organizational Analysis

Principal Investigators: Harry C. Holloway, LTC, MC
Supoch Khwanmitra, MAJ GEN, RTAH

OBJECTIVE: This study will describe the relationships between the formal and informal organizations of the Army Advisory Group of MACTHAI, the mission of this unit, and the transactions between advisors and their counterparts.

DESCRIPTION: The basic assumptions underlying the design of this study are described in detail in previous Annual Reports. The method employed was to observe and interview U.S. advisors and

their Thai counterparts so that specimens of their behavior might be analyzed in terms of the interaction of cultural, formal organizational, small group, and idiosyncratic personal factors.

PROGRESS: Data collection is complete. The U.S. Army principal investigator returned to WRAIR on 16 September 1969. Final analysis will be completed there.

Organizational Analysis of Srithunya Hospital: Community Attitudes towards Schizophrenic Patients in Thailand

Principal Investigators: Professor Phon Sangsingkeo, M.D.
Harry C. Holloway LTC, MC
Chantana Sukavajana, M.D.

Assistant Investigators: Boonarb Panpanya, P.H.N.
Cherdchalong Sivasomboon, R.N.
Chinda Witayarut, P.H.N.
Sukree Tumrongrachaniti, R.N.

OBJECTIVE: To describe and analyze the interaction of the hospital, the non-urban community, and the mental patient in Thailand with the goal of understanding how social institutions influence the rehabilitation of the chronically disabled patient, and within this overall objective, to study that portion of interactions occuring within, and under the control of, Srithunya Hospital, Nonthaburi, Thailand.

DESCRIPTION: Srithunya Hospital is a large (approx. 2,000 bed) psychiatric hospital located in Nonthaburi, about six miles north of Bangkok. It is the largest of five public psychiatric hospitals in Thailand, and treats a disproportionate number of patients diagnosed as "schizophrenic" (an estimated 40% of all admitted schizophrenic patients in Thailand are admitted to Srithunya).

Methods applied to understanding the organization of this hospital include: collection of documents, interview of staff members, observation of procedures and conferences, and appropriate questionnaires. Special attention is given to: admission procedures, ward assignment, method of diagnosis, treatment, record-keeping, changes in ward assignment, staff's concepts of mental illness, discharge decisions, and community relations. (For more detailed description see last year's Annual Report).

PROGRESS: During the period of this report most of the data necessary for the analysis was obtained. Although partial organization of this information will be reported here, most analysis will be done at WRAIR by LTC Holloway and MAJ Russ subsequent to September 1970. It should be understood that the remarks below

reflect only tentative formulations and do not represent final interpretation of the data.

The theoretical model used is based on the work of A.K. Rice. An institution is seen as acting upon raw materials in such a way as to produce a product. There are inputs (in this case we are mainly concerned with admitted patients), primary and subsidiary operations to be performed, and outputs (including discharged patients). There are also various constraints upon the successful performance of organizational tasks.

Srithunya hospital has four major treatment divisions: male inpatient section, female inpatient section, a rehabilitation section (for males only) and the outpatient department. Although these sections are all answerable to the director, the section chiefs have considerable autonomy. But all four treatment sections share two primary tasks: (1) to admit appropriate patients from the community, treat them, and discharge them back to the community; (2) to care for those patients who cannot be returned to the community.

The hospital also has an industrial section (which has as its primary task the production of low-cost hospital beds to be sold to other institutions), maintenance and support sections, and, of course, a director. Although the job of the director has many important internal functions, the main tasks of the office involve the relationship of the hospital with other organizations (including the immediate community and the Thai medical and financial establishments).

The workload of Srithunya has increased markedly during the past ten years. Admissions between 2502 and 2512 B.E. (1959-1969 A.D.) increased by over 200%, but the increase is better shown by soaring rises in outpatient visits and re-admissions (about 500% each) during the same period. (See Chart) During this time the physician staff only doubled. Obviously, the number of professional staff constitutes a serious constraint upon the successful performance of the hospital's primary tasks. Other constraints include: attendant staff of limited training, inadequate funds and equipment, and the necessity of conforming to the requirements of the nation's culture (religion, language, belief, customs).

We found Srithunya to be a successful organization. That is, it

performs its primary tasks both successfully and efficiently. Considering the severely impaired status of the patients admitted, it has a remarkably low retention rate (less than 10% of patients remain in the hospital over one year). The dedication of the professional staff and the beauty of the exterior hospital grounds are striking to those accustomed to chronic psychiatric facilities in the United States.

Further discussion will be divided into input, output, and "throughput" (in-hospital operations). Statements derive from general observations and also from detailed study of 1,030 patients presenting to OPD in a thirty-day period with follow-up of the 347 (33.7%) who were admitted.

INPUT: Srithunya Hospital does not actively recruit patients from the community. Patients come alone or are brought by relatives, friends, police, or on rare occasions are transferred from another hospital. Many of them are brought against their wishes.

Patients can be taken only to the outpatient department for admission. Space there is definitely cramped. Admissions are usually accomplished during the midmorning or early afternoon; very few patients are seen at night or on holidays. OPD is staffed by one full-time physician. Another doctor (drawn from the hospital staff on a rotating basis) also is there during peak hours. One nurse, one assistant nurse, and auxiliary attendants and clerical staff complete the OPD personnel.

Most OPD visits concern possible admission. Scheduled return visits for explicit outpatient care occur, but are not the rule. The number of patients seen during a working day ranges from twenty-five up to one hundred, with an average of about fifty. The heaviest loads are on days following holidays or weekends. Overall, about one-third of patients brought to the OPD are admitted. Decisions to admit can be made only by the doctors on duty at OPD.

No formal legal procedures are necessary to detain patients who do not wish to be treated, but such action is almost always taken with the active cooperation of the individual's relatives or the police. Patients accompanied by the police are most likely to be admitted (about 95%). Those coming alone are least likely to stay (about 10%). Persons travelling a long distance (over 200 km.) to reach the hospital are more likely to be kept. The most common causes for bringing a

patient to Srithunya are frightening or disturbing the family or the neighbors. Relatively few individuals are brought for treatment of symptoms per se. Many have previously sought help from other institutions.

The likelihood of admission varies by age and shows markedly different patterns for the two sexes:

Percent of Each Age Group
Admitted Among Patients Seen at OPD (1,030 successive visits)

	20 or less	21-40	41-60	61 or over
Male	27.08%	38.84%	34.23%	34.62%
Female	41.51%	32.47%	26.21%	8.33%

It seems likely that this difference reflects differential cultural attitudes towards admitting men and women, rather than differences in the prevalence of psychopathology. For example, psychotic young women may be admitted to protect them from wandering and possible sexual assault. But the age distributions of patients brought to OPD are almost identical for both sexes.

More than three-quarters of patients seen at OPD are diagnosed as schizophrenic, and the proportion of these admitted is slightly higher than the average. Data concerning the influence on admission of their occupation, economic status, marital status and ethnic group are not yet analyzed.

THROUGHPUT: The male and female sections share this pattern: admission to an acute treatment "admission" ward from OPD; treatment primarily by drugs and ECT (electroconvulsive therapy); and relatively rapid discharge of most of the patients. Very few patients are found on the "chronic" wards within the first six months of their admission.

But there are considerable differences between the sections. Because of the different training experiences of the section chiefs, the female section places a greater emphasis on group meetings and "remotivation"; the male section on somatic therapies. There is also evidence to suggest different task definition in the two sections. A sizeable proportion of male patients leave the hospital by escaping, but such

an event is unusual among female patients. (Among the 347 admitted patients who were followed, about one-third of the male patients diagnosed as schizophrenic escaped within sixteen weeks of their admission; female escapees in the comparable category were less than two percent.) Difference in escape rates may relate to the greater tractability of female patients (about one in thirteen female admissions are brought by the police compared to one in seven for males). Women are also given less opportunity to escape, being confined more than males within their section compound. Patients tend to be kept on the female admission ward longer than on the male ward, from which they are usually transferred within two weeks to make room for new admissions. But more than half of all admitted patients are discharged (or escape) within eight weeks.

All major treatment decisions are made by doctors on both sections, although they are heavily dependent upon nursing reports for information. The small number of physicians (ten, during most of 2512 B.E.) in relation to the patient load results in patient-physician contact of less than three hours in the course of a mean hospital stay of about four months. (As has already been noted, the median is even shorter; less than two months.)

The nurse is central to patient management in both sections. But although nurses tend to see their task as direct patient care, their role is actually more that of a general ward manager, housekeeper and troubleshooter. Most direct patient care must be done by practical nurses and attendants, many of whom are hampered by limited training and experience.

Srithunya Hospital also has a staff of social workers, approximately equal in number to the physician staff. Their responsibilities include: (1) taking intake histories from new patients at OPD (one social worker is on duty there); (2) follow-up of admitted patients on the wards; (3) contacting the patient's relatives about discharge; (4) helping the patient maintain written correspondence with his family; (5) on certain wards, participating in group therapy and recreation programs; and (6) arranging to send medication by mail to some discharged patients living in distant provinces (and receiving payment by return money order). The social workers are hobbled by lack of mobility; funds and transportation are not adequate to support many home visits.

There is a hospital psychologist and several assistants who perform requested psychological testing. However, these services do not yet seem to be fully integrated into the hospital's diagnostic and therapeutic programs.

OUTPUT: The criteria for discharge are realistic. Complete recovery is not expected and is seldom claimed (less than 2%) but most patients are discharged when "improved" to some degree. Patients considered otherwise dischargeable often must remain in the hospital until a responsible person (usually a relative) can claim them; they are seldom discharged to their own care. Some preliminary investigation suggests that this represents "transfer of responsibility" rather than "termination of responsibility," and may be a function of Thai cultural norms. (Such a practice interestingly resembles current recommendations for community psychiatric practice in the United States and other occidental countries.)

Female patients can be discharged only to the community. Male patients are sometimes transferred first to the rehabilitation village, another treatment section within the larger Srithunya Hospital institution.

The rehabilitation village is separated from the main treatment area of the hospital and surrounded by open land. It has a census of about 125 patients. Originally, it was intended to train otherwise unemployable and undischargeable patients, and still does to some extent. All patients here are required to work, and their tasks include: rice farming, vegetable growing, making fishbaskets, coconut growing, gardening, minor handicrafts, and work necessary for the general operations of the unit (maintenance, cleaning, food handling). At the present time it probably serves a useful training purpose for very few, but is a good place for lodging suitable patients while they are waiting for relatives to come for them. The surroundings are pleasant, and the work constitutes good occupational therapy. Some of the patients are here weaned from medication. But the original 1,000 rai of riceland cannot now be fully farmed, and Srithunya is making other plans for its utilization.

Some patients are directly discharged from the rehabilitation village, but since December 1968 a further intermediate step is available. A "halfway house" has been opened at Rangsit (about fifteen miles

north of Srithunya). This is considered a social welfare institution, not a medical one, but accepts patients directly from the rehabilitation village of Srithunya. In the first six months of 1969 one hundred patients were discharged to Rangsit, of which eighteen later returned home and eight brought back to the hospital.

Other data collected during the period of this report include: descriptions of hospital personnel, conference dynamics, financial and material constraints, conflicting tasks of the industrial unit (making beds and helping patients), staff living conditions, personnel recruitment problems, and problems of task definition and performance. Complete analysis will begin at WRAIR in October 1970.

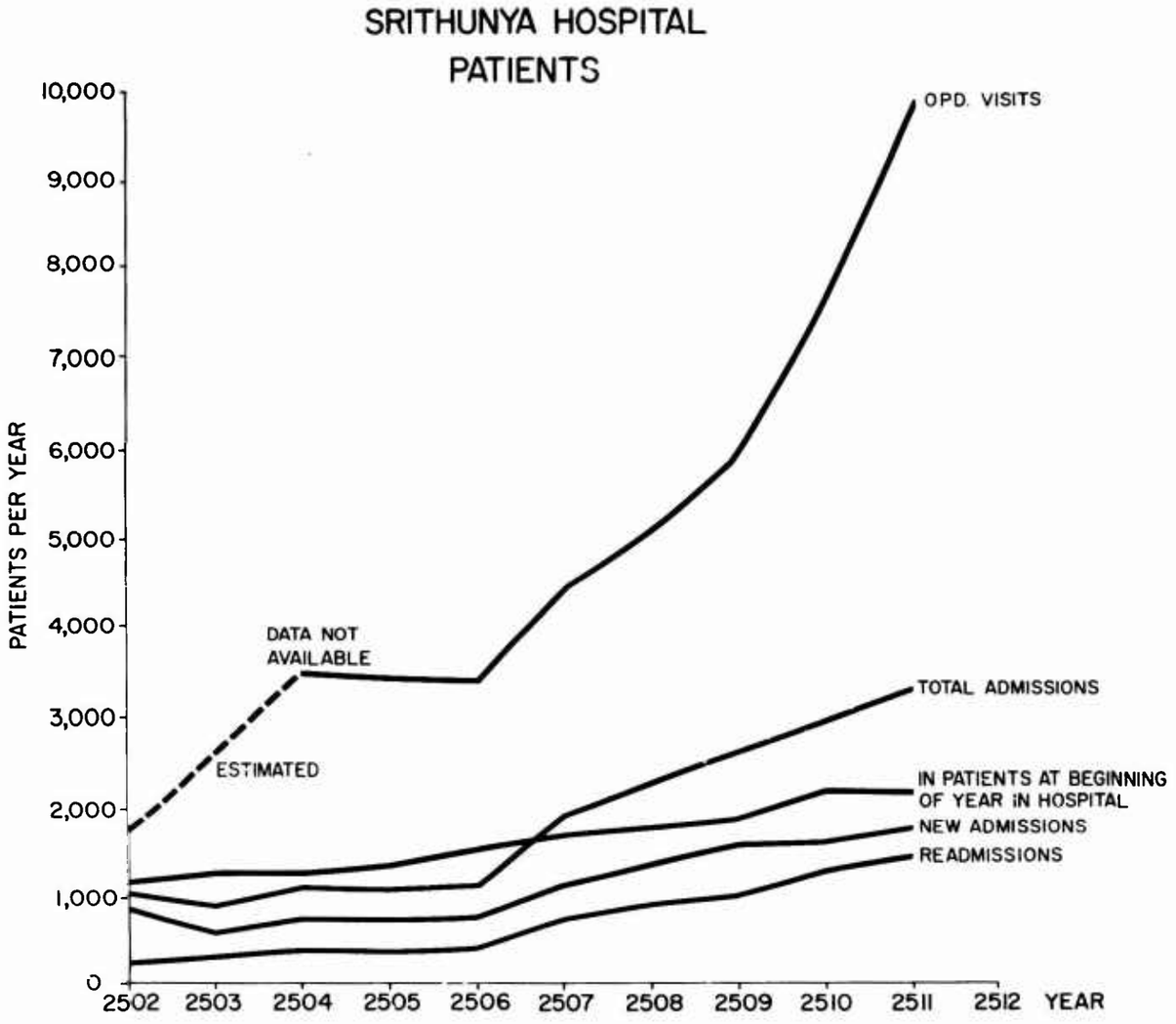
A project studying short-term prognosis in chronic schizophrenic patients (to be done at Srithunya in the period April-September 1970) will supplement the work reported here, and in turn is rooted in this basic study of Srithunya. Information gathered in both studies may be useful in planning a possible future investigation of longer-term prognosis of Thai schizophrenic patients in the community.

SUMMARY: Tentative and partial results of an organizational study of Srithunya Hospital are reported. Special emphasis is given to describing admission, treatment, and discharge of schizophrenic patients. The relation of this work to planned and possible future studies is described.

Psychiatric Evaluation of North Thai-Lua' People: How this Evaluation is influenced by the Experience and the Culture of the Observers

Principal Investigators: Phon Sangsingkeo, M.D.
Chira Sitasuwan, M.D.
Pricha Singharaj, M.D.
Marvin H. Firestone, M.D., MAJ, MC
Jonathan J. Russ, M.D., MAJ, MC

Associate Investigators: Utaiwan Pongsukree
Apakorn Kuntatun
Kriangsak Rittaporn
Tien Jaiboonma
Umneuy Sriroongroj


SRITHUNYA HOSPITAL
PATIENTS
PATIENTS PER YEAR
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
OPD. VISITS
DATA NOT
AVAILABLE
ESTIMATED
TOTAL ADMISSIONS
IN PATIENTS AT BEGINNING
OF YEAR IN HOSPITAL
NEW ADMISSIONS
READMISSIONS
2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 YEAR

Sanchai Bunlungpoh
Vivat Chavengchaiyong
Surasak Laosuwan
Siriwat Sothornwit
Winaisuk Kattipattanapongse

Assistant Investigators: Boonarb Panpanya, P.H.N.
Chinda Witayarut, P.H.N.
Aporn Surintramont, B.A.
Vacharee Jan-Aiem

OBJECTIVES:

1. To psychiatrically study in further detail a segment of a predominantly Lua' and North-Thai lowland urban community in Amphoe Mae Sariang, Changwat Mae Hong Son, where a previous survey had identified individuals with severe psychiatric illness;
2. To study transcultural influences on the psychiatric evaluation of this segment of the population;
3. To study the effects of training and experience in psychiatric field research on a group of third and fourth year Chiang Mai University medical students;
4. To study the influence on diagnoses offered by medical student observers of the affectual reactions they experienced during interviews;
5. To provide medical students experience in doing psychiatric field research and to introduce them to a standardized mental status interview schedule;
6. To acquaint SEATO Medical Research Laboratory investigators with problems and techniques in psychiatric field research in rural Northern Thailand;
7. To assess the effects of doing extensive psychiatric interviewing on the community.

BACKGROUND: A previous cooperative research project surveying disease morbidity and culture in the Mae Sariang area (see previous Annual Report) was accomplished under the coordination of LTC Harry C. Holloway, MC. At that time a survey accomplished by Dr. Pricha Singharaj found a number of members of the community identified as having psychiatric illness by the heads of their households. No details were obtained regarding the psychiatric illness, but responses indicated a prevalence of 2.8% in the segment of the population surveyed.

Mae Sariang District has a population of 50,000, 30,000 of whom are hill tribes; 80% of the hill tribes are Karen and 8% are Lua'. Mae Sariang town has a high percentage of Lua' emigrees. The town is located west of Chiang Mai and can be reached by plane or via road through the mountains from Chiang Mai. Located in Mae Sariang town is a local health facility run by the Thai government, a Christain Mission Medical Unit, a Buddhist monk who acts as a lay healer, a Catholic missionary who acts as a medical liaison to his mission hospital in Chiang Mai, and an Australian missionary nurse.

DESCRIPTION: Two Thai psychiatrists, two American psychiatrists, and ten medical students from Chiang Mai University Medical School interviewed the subjects who were identified as being psychiatrically ill by the previous SEATO Lab study. Control subjects were also interviewed; one selected from the same household and two from adjacent households. Selection of the control subject in the same household was decided first on sex, second on age, third on place in sibship. Control subjects from the adjacent households were selected on the basis of the same criteria, plus the necessity that they be from the same ethnic background.

Prior to the data collection phase, the medical students were trained in giving a standardized Spitzer Mental Status Schedule interview at Suan Prung Hospital, Chiang Mai. The Spitzer Mental Status Schedule was translated into North-Thai language in preparation for the data-collection interviews.

Before training, the medical students were given a questionnaire to test their knowledge and attitudes in the areas of psychiatry, research, and knowledge of the Mae Sariang region. They were again given this questionnaire after data collection in order to assess any changes resulting from their experiences during the project. Anonymity of the participating medical students in answering these questionnaires was maintained throughout the project.

PROGRESS: A. Phase 1: Training of medical students (18 November-24 November)

Prior to going to the target area the medical students participated in training sessions for a total of 28 hours at the Suan Prung Hospital, Chiang Mai, under the supervision of Dr. Chira Sitasuwan, Director

of Suan Prung Hospital, and Major Marvin H. Firestone, MC, Chief, Department of Neuropsychiatry, SEATO Medical Research Laboratory. It was accomplished using modelling techniques, observing interviews through a one-way viewing mirror, and discussions of the mechanics of the Spitzer Mental Status Schedule instrument. Students received further training in the use of a Multiple Affect Adjective Inventory, in order to sensitize them to feelings stimulated in them by interviewees. During training sessions they were given literature and lectures on psychiatric field research, anthropology, and an orientation to the Mae Sariang area and its population.

B. Phase II: Selection of subjects and controls (25 November - 29 November)

Dr. Pricha Singharaj, Public Health physician, with the assistance of a public health nurse and North Thai interpreter, selected subjects and controls based upon previous personal knowledge of the area and data collected by the previous SEATO study. Five subjects were selected based on data from the previous survey and five additional subjects were identified by a reliable informant. Throughout the project, Dr. Pricha maintained secrecy of the population data and insured that experimental safe-guards were observed. Three Lua' interpreters assisted in this phase, as well as in the data-collection phase.

C. Phase III: Interviews (29 November-4 December)

On 29 November, the medical students, staff of the Department of Neuropsychiatry, Dr. Phon and Dr. Chira began orientation in the Mae Sariang area. On 30 November, interviewing of subjects and controls was begun. An average of two interviews per medical student and psychiatrist were accomplished each day for 5 days; during the same period, a neuropsychiatric technician administered Porteus Maze and Digit Span tests to those subjects and controls not given these tests in the previous study. Immediately after interviews, the medical students and psychiatrists completed a Multiple Affect Adjective Inventory.

Because the local rice harvest was taking place during the period of Phases II and III, several difficulties arose which were solved by altering our interview procedures to include several interviews

at subjects' homes in the early morning and late evening. Except for this complication, the planned tasks were accomplished by the research team quite efficiently and with good cooperation from the community.

D. Phase IV: Scoring and Partial Analysis (6 December - 15 February)

After data collection, Spitzer Mental Status Schedules, Porteus Maze results, and Digit Span tests were scored. The medical students, Dr. Chira, and Dr. Firestone met to discuss the findings at Suan Prung Hospital on 6 and 7 December, on 10 and 11 January, and on 14 and 15 February. At these meetings the mental status examinations and other data collected were ordered and partially analyzed.

E. Phase V: Follow-up (12 February-13 February)

On 12 and 13 February a follow-up visit to the Mae Sariang area was accomplished, at which time the local health authorities, subjects, and controls were interviewed in order to ascertain effects of the research procedures.

F. Phase VI: Final Data Analysis (15 February-present)

All data have been classified, scored, and partially analyzed. The data presently are undergoing more refined analysis at the Department of Neuropsychiatry. Further analysis of data collected is underway, and publication to include discussion is anticipated.

RESULTS: Thirty-nine subjects and controls were interviewed by the research team, of whom thirty-three were interviewed by a medical student, a Thai psychiatrist, and an American psychiatrist. Six were interviewed only by a medical student. Spitzer Mental Status examination was done by medical students on all thirty-nine subjects.

A. Subjects and Controls

In the original survey done by Dr. Pricha, five subjects were identified by the heads of their households as having severe psychiatric illness. All five of these subjects were determined to have severe psychiatric illness when interviewed in our single-blind design.

In Dr. Pricha's second survey five additional subjects were identified by gathering information from a key informant living in the village; our interviews found only three of these five to have severe psychiatric illness.

B. Diagnostic Correlation

An accurate diagnosis can be considered: (1) If a diagnosis made by one of the three examiners is confirmed by the results of the Mental Status Schedule scores and psychological tests; or (2) If there is agreement between the two psychiatrists examining the subject independently.

If these criteria for diagnosis are used, of the thirty-three subjects and controls interviewed by all three examiners: twenty subjects and controls were normal or manifested only mild personality disorders; six were neurotic; five were psychotic; and two showed signs of mental retardation. In the group of normal subjects and controls there was agreement between Thai and American psychiatrists in eleven out of the twenty, or 55%. In the group of neuroses, there was agreement between Thai and American psychiatrists in four out of the six, or 66 2/3%. In the group of psychoses, there was agreement between Thai and American psychiatrists in two out of the five, or 40%. In the group of mental retardation, there was agreement between the Thai and American psychiatrists in two out of two, or 100%.

For the thirty-nine subjects interviewed, although there were differences in various diagnostic sub-entities, there was very good agreement within the major categories of psychiatric diagnosis: retardation, psychosis, neurosis, and normal. In fourteen of the subjects and controls there was agreement in the diagnoses of the medical student, the Thai psychiatrist and the American psychiatrist. In this category there were ten normals, one retard, one psychotic, and two neurotics. In five more subjects and controls there was concurrence in the diagnoses of the Thai and American psychiatrists, but not that of the medical student. In this category were two neurotics, one retard, one psychotic, and one normal. Eight subjects and controls were diagnosed similarly by the Thai psychiatrist and the Thai medical student, but not by the American psychiatrist. In this group were seven normals and one neurotic. Three were diagnosed

similarly by the North Thai medical student and the American psychiatrist. In this group were two normals and one neurotic. In three subjects and controls interviewed there was no agreement between any of the interviewers. The results of the Spitzer Mental Status examinations suggest that two of these three subjects were psychotic and one was normal. Six subjects were interviewed only by a medical student; it seems likely that three of these had psychiatric illness, two normal, and one for whom the Spitzer results were not definitive.

C. Diagnosis of Controls

Among the twenty-nine controls twenty-one were diagnosed normal (72%), five neurotic (17%), one psychotic (3%), and two mentally retarded (7%).

D. Affect and Diagnosis

Adjectives reflecting anxiety, hostility and depression were tabulated by psychiatrists and medical students at the termination of each interview, and each interviewer's scores on these affects were totaled. It was found that in totalling the three scores, that the American and the Thai psychiatrists averaged the same amount of affect; this average was one point higher than the affect score of the medical students. Taking the mean for the total affect for both groups and comparing this mean score with diagnosis, the following results were obtained: the medical students interviewed thirty-nine subjects and controls; the psychiatrists interviewed thirty-three. Higher than average affect was associated with a diagnosis of psychopathology made by the medical students in two; of the interviewees, lower affect than average was associated with a designation of normal in sixteen of the interviewees. For Thai psychiatrists, higher than average affect was associated with a positive diagnosis in nine; lower than average affect with a normal designation in eleven. For American psychiatrists, higher than average affect was associated with a diagnosis in ten; lower than average with fourteen normal.

E. Spitzer Scores and Diagnosis

The mean of the total scores of macro scales I, II and III was calculated. Scores above the mean were expected to be correlated

with a positive diagnosis of psychopathology; those below the mean were expected to be found in normal subjects. For the medical students' diagnoses there was a correlation for twenty-six out of thirty-nine, or 67%. For Thai psychiatrists, twenty-five out of thirty-three, or 76%. For American psychiatrists, twenty out of thirty-three, or 61%.

F. Diagnosis of Mental Retardation and Test Results

There was a good agreement in those cases diagnosed as having mental retardation between the Porteus Maze and the Digit Spans tests. The Porteus Maze and the Digit Span tests agreed overall in twenty-seven out of thirty-eight subjects and controls who were administered both tests.

G. Treatment Recommendations

In all thirty-three cases the Thai and the American psychiatrists made similar recommendations about the use of medication and/or psychotherapy. In the cases for which the medical students recommended treatment, however, there were unrealistic recommendations for psychotherapy. Medical students recommended psychotherapy for eighteen out of the thirty-nine cases interviewed. The Thai and the American psychiatrists both recommended only four.

H. Effect on Target Population of the Project

On 12 and 13 February Mae Sariang town was visited by the responsible investigator, at which time the health facility doctors, the lay healers, and the Australian Missionary nurse were questioned about the effects of the project on the people involved in the study. Health records of the studied segment of the population were reviewed. No effects of any kind were apparent. All of the people involved in the study were asked for their opinion of the study by a member of their village; with only one exception, all of them felt positively about the team's efforts and appreciated participation in the study. The Nai Amphoe of the Mae Sariang district was interviewed. He, also, expressed appreciation of our interest in the people of his district and reflected good will towards our team that he had gleaned from some of the participants.

I. Medical Student's Reaction

Without exception, the medical students considered their experience worthwhile and educational, as reported both orally and in several essay-type questions (answered anonymously) about their experience and knowledge related to the project. Multiple-choice questionnaires administered both before and after the training and field experience showed less definite results. There was, however, greater variation in answers given before their experience than four months after their experience.

SUMMARY: Thirty-nine subjects and controls living in a North-Thai Lua' community were psychiatrically evaluated independently by Thai and American psychiatrists and Chiang Mai University medical students. Psychological tests and standardized mental status examinations were also done. The results of the evaluations and testing were compared. The educational experience and interview-associated affects of the medical students participating in the project were studied, and the effects on the community of the study were appraised.

Literature Cited.

WHO criteria, in WHO Technical Report Series 182, Geneva, 1959.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6459	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCES:	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	304
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)
(U) Field Military Medical Research in a Combat Zone (VS)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	9	105
FISCAL YEAR CURRENT 71	7	90

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Medical Research Team - Vietnam
ADDRESS: Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Cutting, LTC Robert T.
TELEPHONE: NA
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: DA
NAME:

21. GENERAL USE
Foreign Intelligence Not Considered.

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Field Medical Research; (U) Field Teams; (U) Trauma; (U) Dermatology; (U) Plague; (U) Enteric Pathogens; (U) Leptospirosis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To investigate actual and potential medical problems faced by US Army personnel and, to a lesser extent, other populations of interest in Vietnam. Current studies by special teams included trauma; griseofulvin prophylaxis of fungal infections and epidemiology of cutaneous infections, plague and enteric pathogens, methemoglobin, encephalitis.

24. (U) Through teams organized for particular studies, and supported by a base laboratory. Conventional procedures with modifications dictated by local conditions are employed.

25. (U) An analysis of chest wounds with definition of criteria for open thoracotomy; IV hyperalementation, hypertonic glucose response to shock was completed. Studies on methemoglobinemia production by antimalarials also completed. Study on pneumonia implicated mycoplasma as causative agent in 40% of cases. Griseofulvin prophylaxis reduced incidence of fungal infections by two-thirds. Enteric studies indicate new series of agents in diarrheas. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 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

BLANK PAGE

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 304, Military medical research program SEA, United States Army Medical Research Team, Vietnam

Investigators.
Principal: LTC Robert T. Cutting, MC
Associate: MAJ Keith Arnold, MC; MAJ William H. Fleming, MC; CPT John C. Bowen, MC; CPT Joseph M. Ballo, MC; CPT Michael Benenson, MC; CPT Charles H. Evans, MC[1]; CPT Tran Huu Phuoc[2]; Nguyen Ngoc Linh[3]; LTC Thomas J. Smith, MC[4]; MAJ Thomas Kilbridge, MC[5]; Nguyen Thi Nhu Tuan[6]; COL Sung Ho Paik[7]; CPT Hyen Tai Yeau[7]

1. Causes of Death in Hospitalized U. S. Military Personnel in Vietnam in 1969.

a. Statement of the problem: The survey was needed to assist in establishing priorities for future research projects. No comprehensive review of all U. S. deaths in hospitals had been heretofore compiled by a single group personally examining the sources of data.

b. Background: Impetus for the study was a pilot review by McNamara and Stremple of 100 consecutive non-head injury deaths at the 24th Evacuation Hospital. This focussed attention on the life-threatening pulmonary problems. It was judged important to gather data concerning all U. S. deaths in hospitals in order to contribute quantitative information concerning those factors associated with the event of death.

c. Approach to the problem: A team composed of experienced officers and sergeants devised a form to record the pertinent information on each death and conducted a pilot study. The source of the data was the hospital record. The form was improved and the survey was extended to all American military hospitals and hospital ships, most of which were visited twice. The hospital (medical) record for each patient dying between 1 January 1969 and 31 December 1969 was reviewed. Information concerning the number of deaths for each hospital as well as the number and type of admissions was obtained from the Monthly Morbidity Reports, the Daily A and D sheets, and from other logbooks maintained to varying degree by the Registrar's offices.

[1]Office of the Command Surgeon, Military Assistance Command, Vietnam
[2]Ministry of Health, Vietnam
[3]Cho Quan Infectious Disease Hospital, Saigon
[4]SEATO Medical Research Laboratory, Bangkok
[5]Third Field Hospital, Saigon
[6]Institut Pasteur, Saigon
[7]Office of the Chief Surgeon ROKF-V, Saigon

d. Results: There were twenty hospitals visited (fifteen Army) including some which closed during the period under study. During this period there were 132,996 admissions of which 55,177 (46.3%) were surgical.

There were 1,253 deaths of which 92.7% occurred in surgical patients. Overall, 2.1% of surgical admissions died. The hospital with the highest surgical death rate, 6.1%, was a neurosurgical referral center and reflected the high mortality rate for head injuries.

Five conditions accounted for 87.3% of the surgical deaths. These were head injury, 42.5%; hemorrhagic shock, 23.9%; septic shock, 11.7%; respiratory problems, 6.1%; and burns, 3.1%.

The principal cause of the 91 medical deaths was malaria with 12 cases.

Most deaths (61.1%) occurred within 24 - 36 hours of admission and 13.6% occurred 2 - 5 days after admission. Nearly one-fifth (18.4%) occurred after the fifth day.

A detailed analysis of each hospital's deaths was prepared.

e. Discussion: It was very frequently observed that the death certificates were poorly completed. Unfortunately, the hospital (medical) records also were often incomplete or poorly prepared. Thus, in many patients, the cause of death was obscured or at least far from obvious. Occasionally, the attending doctor was contacted and contributed clarifying information. However, the records examined were of sufficient detail to afford conclusions in the overwhelming majority of instances. By far the most detailed records were obtained from the hospital ships.

Three-fifths of the deaths occurred roughly during the first day. It is felt that a high proportion of these reached the hospital alive only because of the rapid aeromedical evacuation system.

Head injuries accounted for two-fifths of all surgical deaths. Better preventive measures (i.e., wearing the helmet or a better helmet) would undoubtedly have reduced this toll. Measures to prevent or reduce intracranial swelling might save a small proportion.

Hemorrhagic shock appears to be more than mere loss of circulating volume. The phenomenon of irreversibility is poorly understood. The qualities of the replacement transfusions may be of importance equal to the quantity.

Sepsis is a problem in every hospital. Better antibiotics are not necessarily the solution. More attention needs to be directed to host factors particularly at the cellular level.

Pulmonary problems were difficult to sort out because they occurred in conjunction with severe trauma, blood replacement, anesthesia and surgery, ventilatory assistance, fat emboli and infection.

f. Conclusions: Events associated with trauma were the causes of death of 92.7% of the 1253 U.S. deaths occurring in military hospitals in Vietnam during 1969. Head injury accounted for the greatest proportion (42.5%) followed by hemorrhagic shock (23.9%), septic shock (11.7%), respiratory problems (6.1%) and burns (3.1%). 2.1% of surgical admissions died. Medical admissions accounted for slightly over half the 132,996 total admissions but for only 91 deaths (7.3% deaths). Three-fifths of the deaths occurred during the first 24 - 36 hours but one-fifth died after the fifth day.

g. Recommendations: More detailed epidemiological studies are necessary in order to discover means of markedly reducing the toll from head injury. Studies on cellular respiration in shock and following massive transfusion are needed, as well as studies of pulmonary mechanisms. In sepsis, investigation of host defense mechanisms at the humoral or cellular level are necessary; these could run concurrently with prospective studies of the epidemiology of surgical infections.

2. A Study of Pneumonia Patients Admitted to a U. S. Military Hospital in Vietnam.

a. Statement of the problem: Clinicians on the medical service at the 3rd Field Hospital had observed many patients with clinical and x-ray diagnosis of pneumonia who failed to respond to the antibiotic indicated by sputum culture or else had negative cultures. They requested assistance.

b. Background: Each of the clinicians on the medical service at the 3rd Field Hospital had observed several perplexing cases of pneumonia, usually moderately severe and with a protracted (10 - 14 day) course in spite of appropriate antibiotic therapy. Most had negative sputum cultures in the absence of prior antimicrobial therapy. Two questions arose whether this disease was a specific clinical entity with distinguishing clinical features, and if the disease was due to a single agent or combination of agents.

c. Approach to the problem: All pneumonia patients (civilian and military) admitted by the medical service of the 3rd Field Hospital were studied. Criteria for diagnosis were pertinent clinical findings plus x-ray evidence of a pulmonary infiltrate. Patients were not included if they were on the surgical service or if they were admitted with renal failure, drug overdose or in coma.

Clinical information was recorded by the 3rd Field Hospital physicians.

Sputum was collected and examined for culture and sensitivity at the hospital laboratory and at the 9th Medical Laboratory.

An AFB smear and culture and a fungus culture were performed at the 9th Medical Laboratory.

Virus and mycoplasma isolations were performed at the SEATO Medical Research Laboratory in Bangkok.

Acute and convalescent sera were tested for titer rises by the 9th Medical Laboratory against leptospirosis, melioidosis, scrub and murine typhus, psittacosis, and for cold agglutinins.

Acute and convalescent sera were also tested by the SEATO Medical Research Laboratory against influenza A_2 and B, para-influenza I and III, adenoviruses, RS virus, and mycoplasma. The sputa and sera were stored at -70° C., and shipped to Bangkok under dry ice.

d. Results: In four months Nov 70 through Feb 70) 73 patients entered the study. Detailed clinical information was obtained on only 66 patients. Acute and convalescent sera were obtained on 58 and sputum for viral isolation from 50.

Preliminary serology results were negative for leptospirosis, typhus, psittacosis and melioidosis in all; positive cold agglutinins in 10 patients; high acute and convalescent titers to influenza A_2 in three patients.

Sputum virus isolation was negative in 46 patients; influenza A_2 grew in one specimen and an unidentified virus in another. Six specimens are still being examined.

The CF test for mycoplasma (SEATO Laboratory) was positive in 21 of 58 serum pairs tested (36.7%).

Routine culture and sensitivity revealed mainly normal flora. Two patients had bacterial pneumonia and two had positive AFB smears and were evacuated. Fungus cultures were positive for Candida in a large number of cases.

The disparity between the mycoplasma CF tests and cold agglutination is apparent.

e. Discussion: Cold agglutinins are fairly nonspecific; additionally if the red cells are not separated from the serum before refrigerating the blood, the test will be negative. The CF test is reliable but is more apt to be positive if the serum was collected later in the course of the illness than was obtained in this study. Thus, the incidence of pneumonia due to Mycoplasma pneumoniae could actually have been higher than was observed.

Correlation of the laboratory with the clinical data is in process.

f. Conclusions: Mycoplasma pneumoniae is an important etiologic agent in patients with pneumonia admitted to the 3rd Field Hospital in Saigon during this period. Inasmuch as erythromycin has been shown to be effective in Mycoplasma pneumoniae patients of the same age group, this antibiotic should be considered for use in suspected cases.

g. Recommendations: An epidemiologic study of Mycoplasma pneumoniae patients should be carried out, perhaps starting in the Saigon-Long Binh area. Serological studies should be carried out in representative areas throughout Vietnam. The availability of laboratory methods should increase, particularly of cold agglutinins and CF tests. A Mycoplasma culture capability should be made available, perhaps with the assistance of the 406th Medical Laboratory in Japan. Antibiotic sensitivity patterns should be developed in order to compare SE Asian sensitivity patterns with those found elsewhere.

3. Recovery of Unpassaged Dengue Virus.

a. Statement of the problem: Unpassaged dengue virus material from Vietnam is needed for primate studies, antigen characterization, and for reference seed stocks.

b. Background: For two years the Dengue Task Force of the Commission on Viral Infections of the Armed Forces Epidemiological Board had voiced the need to obtain large quantities of fresh (i.e. unpassaged) dengue virus from Vietnam. The Dengue Task Force discussed the appropriate methodologies for collection. To assist in obtaining USARV approval, the AFEB sent a formal recommendation to The Surgeon General stating that the withdrawal of a unit of blood would not affect the course or clinical outcome of any person with a disease resembling dengue.

c. Approach to the problem: Arrangements were made with the medical services of the 3rd Field Hospital in Saigon, the 93rd Evacuation Hospital in Long Binh, and the 15th Evacuation Hospital in Cu Chi to collect material. A clinical summary sheet was prepared to be used in each case.

Patients selected had symptoms compatible with dengue for less than 72 hours at the time of blood collection. Blood donation was voluntary. Venous blood was collected (300 - 500 ml) into sterile bottles and allowed to clot. The serum was separated aseptically and transferred into sterile containers, stored at -70° C., and was shipped in batches under dry ice to the SEATO Medical Research Laboratory in Bangkok for virus isolation.

d. Results: In the three hospitals during the 3½ month period (19 Sep 69 - 7 Jan 70) only 22 patients were admitted with dengue included in the differential diagnosis who had had symptoms for less than

72 hours. Blood was obtained in each of these. However, no dengue viruses were isolated.

Convalescent sera were obtained in only 4 of the 22 cases. These were tested by HAI for group A and B Arboviruses at the 9th Medical Laboratory and all were negative.

e. Discussion: It has long been assumed that dengue is a fairly common disease in Vietnam and that it coincides with the rainy season (June to early December). Yet this study collected only 22 patients from three large military hospitals during a 3½ month period and none of these resulted in an isolation; four showed no serological rise.

The reasons for the small number of patients are conjectures. Cooperation certainly was good on the part of the hospitals. It is unlikely that the dengue season peaked early in 1969 because the rainy season began late that year (third week in June). Another possibility involves the difficulty in making the diagnosis since the symptoms are nonspecific. Another possibility is that physicans fail to include dengue in the differential diagnosis of a patient with fever; this is unlikely because they knew we were looking for patients. A more reasonable explanation is that for some unknown reason there was very little dengue virus circulating in the "community". Unfortunately, there are no records of confirmed cases at the Saigon civilian hospitals for these serological procedures are not available. The 9th Medical Laboratory reports Group B arboviruses only as a group; it uses a dengue II antigen and in its system obtains higher titers from Jap B encephalitis than from dengue. During the period the 9th Medical Laboratory reported scattered cases of Group B arbovirus disease but very few from the III and IV CTZ.

If some of the patients actually had dengue but we were unable to collect and isolate it, there are at least three possible explanations. First, the virus may be present in the blood (in Vietnam dengue) considerably less than 72 hours; by the time the patient has reported sick to the battalion surgeon, been observed for a day or so with negative malaria smears at the division clearing company, and then transferred to a hospital, 48 - 72 hours or more have have elapsed and the virus may no longer be circulating. Secondly, the virus could have been lost in the collection, separation, transportation and storage of the material in Vietnam. This is more likely to have occurred, if at all, with the patients from the 12th Evacuation Hospital; it was least likely to have occurred with patients from the 3rd Field Hospital in Saigon. Thirdly, the isolation techniques were at fault. The latter is the least likely explanation because the SEATO laboratory has had a vast experience in this regard. It is our judgment that the most likely explanation is the first.

f. Conclusions: Obtaining unpassaged dengue material from U. S. patients was unsuccessful. The probable reasons were a very low level

of virus transmission during the study period, the difficulty in diagnosing the infection, and the time lapse between initial symptoms and arrival through the chain of evacuation to the hospital.

g. Recommendations: The 9th Medical Laboratory data should be reviewed to confirm the season of group B arbovirus disease for each hospital.

Continuous liaison is necessary between the investigators and the staffs of the medical services of the hospitals. In a study where the input is sparse, continued cooperation and interest of the attending doctors is difficult to maintain at a high level.

The most likely method of success would involve instant communication of the fact of a serologically confirmed outbreak of dengue (or group B disease) and then to visit the area and obtain specimens while the patients are still in the battalion aid station or in the division clearing company.

4. Plague Epidemiology and Surveillance

a. Statement of the problem: There is a continuing requirement to maintain laboratory surveillance over the epidemiological aspects of plague in South Vietnam. Most importantly there is a strong need for a single agency interface with the multiple private, governmental, volunteer and military medical and public health organizations, both U. S. and Vietnamese. This is a report of such activities for the two year period 1 May 1967 - 30 April 1970.

b. Background: Plague was first described in Vietnam in 1898 in Nha Trang and since then has had a variable periodicity although there is probably a continuous low level of endemicity. Vivona, Marshall and Cavanaugh documented the current outbreak of plague (USAMRT Annual Progress Report, 1 Sep 65 - 31 Aug 66). With Teschan they established a plague laboratory (Laboratory de la Peste) in a new building located at the Institut Pasteur in Saigon. The current work is a continuation of the efforts of these earlier workers. The original goals were to (a) provide laboratory surveillance for P. pestis among Vietnamese civilian and military personnel and American and Free World forces; (b) train Vietnamese technicians in plague laboratory techniques; (c) act as a central collecting agency for positive P. pestis cultures for research in the U. S.; and (d) monitor antibiotic sensitivities to insure that resistant plague has not been introduced.

c. Approach: A system for the receipt of human specimens from military and civilian sources throughout the country had been well established. These specimens were processed in accordance with standard laboratory procedures. The majority of human specimens were sent by the Cho Quan (Infectious Disease) Hospital in Saigon.

Rat trapping was performed semi-systematically throughout the Saigon area. Each afternoon (Monday - Friday) Institut Pasteur personnel would set 20 - 30 rat traps and recover them the next morning. Trapping locations and number and species obtained were recorded and mapped. Rodents were brought back to the I.P. where they were identified and combed for ectoparasites. Serology and cultures were performed using heart blood and spleen pools. Cultures were also prepared from flea pools.

Rodents were regularly received from U. S. military installations throughout Vietnam, but largely from the Saigon-Long Binh area and the port cities.

d. Results:

(1) Human Specimens

No. specimens received	3229	
No. patients	2399	
No. specimens positive by culture	624	(18.7%)
No. patients positive by culture	568	(23.7%)
Source of Specimen for culture		
Bubo aspirates	1789	
No. positive	490	(27.4%)
Blood	630	
No. positive	118	(18.7%)
Throat	798	
No. positive	9	(1.1%)
Spinal fluid	12	
No. positive	7	(58.5%0

Antibiotic sensitivities were performed on all positive cultures. There were no startling departures from the usual sensitivity patterns.

(2) Rodent Trapping in Saigon

Total No. rodents	7998	
Total No. fleas	8875	
Rattus norvegicus	4702	(58.8%)
Rattus norvegicus fleas	6379	(71.8%)
Rattus norvegicus flea index	1.36	
Rattus exulans	1887	(23.6%)
Rattus exulans fleas	1550	(17.4%)
Rattus exulans flea index	0.82	
Suncus murinus	1409	(17.6%)
Suncus murinus fleas	946	(11.8%)
Suncus murinus flea index	0.67	

(3) Rodents trapped at U. S. Military Installations

Total No. rodents	6975	
Total No. fleas	7995	
Rattus norvegicus	2095	(30.0%)
Rattus norvegicus fleas	5971	(74.6%)
Rattus norvegicus flea index	2.85	
Rattus rattus	2273	(37.5%)
Rattus rattus fleas	1256	(15.7%)
Rattus rattus flea index	0.55	
Bandicoot species	1388	
Bandicoot species fleas	324	(4%)
Bandicoot species flea index	0.23	
Rattus exulans	705	(10.1%)
Rattus exulans fleas	183	
Rattus exulans flea index	0.26	
Suncus murinus	514	(7.3%)
Suncus murinus fleas	272	(3.4%)
Suncus murinus flea index	0.53	

(4) Positive Cultures from Flea Pools

Rattus norvegicus	9
Suncus murinus	3
Rattus rattus	1
Total:	13

(5) Positive Cultures from Spleen Pools

Rattus norvegicus	2
Rattus rattus	2
Rattus exulans	1
Total:	5

e. Discussion: The human specimens were received from areas throughout Vietnam. Occasionally they would be a week or more old at receipt, but if transported in Cary-Blair or similar media, they were usually satisfactory.

All technical phases of the work were performed by Institut Pasteur personnel working under the supervision of USAMRT non-commissioned officers. There was no commissioned laboratory officer present during most of the two-year period. During the second year it became apparent that the Fraction I antibody positively rates were inconsistent and low,

in the order of 1:200. Although minor technical errors were corrected, it became apparent that the difficulty was probably the antigen which had been stored for several years. Because of the lack of confidence in the Fraction I serology, no results are reported.

The method of trapping does not yield confidence in the results as an indicator of the magnitude and distribution of rodents in the Saigon area. The Institut Pasteur personnel give the impression of haphazard placement of traps, their precise locations governed by convenience. Another problem is trap thefts. Consequently, the pattern has evolved of baiting the trap and merely handing it to a householder. A common observation is the collection of a sprung trap (minus the bait) the next morning. Vietnamese fondness for rat meat has been frequently alleged as a barrier to successful rodent collection.

The most common rat in RVN is Rattus norvegicus. In numbers it constituted three-fifths of the rats trapped in Saigon. Yet it constituted only one-third of the rats trapped on U. S. military installations where it was equalled in numbers by Rattus rattus. It is curious that not one Rattus rattus was trapped during a two-year period of trapping in Saigon which caught 8,000 rats. The same Vietnamese personnel were making the identifications.

Flea indices by area and by rodent species were maintained at monthly intervals. The natural monthly variation was not influenced during the time frame of the increased incidence of plague in the Saigon area (Jan - Feb 1970). The heaviest concentration of plague patients during this period came from Hau Nghia province, to the immediate northwest of Saigon, but no trapping was performed in this province. Rattus norvegicus also had had the highest indices, both in Saigon as well as U. S. military installations. These patterns are consistently observed in the areas of Vietnam monitored by the KOPREM teams.

All fleas identified were Xenopsylla cheopis. Of several hundred flea pool cultures, only 13 were positive during the two-year period and 9 of these were from Rattus norvegicus. Only five spleen pools were positive. The magnitude of this effort in the face of so little return suggests that these procedures be evaluated for discontinuance, their role being relegated solely to special investigation rather than routine surveillance.

f. Conclusions: There is a continuing need to maintain a strong laboratory support of plague surveillance activities. The laboratory should possess, in addition to high technical competence, ease of administrative and geographic access to contributing organizations. Rapidity of feedback of information to the healthy workers in the field is a neglected feature of importance.

A continued public health laboratory diagnostic capability is most important. Over two years 3329 specimens for culture were received

from 2399 patients. Only one-fourth of the 1789 bubo aspirates were positive, indicating the difficulty of obtaining the specimen plus the drying out if transportation was delayed.

Eight thousand rodents were trapped, identified, and combed for fleas. Rodent "censuses" by geographic area and flea indices by month were maintained. This information was not observed to be applied in any practical fashion during the second year of the report period.

The small number of positive flea pools (13) and spleen pools (5) causes the question to be raised concerning the practicality of continuing these procedures.

Results on Fraction I serology performed on the rodents' heart blood were untrustworthy, probably due to deterioration of the antigen.

Rattus norvegicus was the most common rodent and also had the consistently highest flea index.

g. Recommendations:

(1) A plague diagnostic capability must continue, preferably as part of a large public health laboratory.

(2) Rodent trapping should be conducted in a more systematized fashion.

(3) Methodology and reagents for Fraction I antibody serology should be corrected.

(4) Except for special studies, culture of flea pools and spleen pools should be discontinued.

5. Bacterial Enteropathogens in Vietnam

a. Statement of the problem: Vietnam is an area where bacterial diarrheas and cholera are endemic and epidemic. Diarrhea causes a continuous drain on manpower and efficiency in Vietnam.

b. Background: In 1965 Vivona et al. established diagnostic and surveillance procedures for bacterial diarrheas and cholera (see USAMRT Annual Report, 1 Sep 65 - 31 Aug 66). The purpose was to (a) gain information concerning the distribution of various enteropathogens in the Vietnamese civilian population that might affect U. S. troops; (b) assist American medical personnel with a specialized enteric bacteriology laboratory; and (c) train Vietnamese civilians in modern methods of culture and identification of enterobacteriaceae. During its early days the laboratory was a diagnostic center for American military doctors, and later became a reference center. Its primary purpose of gaining experience with diarrheal flora of Vietnamese was gained through receipt

of specimens from two sources Chc Quan (Infectious Disease) Hospital and Nhi Dong (Children's) Hospital.

c. Approach to the problem: The current work is a continuation of that previously reported by Vivona and subsequent authors. The methodologies for isolation, identification, and antibiogram studies are those previously established. The current report period is 1 May 1969 to 30 April 1970.

d. Results: The enteric pathogens laboratory received 2799 diarrhea specimens from Vietnamese patients with diarrhea. The source was principally the Cho Quan Hospital in Saigon. Only during the first three months did the laboratory receive specimens from Nhi Dong (Children's) Hospital. An enteric pathogen was isolated in 879 (31.4%) of the specimens.

	No.	% of Pathogens
Shigella sp.	219	24.9
Proteus sp.	182	20.7
Salmonella sp.	141	16.0
V. cholerae	103	11.7
Providencia	90	10.2
Path. E. coli	80	9.1
Pseudomonas	55	6.3
Alkalescens-dispar	9	1.0
	879	99.9

It should be noted that the Pseudomonas, E. coli and Proteus were isolated during the months of May, June, and July when the laboratory was receiving pediatric specimens. The 80 pathogenic E. coli included 11 different serotypes; 025, B19, B23 were the most common. P. mirabilis was the most common Proteus species, followed by P. morganii. Proteus was reported out only in children under 15 years of age.

The 141 Salmonella isolates included 23 species, two of which were untypable and were forwarded to WRAIR Washington for further study.

Salmonella Group	No.
A	2
B	91
C	6
C2	5
D	15
E	6
E1	16
Total	141

In the group B Salmonella, S. java was most common (50 isolates) followed by S. paratyphi B (27 isolates).

Seven species of Salmonella were isolated for the first time: S. stourbridge, S. tshiongwe, S. canada, S. essen, S. stanleyville, S. anaterm, and S. cairina.

The 219 Shigella isolates were:

S. flexneri	160
S. sonnei	37
S. dysenteriae	16
S. boydii	6
	219

d. Discussion of results: The quality of the laboratory work was unquestionably of a very high order. Miss Nguyen Thi Nhu Tuan, the technician in charge of the laboratory, deserves special recognition for her skill and competence. While the results are valid, their interpretation and utility from a public health viewpoint are open to question. The laboratory serves as a public health diagnostic center for Saigon and surrounding regions. Unfortunately it received specimens primarily from two hospitals during the first four years and primarily from one hospital during the last nine months. Thus it hardly portrays the true picture of diarrheal disease throughout the Saigon area. Nevertheless, the laboratory provides an extremely important service function and should be continued although its support should be assumed by a Vietnamese civilian public health agency. The chief value of the laboratory to WRAIR is that it maintained a highly skilled capability into which various diarrheal investigations could be (and were) thrust at virtually no additional expense beyond media supplies.

e. Conclusion: During the period 1 May 1969 to 30 April 1970 the Enteric Pathogens Laboratory received 2799 specimens with 879 (31.4%) positive for enteric pathogens. There were 103 cholera isolates (all El Tor Inaba). Pathogenic E. coli, Proteus sp. and Pseudomonas sp. were commonly observed in specimens from children, but these were sharply reduced when the Nhi Dong (Children's) Hospital developed its own bacteriology laboratory.

The results of the Enteric Pathogens Laboratory possess confidence but their public health significance is questionable because they represent a small and uncertain proportion of the diarrheal disease in the Saigon area.

f. Recommendations: The enteric pathogen public health laboratory function be assessed by a Vietnamese public health agency.

6. An Outbreak of Leptospirosis among Korean Troops in Vietnam

a. Statement of the problem: To further define and investigate leptospirosis as it exists in the Republic of Vietnam.

b. Background: Leptospirosis is endemic in Vietnam but its spotty distribution is poorly understood. Although French troops experienced a great deal of difficulty with this disease, it has not been a major problem to U. S. troops in terms of either its severity or its frequency. This study is a retrospective clinical and laboratory analysis of an outbreak experienced primarily by two companies on a regimental-sized operation of Korean troops.

c. Approach: Hospital charts of 35 patients were made available and were translated. Of these, 30 had had serological studies performed at the 9th Medical Laboratory drawn during their illness. All patients were hospitalized at the 102nd Evacuation Hospital in Nha Trang.

d. Results: Of the 35 patients, 3 died, 35% had hemorrhagic manifestations and 40% exhibited some degree of jaundice. Most had respiratory symptoms.

Twenty had serological evidence of leptospirosis. Unfortunately sera were not examined for specific serotype.

A report is being prepared describing the clinical and epidemiological findings.

e. Conclusion: Leptospirosis can be a severe disease in Vietnam. The total number of cases involved in this outbreak is probably 50 - 75, which would place the case fatality ratio at 4 - 6 per cent. Pulmonary, hemorrhagic and icteric features were present in a large proportion of the patients.

f. Recommendations: A laboratory capability for the study of leptospirosis should be available to investigators in Vietnam. This capability, currently being furnished by the 406th Medical Laboratory in Japan, must include agglutination lysis tests to establish specific serotypes. Epidemiologic studies and seasonal geographic mapping of the disease are needed.

7. Plague in Vietnamese Civilians -- Clinical and Laboratory Study.

a. Statement of the problem: Although a great deal of clinical information concerning plague had been obtained, there is a striking lack of biochemical information correlated with clinical findings.

b. Background: Experience during the past four years has failed to confirm initial fears that plague might significantly affect American military personnel in Vietnam. The number of cases in U. S. personnel

has been restricted to a mere handful, and antibiotic resistant strains have failed to emerge. Nevertheless, the possibilities of antigenic shifts, antibiotic resistance and introduction into U. S. port cities remain at least a theoretical consideration.

c. Approach to the problem: The only hospital in Saigon which cares for plague patients is the Cho Quan (Infectious Diseases) Hospital. Arrangements were made with hospital authorities to examine and obtain specimens from suspected plague patients. A clinical summary sheet was devised and with the assistance of a senior medical student, Nguyen Ngoc Linh, was completed for each patient. Bubo aspirates were drawn and smears were performed by Cho Quan personnel; cultures were performed at the USAMRT - Institut Pasteur plague laboratory. Blood was drawn for culture, hematology, clinical chemistry and serologic studies. Throat cultures were taken from the patient and from all persons accompanying the patient to the hospital.

d. Results: The study took place during March and April 1970. This was a time of increased incidence of plague in the Saigon area, particularly in the Hau Nghia Province to the northeast.

There were 122 patients admitted to Cho Quan with the diagnosis of probable plague during the study period. Most patients (85.7%) came from Hau Nghia Province and most of these came from the Cu Chi district. There were 57 males and 55 females and 70.5% of the patients were under age 21.

A swelling or bubo was present in 91.1% and was complained of by 86.6%. The bubo was single in 83.3%. The most common site of the bubo was the femoral region followed by inguinal, cervical and axillary regions. Only one patient with generalized lymphadenopathy had bacteriologically confirmed plague. Epitrochlear and axillary involvement of the same arm occurred in four patients with confirmed plague.

Bacterial confirmation of plague occurred in 55 (49.1%) including six of the eight patients who died.

Of the 41 patients who had been previously vaccinated, 16 had confirmed plague. Only 11 had received vaccine (live, manufactured by the Institut Pasteur) 2 weeks to 6 months prior to admission.

Bacteriological Results:

Positive direct smear	25.0%
Positive bubo culture	42.0%
Positive blood culture	24.1%
Positive throat culture	1.7%
Positive throat (contacts)	0

Serology (Fraction I hemagglutination) showed a rise in 22 confirmed and 10 non-confirmed patients.

The mean hematocrit was 35% for both confirmed and non-confirmed patients. The initial WBC was invariably elevated and a few patients had very high counts (40 - 80,000). Serum sodium and potassium levels were normal as was the serum creatinine. However the BUN was elevated in those patients who were clinically dehydrated and vomiting. The SGOT was abnormal (>40 units) in 60% of the confirmed and 41.6% of the non-confirmed cases. The alkaline phosphatase was abnormal (>50 units) in 73.6% of confirmed and 60.0% of non-confirmed patients.

e. Discussion: The clinical features of these patients were similar to previous descriptions of the disease. The abnormal liver function tests (SGOT and alkaline phosphatase) were an interesting finding which may possibly be related to septicemia or to the plague bacillus itself or one of its products. The significance of the abnormal liver findings are not apparent beyond the fact that plague is a disease of profound systemic manifestations.

Only 42% of the bubo aspirates were bacteriologically confirmed. This may relate to the fact that many patients had been started on antibiotics as a home remedy (most antibiotics are readily available, like aspirin, in Vietnam) or to the fact that organisms are located in the lymph gland lying deep within the large inflammatory bubo.

f. Conclusions: Clinical, bacteriological and biochemical studies were performed in 122 consecutive Vietnamese civilian patients admitted to the Saigon infectious disease hospital with the diagnosis of plague. There were 8 deaths. Bacterial confirmation was obtained in only 55 patients. Liver function studies were abnormal in two-thirds of the patients.

8. The Relationships of Malaria with Blood Groups, G-6-PD Levels, and Hemoglobin Patterns.

a. Statement of the problem: The frequency and severity of malaria in various populations have been shown epidemiologically to be influenced by blood groups, by G-6-PD levels, and by hemoglobin patterns. The question arises as to whether these influences might be observed in U. S. troop populations experiencing clinical malaria.

b. Background: An epidemiologic correlation has been shown between a high blood group B gene frequency and the presence of endemic falciparum malaria in the population. Secondly, the severity of a malaria infection may depend in part on the presence of a common antigen, namely blood group A substance, in the parasite. It has been suggested that the naturally occurring anti-A antibody in individuals with blood group B or O might inhibit the invading organism. In addition, in a person with blood group A, antibody may be made against the A substance of the parasite which might combine with host red cells and produce a hemolytic reaction. A high anti-A agglutinin titer has been observed in patients with malaria. Thirdly, as a part of this investigation, data concerning G-6-PD and hemoglobin patterns would be collected and related to malaria.

c. Approach to the problem: U. S. military personnel admitted to the 6th Convalescent Center with a diagnosis of falciparum malaria were studied. A control group consisted of afebrile surgical patients recovering at the same hospital. Vietnamese, Cambodians and Montagnards associated with Special Forces camps were also studied. Each patient had a malaria smear and venous blood was then collected for ABO grouping, Rh typing, G-6-PD determination, isoagglutinin titers, and hemoglobin electrophoresis.

d. Results: The study is in progress. To date, 178 U. S. military malaria patients and 146 controls have been sampled. Also 365 indigenous personnel without malaria have been examined.

e. Conclusions and Recommendations: None at this time.

9. Congenital Malformations, Hydatidiform Moles, and Stillbirths in the Republic of Vietnam, 1960-1969.

a. Statement of the problem: Has the military use of herbicides in Vietnam influenced the frequency of birth abnormalities among Vietnamese people?

b. Background: A 1969 study reported an increased incidence of developmental abnormalities in rats and mice born of mothers which had received 2,4,5-D and 2,4,5-T. Herbicides containing these compounds have been used for military purposes in Vietnam. Subsequent investigations revealed that dioxin, a contaminant varyingly present in the manufacturing process, was a primary cause of the teratogeny. A great debate arose in the lay and scientific press over the use of herbicides and their possible harmful effects.

c. Approach to the problem: A steering committee of high level MACV, MOH, and USAMRT personnel was organized. It determined that a pilot study was necessary to determine the availability and accuracy of obstetrical records in Vietnamese medical facilities and to develop techniques for review and interpretation. The pilot survey was successful and was extended throughout the country.

In all but four hospitals a Daily Summary Ledger, prepared by the chief midwives, served as the primary source document. This contained 15 - 20 pieces of information and always contained at least the following: hospital admission number; patient's name, age, parity and date of admission; presentation, time, and method of delivery; baby's sex, weight, and general condition at birth; placenta weight; estimate of blood loss; and name of person attending. A "remarks" column recorded such information as vacuum extraction, reversion, placenta delivery assistance, cesarean section and indication, blood transfusion, uterine revision maternal or infant complications and results, and congenital malformations. The accuracy of the information in the Daily Summary Ledger was tested in each hospital by comparing it to random samples of individual medical

records; no discrepancies were found in several hundred such comparisons.

At three provincial hospitals no Daily Summary Ledger was kept. Here every individual ob-gyn record available was individually examined.

Tu-Du Hospital in Saigon, the country's largest maternity hospital, employs a system of automated data processing. The system uses manual coding of the information derived from hospital records, transposition to punch cards, and compilation. The system specifies eighteen malformation types; these include all those commonly observed, the remainder being categorized as unspecified.

Abortions were distinguished from stillbirths by a recorded fetal weight of less than 750 grams. There were very few that weighed less than 1500 grams.

Malformations were counted in both liveborn and stillborn infants, but not in abortions. More than one malformation was occasionally recorded for a single infant, but in no case were more than three malformations recorded for a single birth.

Hydatidiform moles were counted only if delivered.

Twenty-two hospitals were visited. All are public hospitals in the MOH system. They include 6 districts, 13 provincial, and 3 capital hospitals representing all geographic areas. Data were collected for the decade 1960-1969 in order to include several years before and after the military use of herbicides.

d. Results: A total of 499,119 birth events were counted. These include 480,087 livebirths, 16,166 stillbirths, and 2,866 hydatidiform moles. In addition, 2,355 malformations of all types were counted.

There is a relative lack of data for the first half of the decade, this period contributing only one-third of the data.

The data have been arranged by hospital, by region and by year in order to compare the annual and geographic variations in incidence rates for stillbirths, moles, and malformations.

Only obvious congenital malformations were recorded. Of the 2,355 malformations, forty per cent were not specified to type in the Ledger nor in the individual medical records. Anencephaly, cleft lip/palate, clubfoot and hydrocephaly accounted for over 80% of all specified malformations. Congenital heart disease was not recorded. There were no autopsy records. Unusual deformities, such as those associated with thalidomide, were not observed.

Information was collected concerning the number of acres sprayed annually in Vietnam. Prior to 1966, there was comparatively little use

of herbicides. Since 1966, over 800,000 acres were sprayed annually. The data was grouped into the two time periods, 1960-1965,and 1966-1969.

During the decade there was a gradual decline in the countrywide stillbirth rates and stable mole and malformation rates, weighed by the Saigon experience which contributed 59% of the data.

Sorting the data into two time periods, before (1960-1965) and after (1966-1969), the large scale military use of herbicides failed to show any influence of herbicides. Rather, a downward trend was observed in all categories of abnormal birth events: The stillbirth rate fell from 36.1 to 32.0, the mole rate from 6.6 to 5.6, and the malformation rate from 5.5 to 4.5 per 1,000 livebirths. These rates are within ranges reported for other Asian populations.

e. Discussion of the results: This study can be criticized because it does not directly test a relationship between herbicides and birth abnormalities. Such an assessment would require prospective studies with an examination of one population before and after exposure, or examination of two comparable populations with only one exposed to herbicides. The study would require information on the precise dose of herbicide received, the week of pregnancy, careful examination of newborns and follow-up appraisal. All stillborn and infant deaths would require post-mortem examination. The dose of herbicides could then be related to embryological development of malformations. Such a study is obviously impossible.

The current study has several biases. First, nearly all the information was derived from population centers and the larger hospitals. Secondly, there are no data from private medical sources. Thirdly, the data are almost exclusively restricted to ethnic Vietnamese.

Untoward events limited the availability of records at many hospitals. These include a mortar round in the record room, a flood, records loaned and lost, administrative disposition of records, and the 1968 Tet offensive.

During the earlier part of the decade some hospitals recorded an unrealistically low number of abnormal birth events. Many personnel at these hospitals were frank in admitting incomplete reporting during those years. Most of the hospital directors had been newly appointed and improvement in record keeping occurred coincident with their assignment. This has resulted in more complete reporting during the latter part of the decade and the data in some of these hospitals suggest an upward trend where, in fact, one may not actually exist.

Changes in local obstetrical referral practices also influence rate changes in specific hospitals.

The feasibility of relating the birth event data to the quantity of herbicide sprayed by year by province was studied. The records for

each aerial spray mission were made available by MACV. However, it was not possible to determine what proportion of any province's total yearly birth events had been collected by the study. Our inability to obtain a consistent sample of birth events by province precluded meaningful correlation between herbicide and birth event data.

f. Conclusions: Medical records at Vietnamese medical facilities (MOH public hospital system) were sufficiently complete and accurate to compile birth events data for the decade of the sixties.

The study collected information on 480,087 livebirths, 16,166 stillbirths, 2,866 moles and 2,355 malformations of all types from medical records maintained at 22 hospitals representing the Coastal, Interior, Capitol and Delta geographic regions.

Sorting the data into two time periods, before (1960-1965) and after (1966-1969), failed to show any influence attributable to herbicides. The stillbirth rate fell from 36.1 to 32.0; the mole rate from 6.6 to 5.6 and the malformation rate from 5.5 to 4.5. These rates are within the ranges reported for other Asian populations.

g. Recommendations: Further expansion of this study to more cities and more hospitals would add little useful information and is not warranted considering the enormous effort required.

Interest in abnormal birth events was stimulated wherever the study team visited. Upon revisits, hospital personnel repeatedly informed us that they were now very careful to record all abnormalities, including less significant ones, and personnel were recording their description rather than merely recording the fact of a malformation being present. This new practice will probably result in artificially higher rates for specific abnormalities and probably for all abnormal birth events during the succeeding years.

10. Methemoglobinemia and Malarial Prophylaxis in Vietnam

a. Statement of the problem: Antimalarial chemoprophylactic drugs in current use have the capacity to produce methemoglobinemia among U. S. military populations taking different antimalarials?

b. Background: Chloroquine, Primaquine and Dapsone all have the potential capacity to produce cyanosis (methemoglobinemia). Conrad collected six American soldiers who became frankly cyanotic while taking antimalarials and found that they had a markedly decreased level of nicotinamide adenine dinucleotide (NADH) methemoglobin reductase in their red blood cells. In normal persons methemoglobin forms after exposure to certain oxidant drugs and other chemicals, but it is continuously converted back to hemoglobin by the enzyme. It is postulated that persons taking certain antimalarials may maintain a continuous low level of methemoglobinemia and that this may, in some individuals, build to higher

levels depending upon the relative deficiency (or incomplete penetrance of the gene) of NADH methemoglobin reductase in heterozygotes. The incidence of the enzyme deficiency is unknown. The prevalence of subclinical levels of methemoglobinemia in combat troops in long-term prophylaxis is also unknown and may be significant.

c. Approach to the problem: Four groups were studied:

(1) Control group on no medication
(2) Chloroquine-Primaquine (C/P) alone
(3) Chloroquine-Primaquine plus Dapsone
(4) Chloroquine

A history card was prepared on each subject. Because the C/P tablet is administered on Mondays to American troops, all bloods were drawn on Tuesday mornings. Specimens were drawn in EDTA vacutainers, kept on ice, and determinations were run the same day. The method of Evelyn and Malloy (1938) as modified by Leahy and Smith (1959) was used. The upper limit of normal is 2.5% by this method.

Initially several hundred specimens were obtained and frozen, stored under dry ice, and determinations performed at the conclusion. All the readings were unsatisfactory because of interference caused by the formation of some hemoglobin-albumin complex which caused extremely high readings. Thus the study had to be repeated.

d. Results:

(1) Controls - 90th Replacement arrives who had never taken any antimalarials.

No. drawn	0%	1.0 - 2.5%	2.6 - 4.9%	5.0%
68	54	13	1	0

(2) C/P Alone - 5/60 Inf Bn, 9th Div. This division had never been on Dapsone and consistently had the best malaria record of all major units in Vietnam.

No. drawn	0%	1.0 - 2.5%	2.6 - 4.9%	5.0%
117	64	53	0	0

Examination of the fidelity (history) statements revealed that of those soldiers who were reasonably faithful, one-half have no measurable MetHb and one-half have measurable levels but within normal limits.

(3) Long-term C/P Plus Dapsone - Tropic Lightening Training Center (TLTC) 25th Division (similar to an NCO academy) and D 2/14 Inf,

25th Div. This division consistently has had the best malaria record of all major units experiencing falciparum malaria.

	No. drawn	0%	1.0 - 2.5%	2.6 - 4.9%	5.0%
TLTC	85	20	35	28	2*
D 2/14	102	28	46	25	3*

7.4, 7.8, 6.7, 8.5, 9.3

The combined experience is that 26% show no MetHb, 43% show measurable amounts but WNL, and 31% show abnormally high levels.

Examining the fidelity statements is revealing:

	Faithful (N=75)	Careless (N=112)
No MetHb	21%	29%
WNL levels	40%	45%
Excessive levels	39%	26%

(4) Short-term C/P Plus Dapsone - 7/17 Air Cav Sq, 1st Aviation Bde. This unit had been on haphazard and relatively unsupervised malaria prophylaxis. Beginning 22 days before our visit an intensive campaign was mounted by the local medical staff to assure daily Dapsone and weekly C/P administration to include a medic standing in chow line handing out the tablets and checking off a roster.

No. drawn	0%	1.0 - 2.5%	2.6 - 4.9%	5.0%
168	103	49	15	1*

*6.7

Only 39% had measurable amounts of MetHb in spite of the deliberate and concentrated efforts to assure administration of chemoprophylaxis. This is contrasted with the 25th Div experience:

	25th Div	7/17 Dav
No MetHb	26%	61%
WNL levels	40%	29%
Excessive levels	39%	10%

Examining the fidelity cards reveal that 140 (83%) were in the faithful categories. Yet of this group of 140, 59% showed no MetHb at all!

(5) Non-U.S. Forces - Chloroquine plus Dapsone. Earlier studies by USAMRT personnel had shown a varying but occasionally high degree of

deficiency of G-6-PD and other RBC respiratory enzymes in different native Vietnamese groups. This information aroused a certain reluctance to administer malaria prophylaxis because of the significant amount of hemolysis to be expected in various segments of this enzyme-deficient population.

Because of planned recommitment to an extremely highly endemic area in War Zone D, the B-36 Mobile Strike Force was placed on Chloroquine and Dapsone. Two months earlier it had experienced a severe epidemic of falciparum malaria in the same region.

	No. drawn	0%	1.0 - 2.5%	2.6 - 4.9%	5%+
a. Recon Co.	106	77	23	5	1*
After 14 das of C + D	62	37	29	2	0

*5.1

Recon company had had by far the lowest attack rate of malaria during the earlier outbreak. It had been on Dapsone alone since April, but primarily when in the field. Of the 61 serum pairs collected, 28 remained negative throughout, 26 showed a rise in MetHb and 7 showed a decrease. This unit was comprised mostly of ethnic Cambodians.

b. Co A B 36 III MSF

No. drawn	0%	1.0 - 2.5%	2.6 - 4.9%	5.0%+
96	21	36	29	10*

*5.3(2), 5.4, 5.5(3), 5.7, 6.6(2), 6.7

This unit had been on C+D for three days. However, one-half its members had had clinical falciparum malaria (Rx with quinine) two months previously. This unit was composed solely of ethnic Cambodians.

c. Co B 3-36, III MSF

No. drawn	0%	1.0 - 2.5%	2.6 - 4.9%	5.0%+
97	52	36	8	1*

*14.6

This unit had been on C+D malaria prophylaxis for 2½ weeks. Yet 54% had no MetHb and only 9% had excessive amounts. The unit was 2/3 Vietnamese and 1/6 Chinese and Cambodian each. The MetHb distribution pattern was similar in each group. The person with 14.6% MetHb was a Vietnamese and completely asymptomatic.

e. Discussion of the results: With the possible exception of the individual in Co B, III MSF, it is probable that the methemoglobin levels measured were merely the product of an oxidant drug rather than a deficiency of NADH methemoglobin reductase. None of the individuals were cyanotic and none had any complaints with reference to breathing difficulties.

It is reassuring to demonstrate that C/P can be given to 117 U. S. troops and C/P + D to 355 other U. S. troops - all without difficulty. The 255 Vietnamese (mostly ethnic Cambodians) studied also revealed that C + D can be given to some groups without difficulty.

The significance of these low but definite levels of MetHb is difficult to pinpoint. No behavioral (i.e., memory, vigilance, tracking) or physical tests were administered. It is doubtful whether those persons with the higher measured levels would have fared poorly on such tests. The MetHb levels are not of major importance in themselves. Probably the oxidation of respiratory ferroporphyrin enzymes of RBCs is more important, just as it is their depletion in iron deficiency and not the mild anemia which results that causes symptoms and impaired bodily function.

f. Conclusions: Oxidant drugs, such as antimalarials, can result in measurable levels of methemoglobin in otherwise normal individuals.

The following is a general set of rules drawn from this study:

Category	0%	Measurable	% Expected Elevation	High
No drugs	80%	15%	5%	0
C/P	50%	45%	45%	0
C/P $\pm$ D	25%	45%	25%	5%
C + D (Vietnamese)	40%	40%	15%	5%

The above assumes reasonable or average level of fidelity of prophylaxis, and is intended to be a rough guide concerning the levels of MetHb to be expected under similar conditions.

g. Recommendations: Controlled studies should be accomplished, particularly utilizing the more complex behavioral tasks, in order to determine if the levels of methemoglobin observed in this study, particularly in the range of 5 - 10% (600 - 1500 mg) are associated with a performance decrement. If there is a decrement, then the combination of C/P with D should be avoided by certain groups, such as aviators, for routine prophylaxis even in endemic areas.

11. A Field Trial of Griseofulvin in the Prophylaxis of Dermatophytoses. 1969.

a. Statement of the problem: The high incidence of dermatophytosis among U. S. combat troops operating in the Mekong Delta has strongly indicated the need for an effective preventive measure.

b. Background: Skin diseases are the most significant cause of man-days lost from duty in one's proper MOS, as has been reported in the Command Health Report of every combat unit operating in the Mekong Delta. The wet terrain, particularly during the monsoon season, and the requirements of the tactical situation prevents the regular drying-out of the skin needed to prevent fungal disease.

The principal areas of involvement are the feet, groin, and buttocks followed by the belt line, axilla, trunk, arms and face. The most incapacitating areas of involvement is the boot-sock area because of the frequent secondary bacterial infection and pyoderma.

Griseofulvin has proven of value in the treatment of dermatophytosis since its introduction in 1958. All species of dermatophytic fungi have been found to be susceptible including that commonly found in the Mekong Delta, Trichophyton mentagrophytes. The recommendation that griseofulvin be tested as a causal prophylaxis was first made by Blank in a 1967 report to the Armed Forces Epidemiological Board.

c. Approach to the problem: A maneuver battalion (3/47 Infantry) was selected for the study, which was underway four weeks when the battalion was abruptly sent back to the U. S. A second battalion (5/60 Infantry, also in the 9th Division) was then selected. The study ran for a period of two full months, July and August 1969, during the monsoon season.

Four rifle companies were involved in the study. Two of the companies were placed on a daily dose of two 500 mg griseofulvin tablets and two companies were placed on placebo tablets made by the same McNeil die. Only combat MOS's were involved. All four companies were engaged in the same type of tactical operations with the same frequency. They spent the same amount of time in the field and were exposed to the same type wet terrain. Treatment and control groups were very similar in terms of specific missions (day or night ambushes, checkerboards, insertions and reinsertions, sweeps and patrols).

The study began with a thorough inspection of all field troops and an initial form was completed. Any soldier with dermatophytosis was excluded from the study. The remainder were each given a small plastic vial containing enough tablets, active or placebo, to last about two weeks.

Each time a company came in for "stand down", about once every 6 - 10 days, the troops were thoroughly inspected and vials refilled. Any

observed lesion was recorded on the body diagram form and was cultured on Dermatophyte Test Medium (DTM); the soldier was placed on treatment and was removed from the study.

Cultures were identified and shipped to the University of Miami for confirmation.

d. Results: Since griseofulvin requires about fourteen days to reach maximum levels in the stratum corneum, the data collected during the first two weeks of the study were excluded. Results are:

	No. troops	No. cases	% involved	Man-days	Cases/1000 man-days
Treatment	159	15	9.5	4425	3.4
Control	154	43	27.9	4285	10.0
Total	313	58	18.5	8710	6.7

Ninety-five per cent of the positive cultures grew T. mentagrophytes. There were no side effects.

e. Discussion: The study showed a three-fold reduction in the incidence of dermatophytosis in troops on prophylactic griseofulvin. Yet it was the general consensus that both groups exhibited a marked drop in the incidence of all disabling skin diseases. This same pattern of disease reduction had been previously observed in other battalions and by other investigators. Under these circumstances it is difficult to sort out the improvement due to the treatment from improvement due to an uneven "Hawthorn effect." The study can be criticized for its poor control design. Admittedly, the allocation of individuals into treatment and control groups by serial number would have been a superior course of action.

Fidelity of pill-taking was probably optimum. The study team lived with the battalion throughout the period and occasionally accompanied the troops on short operations. Rapport was excellent with the hierarchy in each company, and the study aroused the interest and the cooperation of the senior sergeants. Thus it can be said that this study was not a fair trial of the entire system, but rather represented a set of fairly favorable circumstances to measure the activity of an agent under field conditions.

f. Conclusions: Administration of 1 gm griseofulvin as a daily prophylaxis resulted in a three-fold reduction in the incidence of dermatophytosis in combat troops in the Mekong Delta. The study was inadequately controlled and occurred under favorable conditions. All skin problems in both treatment and control groups were reduced in number and frequency during the study.

g. Recommendations: The study should be repeated with emphasis on testing the entire system under more natural conditions, i.e., without

the direct influence of the presence of the WRAIR investigators stimulating all parameters to improve. Additionally, the study should be better controlled.

12. A Field Trial of Griseofulvin in the Prophylaxis of Dermatophytoses - 1970.

a. Statement of the problem: A more rigidly controlled study of the entire griseofulvin-soldier-environment system is needed to better assess its capability to prevent dermatophytosis.

b. Background: See previous study, this report.

c. Approach: Two battalions were selected, 2/60 and 5/60 Infantry, 9th Division, located in the Mekong Delta. The study was again limited to line companies, some 500 men per battalion initially eligible. Assignment to treatment or control group was by SSAN. The same procedures were used (or were attempted) as in the 1969 study. The study began in one battalion in March and the other in April 1970 in order to bracket the rainy season expected in mid-June.

Skin biopsy material was obtained for griseofulvin analysis.

d. Results: Only 491 individuals completed the study period of at least 6 to 8 weeks.

	No. Completed	No. Cases	%
Total			
Treatment	238	53	22.3
Control	253	67	26.5
2/60 Infantry			
Treatment	106	24	22.6
Control	99	28	28.3
5/60 Infantry			
Treatment	132	29	22.0
Control	154	39	25.3

It would appear that under these conditions that griseofulvin does not appear to exert any protective effect. The data are being further refined and the biopsies processed.

Surprisingly, not one side effect was discovered.

e. Discussion: The study was interrupted by frequent moves of the battalions and made difficult by the scattered locations of the companies. Much of the study occurred during the Cambodian campaign of May-June 1970 and not in the Mekong Delta. The weather was much hotter in Cambodia than in the Delta and there were many more hardships. Analysis of biopsies will

reveal whether the administration of the drug did not work because it was not taken (actually, did not get into the skin) or because its presence in the skin did not protect.

f. Conclusions: A severe field trial of the entire griseofulvin system conducted in two battalions failed to reveal any protective effect of the drug.

g. Recommendations: Griseofulvin should not be adopted as routine prophylaxis for dermatophytosis. Additional studies should be conducted, including field trials under other circumstances, to more precisely define its value.

13. A Comparative Assessment of Pressure-Limited and Volume-Limited Respirators for Long-Term Ventilatory Support.

a. Statement of the problem: Objective data comparing pressure versus volume-limited respirators is sorely needed in order to determine appropriate criteria for selection of the type of respiratory assistance apparatus.

b. Background: Pressure-limited respirators (Bird and Bennet) had been in common use at U. S. military hospitals in Vietnam, and volume-limited respirators (Emerson and Engstrom) were introduced on a limited basis. The selection of the type of respirator was usually determined by availability rather than by objective criteria. The clinical observation was soon made that some patients appeared to do better on Emerson (volume) respirators.

c. Approach to the problem: All patients at the 24th Evacuation Hospital requiring prolonged ventilatory support entered the study. In all there were 128 patients of whom 81 were studied in detail. Of these, 42 patients were switched back and forth from volume to pressure-cycled respirator and vice versa on 65 occasions.

All patients were carefully monitored clinically and were followed with blood gas and pulmonary mechanics studies.

d. Results: Data collection is complete and is being analyzed. Rough preliminary results confirm the earlier clinical impression that volume-limited respirators are superior in certain patients.

With total chest compliance as an indicator:

	No. of Patients		
	Improved	Worsened	Total
Switch			
Pressure to Volume	28	4	32
Volume to Pressure	6	27	33

With arterial oxygenation as an indicator:

	No. of Patients		
	Improved	Worsened	Total
Switch			
Pressure to Volume	26	6	32
Volume to Pressure	7	26	33

e. Conclusions and Recommendations: None, as yet. It is anticipated that analysis of the data will provide guidelines for which type of respirator is most suitable for several situations, and also indicate those situations in which either type is suitable.

14. Analysis of Liver Injuries in Combat Casualties.

a. Statement of the problem: Experience and review of a large number of liver injuries are needed in order to assess the influence of various operative and postoperative care procedures upon morbidity and mortality.

b. Approach to the problem: All liver injuries admitted to the 24th Evacuation Hospital were followed prospectively to determine what factors influence morbidity and mortality. Clinical material was collected as well as liver function studies.

c. Results: Data collection is complete and is still being analyzed. There were 81 patients in the study. Sixty per cent of the liver injuries were due to low velocity gunshot wounds or to shell fragments; forty per cent were due to high velocity missiles or to blunt trauma.

Multiple injuries were involved in 76 (94%) of the patients. There were 21 deaths in the series (26%) including five intraoperative deaths.

Major liver resections were performed in fourteen patients (18%) with an operative mortality of 57% (8 of these patients died). These 14 patients received an average of 27 units of blood. No patients survived who received over 30 units of blood. Conversely, there was only one death in the patients who received less than 20 units.

External biliary drainage was employed in 22 (27%) of the patients. Bleeding stress ulcers developed in 7 (32%) of these 22 patients. Conversely, only one patient (1.9%) developed stress ulcers of the remaining 54 who survived the operation but were not drained exteriorly. The severity of the overall injuries was the same in both groups, their only difference being that the group who received T-tubes had more severe liver injuries.

There were four complications due to the T-tube and two of these patients died of hepatic insufficiency.

d. Conclusions: The most effective methods for the rapid control of hepatic hemorrhage are being compiled for each type and degree of injury.

The data suggest a striking correlation between the diversion of bile by external drainage and the production of bleeding stress ulcers in severely injured patients.

e. Recommendations: The association of external biliary drainage with stress ulcers should be looked for in other series of patients and followed up in the laboratory.

15. Use of Diuretics in the Treatment of Early Wet Lung Syndrome.

a. Statement of the problem: Wet lung syndrome (WLS) has been a major factor in the morbidity and mortality of severely wounded patients. The goal was early identification of those patients developing WLS and demonstration of an effective therapy.

b. Background: There have been numerous descriptive reports of WLS, the common factor being a major insult, traumatic or surgical. The mechanisms for the development of WLS are obscure and perhaps multiple. Isotopic techniques have recently shown that these patients have an apparent increase in pulmonary interstitial fluid. Earlier workers at this unit reached the conclusion that in many instances WLS may be the result of excessive fluid given during resuscitation. Certainly most of the patients who develop WLS have been severely injured and have received large volumes of replacement fluid or colloid. It is extremely difficult to determine the actual volume of fluid lost.

c. Approach to the problem: During the period 1 January - 1 July 1970 the 24th Evacuation Hospital performed major surgery on over 2000 patients of whom 127 received postoperative ventilatory assistance. Ten patients entered the study. Determinations were made twice daily for two days, then daily for three days, then every other day. These included arterial gases, minute volume, tidal volume, and peak inspiratory pressure, from which the pulmonary compliance was calculated. All the ten patients were receiving ventilatory support via tracheostomy and all had positive tracheostomy cultures (Klebsiella, aerobacter and/or pseudomonas). All patients were on antibiotics. None had thoracic injuries.

Early in the study it was noted that when pulmonary compliance drops, a reason should be vigorously sought. As a rule, this was found to be due to heavy secretions, tracheostomy tube position, atelectasis, consolidation, or pneumothorax. However in a few patients there was a rapid decline in compliance for no apparent reason; it was felt that this represented incipient or early WLS. All ten patients had audible rales and two were in pulmonary edema.

The amount of initial fluid replacement and the fluid balance from time of surgery were monitored. The ten patients averaged over 11,000 cc

initial replacement (6,500 - 16,000 cc). Thereafter all but one was in positive fluid balance until the onset of WLS. The onset of WLS occurred 2 - 15 days post-injury with a mean of 5.2 days.

Upon diagnosis of WLS, CVP and chest x-ray were taken. Furosemide, 40 - 80 mg., was administered intravenously (one patient also received 25 mg ethacrynic acid). Urine volume was measured over the next two hours and all determinations were repeated 1½ - 2 hours following therapy.

d. Results: All patients responded dramatically with a striking increase in compliance within 1½ - 2 hours. In every instance this was accompanied by an increase in arterial oxygenation. There was no change in CVP. Urine output in the first two hours averaged 2,165 cc with a range of 1,155 - 3,800 cc. Both patients in pulmonary edema promptly cleared and rales decreased in 7 of the remaining 8 patients. The eighth patient had pneumonitis. Two of the patients were re-treated for similar problems 1 and 4 days later with similar results. Seven of the patients were long term survivors, breathing room air and maintaining arterial pO_2 greater than 80 mm Hg. Three died of sepsis and head injury. Autopsy in two were negative for fat stains.

This therapy was applied to 3 cases of blast injury to the lung and was found to be without benefit.

e. Discussion: Auscultation and chest x-ray showed a picture compatible with fluid overload, yet the CVP was normal in each. This led to the postulate that in some patients, for reasons that remain obscure, the left ventricle may fail before the right thus allowing fluid to accumulate in the lungs without a concomitant rise in CVP. If this situation does occur, the clinician who relies heavily on CVP to guide fluid administration could easily administer an excess with eventual mobilization and accumulation in the lungs.

Prior to this study, the 24th Evacuation Hospital had an average of over 2 deaths per month attributed to WLS. During the six months of the study and the three months following there were no deaths attributable to WLS.

f. Conclusions: In patients with normal CVP a decrease in pulmonary compliance and arterial oxygenation heralds the onset of WLS. Treatment of ten patients with Furosemide resulted in striking improvement in compliance and arterial oxygenation and diuresis of 1,155-3,800 cc -- all in 1½ - 2 hours. Fluid balances in these patients suggest overhydration to be the etiology, or at least a component, of WLS.

g. Recommendations: The routine measurement of pulmonary compliance is suggested as a simple, inexpensive and non-invasive method of detecting the early onset of WLS, as well as other problems in patients requiring ventilatory assistance. Therapy with strong diuretics is indicated when WLS is suspected.

Further studies on pulmonary artery and left atrial pressures in WLS may contribute to our understanding of this problem.

16. A Prospective Study of the Etiology and Mechanisms of Stress Ulcer.

a. Statement of the problem: Bleeding stress ulcer is a disastrous complication occurring in 6% of the severely wounded (SI and VSI) admitted to the 24th Evacuation Hospital. One-third of these patients die.

b. Background: This study is a follow-on to earlier work performed by this unit. The etiology of the disease is not understood, and a rational approach to prevention and treatment awaits clarification of the complex mechanisms involved.

c. Approach: Injured patients were studied prospectively with sequential biochemical and endocrine studies. These include histamine, serotonin, renin, angiotensin, gastrin, cortisol, urinary 17-OH ketosteroids and VMA. Selected patients were examined by gastroscopy.

d. Results: A total of forty patients were studied. Laboratory determinations are in process. Fifteen patients were gastroscoped and photographed.

e. Conclusions and Recommendations: None, as yet.

17. Arterial Hypoxemia in Patients with Infectious Hepatitis - Apparent Pulmonary Shunting.

a. Statement of the problem: In an earlier study of in-flight evacuation problems in surgical patients, hepatitis patients utilized as controls were observed to have significantly decreased arterial oxygenation.

b. Approach to the problem: Serial blood drawings were obtained on patients hospitalized with infectious hepatitis. Blood gas levels, oxygen-hemoglobin dissociation curves, and 2-3 DPG levels were determined.

c. Results: Material has been collected from 26 patients and is being analyzed.

d. Conclusions and Recommendations: None, as yet.

18. The Role of 2-3 Diphosphoglycerate in Oxygen Transport Following Massive Blood Transfusion.

Statement of the problem: Banked blood has low levels of 2-3 DPG which is necessary for oxygen transport from the hemoglobin molecule to the cell. Massively transfused casualties may have a low level of circulating 2-3 DPG regeneration. Similar studies were performed in patients before, during, and after air evacuation to Japan.

c. Results: Laboratory samples on 32 patients are being processed at the University of Pennsylvania and at USAMRL, Fort Knox, Kentucky.

d. Conclusions and Recommendations: None, as yet.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 304, Military medical research program SEA, United States Army Medical Research Team, Vietnam

Literature Cited.

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OA 6462	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	305
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(A			

11. TITLE (Procede with Security Classification Code)*

(U) Military Medical Research Program, SEASIA, WRAIR - Zoonoses (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	3.5	60
FISCAL YEAR CURRENT 71	2.5	45

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL NAME: Meroney, COL, W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Division of Veterinary Medicine
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Asbill, COL, S. G.

TELEPHONE: 202-576-5193

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Alexander, Ph.D., A. D.

NAME: Rogul, Ph.D., M. DA

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Zoonoses; (U) Leptospirosis; (U) Melioidosis; (U) Epidemiology; (U) Serology

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) To study militarily important diseases transmissible from animals to man. Specific emphasis is on the improvement of treatment and control measures for melioidosis and leptospirosis, improvement of laboratory diagnostic procedures for melioidosis, determining natural history and occurrences of these diseases.

24. (U) Conventional and improvised microbiological and chemical technics are used.

25. (U) 69 07 - 70 06 To date, in vitro tests in single or combined drugs have provided no new approaches for therapy of melioidosis. Reported high efficacy of rifampin for melioidosis was not verified. Factors relating to development and characteristics of smooth and rough colonial types of melioidosis organisms have been resolved and will serve to improve laboratory diagnostic procedures. Approximately 300 cases of melioidosis in servicemen have been affirmed at WRAIR since the winter of 1965-1966. Ampicillin has high therapeutic efficacy in experimental leptospiral infections in hamsters and appears to be a promising drug for treatment and prophylaxis. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 305, Military Medical Research Program, SEA, WRAIR - Zoonoses

Investigators.
Principal: COL Stephen G. Asbill, VC
Associate: A. D. Alexander, Ph.D.; M. Rogul, Ph.D.; L. Evans, B.S.; A. Warner, Jr.; S. Schwarting, M.S.; L. Williams

1. Leptospirosis.

Two newer antibiotics, rifampin and ampicillin were examined for antileptospiral activity using a previously developed hamster-infectivity system (WRAIR Annual Report, 1966-1967). Rifampin had no activity, whereas, ampicillin had high antileptospiral activity. The activity of ampicillin was compared to that of penicillin G and aureomycin using the bataviae hamster-infectivity system. In the initial tests, weanling 30 to 40 gram hamsters were infected with 75,000 organisms. Test drugs in a peanut oil base were given s.c. in a single dose 48 hours after infection using 5 animals per dose level. Findings are summarized in Table 1.

Table 1. Therapeutic Effect of Ampicillin, Penicillin G and Chlortetracycline for Weanling Hamsters Infected with Serotype bataviae (1415)*

Dose mg/Kg	No. dead / No. tested (MST)** when treated with Ampicillin	Penicillin G	Chlortetracycline
640	2/5 (13.0)	3/5 (6.0)	0/5
160	1/5 (7.0)	5/5 (5.8)	0/5
40	2/5 (7.0)	5/5 (5.8)	0/5
20	2/5 (6.5)	5/5 (5.0)	1/5 (5.0)
10	1/5 (5.0)	5/5 (4.8)	3/5 (5.0)
5	2/5 (5.0)	5/5 (4.8)	4/5 (5.7)

* Animals infected i.p. and treated 2 days later. Drugs given in peanut oil base in single dose s.c.

** Controls: 10/10 (5.1). MST = Mean survival time in days.

The activity of ampicillin was comparable to that of aureomycin and 64 to 128 fold greater than that of penicillin G.

Additional tests were designed to determine the effect of delay in treatment on the efficacy of the ampicillin as compared to penicillin G and tylosin in protecting adult hamsters (120 to 140 grams) with a 8-9 day course of fatal bataviae (1415) infection. For this purpose, preliminary tests were done to determine maximal tolerated doses which were chemotherapeutically effective. Adult hamsters were infected i.p. with approximately 1000 organisms and divided into groups of 5 for treatment regimens. Treatment was started on the third day after infection and continued for 5 days. Drugs were given s.c. twice daily at 6 hour intervals. Tylosin was included to afford comparisons with previous findings (Wood and Alexander, Antibiotics and Chemotherapy 11: 592, 1961). The results (Table 2) again provided evidence that ampicillin had greater antileptospiral activity than penicillin G.

Table 2. Therapeutic Effect of Ampicillin, Penicillin G and Tylosin for Adult Hamsters Infected with Serotype bataviae (1415)

Drug	Dose* mg/Kg/day	Treated No. dead / No. tested (MST)**	Drug controls No. dead / No. tested
ampicillin	24	5/5 (8.2)	3/5
	12	3/5 (9.0)	2/5
	6	1/5 (8.0)	
	3	1/5 (8.0)	
penicillin G	24	0/5	0/5
	12	3/5 (14.0)	1/4
	6	4/5 (13.8)	
	3	4/5 (14.2)	
tylosin	24	0/5	0/5
	12	0/5	0/5
	6	1/5 (14.2)	
	3	1/5 (14.0)	

* Drugs given 2 X daily s.c. for 5 days beginning on day 3 after infection.

** Controls 7/7 (8.2). MST = Mean survival time in days.

In these tests ampicillin and tylosin provided comparable protection of hamsters at dosage levels of 3 and 6 mg/Kg/day. Higher doses of ampicillin were toxic for hamsters.

Tests to determine the efficacy of 3 antibiotics when treatment at 3 and 6 mg/Kg/day was delayed 3 to 7 days were similarly conducted in bataviae-infected adult hamsters. In these tests the infectivity dose was lighter than anticipated. The mean survival time (MST) in

controls was 8.9 days (range 8-10 days) and 3 of 25 controls survived instead of the planned MST of 8 days with 100% fatality. Nevertheless, tests served to demonstrate not only the greater protective efficacy of ampicillin over penicillin G but also its superior curative properties for leptospiruria. To determine leptospiruria, surviving animals were killed on the 22nd day of the experiment for microscopic, and if necessary, for cultural examinations of kidneys. The findings are summarized in Table 3. Ampicillin protected all animals at both doses when treatment was delayed as late as 7 days after infection. The same protection was afforded by tylosin except for one death. Forty-one of 50 hamsters treated with penicillin survived and leptospiruria was found in all groups of surviving animals except those given higher doses on the third day of infection. Leptospiruria was detected in none of the ampicillin treated hamsters. The curative property of ampicillin was also greater than tylosin which had not eliminated leptospiruria when treatment at lower dose was started after the fourth day of infection.

2. Melioidosis.

a. Serological studies of human cases. Continued support was provided to Armed Forces Medical Laboratories for the laboratory diagnosis of melioidosis. During the period of this report, 69 cases of melioidosis were confirmed. Approximately 300 cases of melioidosis in U. S. Armed Forces personnel have been affirmed by this laboratory since winter of 1965-1966. Laboratory diagnosis of melioidosis was also established for 2 veterans who had served in Southeast Asia and in a newborn child at Tripler General Hospital.

Evaluation of hemagglutination (HA) and complement-fixation (CF) tests for melioidosis based on test findings on sera from 185 proved cases were presented in the previous annual report. Findings were extended to include 27 additional cases. New tabulations of data on correlation of HA and CF test findings on distribution and range of titers at different time intervals during and after the course of disease were essentially the same as shown previously. Additional clinical data were obtained to determine the relative occurrence of transient low titer or seronegative HA and CF reactions in patients with various types of infection, and also, to evaluate the possible clinical significance of persistent antibodies. The types of serological reactions observed from 8 days to 6 months after disease onset in serum samples from 198 patients are summarized in Table 4. Appropriate comparative data were available for 198 of the 212 cases. Variable positive and negative reactions in serial serum samples were seen in 8 of 24 patients with localized wound or burn infections and were primarily manifested in HA tests. Disparate HA and CF test reactions occurred in approximately equal frequency in 11% of the patients with localized pulmonary disease. Inconsistent serological reactions occurred in 3 of 25 patients with other forms of disease. Approximately half of the "negative" sera were marginally reactive, viz., partial

Table 3. Effect of Delay in Treatment on Chemotherapeutic Efficacy of Ampicillin, Penicillin G and Tylosin for Hamsters Infected with Serotype Bataviae (1415)*

Days treatment delayed**	Drug dose Mg/Kg/day	Ampicillin		Penicillin G		Tylosin	
		No. dead / total	Leptospiruria in survivors	No. dead / total	Leptospiruria in survivors	No. dead / total	Leptospiruria in survivors
3	6	0/5	0/5	2/5	0/3	0/5	0/5
4		0/5	0/5	0/4	1/4	0/5	0/5
5		0/5	0/5	1/5	3/4	0/5	0/5
6		0/5	0/5	0/5	5/5	0/5	0/5
7		0/5	0/5	0/5	5/5	0/5	0/5
3	3	0/5	0/5	2/5	2/3	0/5	0/5
4		0/5	0/5	0/5	5/5	0/5	0/4
5		0/5	0/5	2/5	3/3	0/5	2/5
6		0/5	0/5	0/4	4/4	0/5	2/5
7		0/5	0/5	2/5	3/3	1/5	2/4

* Controls: 22/25 deaths; mean survival time 8.9 days.

** Drugs given 2 X daily for 5 days.

but less than 50% fixation at 1:4 dilution on CF test or reactive at 1:20 dilution on HA test. Transient seronegative reactions were seen with either HA or CF test but rarely with both tests.

Table 4. Types of Serological Reactions* in Patients with Various Forms of Melioidosis

Type of infection	Number of patients**				
	Total	HA + CF +	HA + CF v	HA v CF +	HA v CF v
Localized pulmonary	100	89	4	5	2
Pulmonary and other organ	15	13		2	
Localized wound	24	16	2	6	
Wound and other organ	5	5			
Septicemia	7	6		1	
Fever undetermined origin	9	8	1		
Other	9	8	1		
Unknown	29	29			
Total	198	174	8	14	2

* 8 days through 6 months after disease onset.

** Symbols: + = positive; v = variable negative and positive.

Thirty-one patients provided serum samples 9 months or more after disease onset. Persistence of HA and CF antibodies was demonstrated in 80-100% of the samples obtained at various time intervals from 9 months to 2-1/2 or more years after disease onset. The data may have been biased by the disproportionately higher representation of patients (20 of 31) who had prolonged or persisting infections of 6 or more months duration. Nine patients in this series had responded well to antibiotic treatment and had a relatively limited clinical course of disease or infection. The disease history on 2 patients was incomplete. The late serological findings in 9 patients with limited and in 20 patients with prolonged infections are summarized (Table 5). Both CF and HA antibodies were detectable in all but 3 patients. The 3 exceptions had variable positive and negative reactions during their course of disease. Within the limitations of the number of patients tested, the geometric mean HA and CF titers of patients with prolonged infection were one and one-half to three-fold higher than those of patients with a limited disease course. Four of 7 patients had recurrent episodes of disease when specimens were taken two or more years after disease onset. Serum from 1 of the 4 patients was obtained seven years after initial disease. The duration of positive serological reactions could not be related to the type of disease. Persistent high titer reactions were seen in patients with subacute disease of several weeks to several months duration, as well as in patients with prolonged complicated infections.

Table 5. Persistence of HA and CF Antibody Titers in Patients with a Limited or Prolonged Course of Infection*

Time after disease onset	No. patients positive/No. tested (GM titer) Limited infection HA	CF	Prolonged infection HA	CF
9-12 months	3/4 (269)	4/4 (54)	9/10 (442)	9/9 (119)
1-2 years	6/6 (254)	5.6 (20)	10/11 (467)	10/11 (50)
➢2 "	2/3 (59)**	3/3 (40)***	6/7 (177)	7/7 (86)

* Patients with limited infection responded well to antibiotic treatment and were free of signs of infection within a 6 month period. The duration of signs of disease or infection in prolonged infections was 6 months or longer.

** Time of sampling: 2 yrs., 1 mo. (HA-neg); 2 yrs., 8 mo.; 3 yrs., 2 mo.

*** Time of sampling: 2 to 2-1/2 yrs. - 3 patients (one HA test negative); 3 to 3-1/2 yrs. - 3 patients; 7 yrs. - 1 patient (chronic case).

b. Intra-strain growth inhibition of P. pseudomallei. Relationships of bacteriocin-like properties of strains with colonial, cultural and physiological characteristics had previously been reported (WRAIR Annual Reports 1968 and 1969). Understanding these relationships is important for laboratory diagnosis and may have a bearing on the pathogenicity and drug sensitivity of strains. It was previously reported that the probable growth inhibitor was D-arginine or D-lysine. This conclusion was derived from the results obtained from thin layer chromatography (TLC) of broth supernatant fluids from cultures of P. pseudomallei strain 165 in Rice's protein-free medium. The non-inhibitory strain 7815 did not elicit a ninhydrin positive spot that was exhibited by inhibitory strains. Subsequently, it was found that this spot was not consistently reproducible and the conclusion was erroneous. The TLC findings could not be corroborated by quantitative amino acid analysis of 3 day old agar culture expressed fluids. However, it was found that the agar fluids of an inhibitor culture contained very large amounts of ammonium ions, whereas, the non-inhibitor strain contained even more. This seemed to contradict the fact that inhibitor cultures had an alkaline pH of 8.6 and non-inhibitors pH 6.8 after 3 days incubation on agar. The only possible explanation was that the rough non-inhibitor culture (strain 7815) was producing a highly acidic metabolite in addition to ammonium ions. This conclusion seemed even more reasonable when the findings of Nicholls (Brit. J. Exp. Path. 11: 28, 1930) were considered. Nicholls reported that the rough strains of P. pseudomallei produce oxalic acid, whereas, the smoo ains do not. We then qualitatively verified Nicholls' finding nhibitor strain 165 and non-inhibitor strain 7815.

The amounts of NH_4^+ produced in Wahba agar and broth cultures were determined, so that natural inhibition and its neutralization could be simulated by using varying amounts of NH_4^+ and oxalic acid incorporated into agar media. The procedure was complicated by the difficulty of assessing NH_4^+ in agar. On the average, the amounts of NH_4^+ accumulating in various cultures after 3 days incubation were as follows:

P. pseudomallei	Wahba agar	Wahba broth
Smooth-inhibitor strain 165	0.025% NH_4^+	0.006% NH_4^+
Rough non-inhibitor strain 7815	0.085% NH_4^+	0.013% NH_4^+

The amounts of NH_4^+ produced in broth were considerably lower than the amounts produced in agar. This was attributed to higher NH_4^+ production in the presence of oxygen. Ammonium hydroxide was incorporated into Wahba agar. A final concentration of 0.085% NH_4OH yielded an agar medium of pH 8.4. Oxalic acid was added to this medium to provide a spectrum of pH from 8.4 to 6.7. It was found that inhibition in both S and R strains occurred at pH 8.4 (0.0% oxalic acid) and pH 8.1 (0.04% oxalic acid) when cultures were streaked across these agar plates. No inhibition occurred at pH 7.8 (0.07% oxalic acid).

Physiologically, the inhibitory action of smooth strain 165 could be overcome by incorporating 3% glucose into Wahba agar. The addition of 3% glycerol did not overcome the inhibitory action of strain 165. In order to facilitate quantitative measurements, an attempt was made to reproduce the oxygen tension of agar cultures in broth cultures. Therefore, 175 ml of Wahba broth was placed into wide bottomed Fernbach flasks of 2800 ml capacity. This provided a very shallow medium (approximately 0.5 cm depth) resulting in a large surface area exposed to air. Another flask was filled the same way with Wahba broth containing 3% glucose. Each flask was inoculated with 0.1 ml of an overnight brain heart infusion broth culture of either strain 7815 or 165. The cultures were placed in a shake incubator at 37 C and allowed to remain stationary for 3 hours. The cultures were shaken overnight. Growth was so heavy the next morning that shaking was discontinued for the remainder of the experiment. Table 6 summarizes the results obtained from this experiment. In general, these results seem very similar to the conditions that occur in agar. As expected, the growth of both strains increased in all media during the first 24 hours. At the end of three days, strain 165 Wahba broth culture had a rise in pH from 7.3 to pH 8.5, the viable cell count was decreased, the amount of ammonium ions was high and there was little if any oxalic acid. In contrast, strain 7815 had a pH decrease from pH 7.3 to 6.8, the viable cell count was still elevated and the amounts of ammonium and oxalic acid were very high. The results from the preceding experiments were conclusive that the inhibitory substance produced by smooth strains on Wahba agar was NH_4^+. It is also evident that rough strains neutralize this toxicity by the production of oxalic acid.

Table 6. Comparison of a Smooth and a Rough Strain of P. pseudomallei Grown in Shallow and Shaken Broth Cultures

Strain	Medium	Viable cells/ml	Colony type	Broth pH	% NH_4* X 10^{-3}	Oxalic acid mg/100 ml
165	Wahba					
	0 hrs	2.1 X 10^5	smooth			
	24 hrs	3.3 X 10^9	smooth	8.2	15.51	2.47
	48 hrs	3.0 X 10^9	smooth	8.1	26.24	0.66
	72 hrs	1.0 X 10^8	smooth	8.5	26.62	4.02
7815	Wahba					
	0 hrs	7.0 X 10^5	rough			
	24 hrs	9.2 X 10^9	rough	7.5	20.00	66.81
	48 hrs	1.1 X 10^{10}	rough	6.9	55.80	172.57
	72 hrs	1.4 X 10^{10}	96% rough**	6.8	74.31	231.52
165	Wahba + 3% glucose					
	0 hrs	2.1 X 10^5	smooth			
	24 hrs	3.3 X 10^9	smooth	5.9	1.23	9.72
	48 hrs	8.9 X 10^8	smooth	4.0	1.48	0.91
	72 hrs	7.1 X 10^5	smooth	3.8	1.14	5.17
Control	Wahba			7.3	1.62	2.29
	Wahba + 3% glucose			6.5	1.89	3.13

* % NH_4 is customarily expressed as grams of NH_4 per 100 gms of sample.

** The variant 4% gave rise to mucoid and very small rough colonies.

Interestingly, when the composition of Wahba broth includes 3% glucose, very little ammonium ions are detected in cultures of strain 165, and little if any oxalic acid is found, but the pH decreases from 6.5 to 3.8. It is obvious that another acidic metabolite is being formed and quite possibly combining with the NH_4^+ for the purpose of synthesis. Although the acidity seems to decrease the viable cell count, it should be remembered that in agar cultures, the 3% concentration of glucose neutralized the inhibitory action of strain 165, even though 3% glycerol did not.

The conclusion that ammonium ions were inhibitory was further verified by comparing the action of pronase and trypsin solutions on agar cultures of the inhibitory strain and Wahba agar brought to pH 8.7 by NH_4OH. In both cases the inhibitory effect was completely neutralized by pronase and partially by trypsin; thus, indicating that these compounds were acting more as protective proteins rather than enzymes.

c. Chemotherapy. Hobby et al. (Am. Rev. of Resp. Dis. 99: 952, 1969) reported that the antibiotic rifampin was highly reactive against P. pseudomallei in vitro (minimum inhibitory concentration 0.04 µg/ml) and in vivo. These findings were based on studies conducted with one strain of P. pseudomallei. In view of current critical needs in chemotherapy of melioidosis, it was deemed important to verify the reported activities of rifampin; extensive in vitro tests were done with 31 recently isolated strains. A broth dilution procedure in microtiter plates was used (Marymount and Wentz, Am. J. Clin. Path. 45: 548, 1966. On day of test, rifampin was dissolved in either a 20% solution of ethyl alcohol or a 10% solution of N-N-dimethyl formamide in a 0.15 M phosphate buffered (pH 7.3) physiological salt solution to provide a stock solution of 1000 mcg rifampin per ml. Stock solution was then diluted 10-fold with phosphate buffer. Further doubling dilutions were made with Mueller-Hinton broth in U-bottomed microtitration plates with a 0.05 ml microtiter loop. Other antibiotics used for comparative tests with rifampin were dissolved in phosphate buffer and diluted in the same way as rifampin. Overnight cultures initially in Mueller-Hinton broth and subsequently in Mueller-Hinton broth containing 0.1% KNO_3 were used as a source of inoculum. The addition of KNO_3 to Mueller-Hinton broth enhanced growth with little or no formation of a surface pellicle. The density of the cultures were adjusted spectrophotometrically to OD of 0.15 at 600 mµ wavelength with the use of 18 X 150 mm pyrex test tubes as cuvettes, and then serially diluted 10-fold with broth to provide various concentrations of organisms used in tests. The number of organisms contained in the culture was determined by conventional agar plating technics.

The desired dilution of culture was added to each dilution of antibiotic in the microtiter wells in 0.05 ml amounts with a calibrated dropper. Microtiter plates were covered with an adhesive transparent plastic, and incubated at 35 C. Readings were made after 24 and 48

hours of incubation. An appropriate dilution of an overnight broth culture of Sarcina lutea was used to affirm the activity of rifampin with the use of the microtitration technic. Diluent growth and sterility controls were included for each test run.

Initially, the sensitivity on 31 different strains of P. pseudomallei to rifampin was determined with the microtiter technic using a concentration of organisms ranging from 1.2×10^5 to 1.1×10^6 per ml. The minimal inhibitory concentration (MIC) of rifampin after 24 hours incubation was 25 mcg/ml for 3 strains and greater than 25 mcg/ml for 28 strains.

Two of the 31 strains which differed in their sensitivity to rifampin at test level of 25 mcg/ml were selected for additional tests to determine if inoculum size had a bearing on drug inhibitory level. Tests were set up in triplicate with 10^{-3}, 10^{-4} and 10^{-5} dilutions of the standardized overnight cultures in Mueller-Hinton broth. The final concentration of organisms of the highest dilutions of the two test cultures were 500 and 650 organisms per ml. The MIC's of rifampin after 24 hours incubation are summarized in Table 7. After 48 hours, MIC values were usually 2-fold higher. The findings would serve to indicate the presence of cells with relatively high rifampin resistance in cultures of P. pseudomallei.

Table 7. Effect of Culture Inoculum Size on Minimal Inhibitory Levels (MIC) of Rifampin for 2 Strains of Pseudomonas pseudomallei

Strain	Final concentration of colony forming units per ml	MIC mg/ml
8202	50,000	> 25
	5,000	25
	500	25
8656	65,000	25
	6,500	12.5
	650	6.25
Control Sarcina lutea	50,000	0.045
	5,000	0.045
	500	0.045

Repeat tests using variable concentration of cells were done on the two strains and on 4 additional strains. The cultures were grown in Mueller-Hinton broth containing 0.1% KNO_3. Three each of the cultures produced predominantly smooth and rough colonies, respectively, when plated on nutrient agar containing 3% glycerol. There was no apparent

relationship between smooth and rough forms of P. pseudomallei and relative sensitivity to rifampin.

Three strains were selected to compare the activity of rifampin to other antibiotics. One of the strains was known to be chloramphenicol resistant. A 10^{-4} dilution of overnight cultures in Mueller-Hinton broth containing 0.1% KNO_3 was used for tests. Results are shown in Table 8. Rifampin had relatively poor activity for P. pseudomallei when compared with tetracycline and chloramphenicol -- drugs now commonly used in the treatment of melioidosis.

Table 8. Comparative Activity of Rifampin and 6 other Antibiotics for 3 Strains of P. pseudomallei*

Drug	Minimal inhibitory concentration (ug/ml) for strains		
	8202	8565	8624
chlortetracycline	12.5	6.25	12.5
tetracycline	12.5	6.25	3.13
chloramphenicol	25.0	25.0	> 100
kanamycin	> 100	50	100
penicillin G	100	50	100
polymyxin	> 100	> 100	> 100
rifampin	> 50	50	50

* A 10^{-4} dilution of a Mueller-Hinton broth culture containing 0.1% KNO_3 was used as inoculum. Concentration of organism in antibiotic-culture mixtures ranged from 1.95 to 2.5 X 10^5.

A chloramphenicol-resistant and chloramphenicol-sensitive strain was used to test effects of various combinations of antibiotics. The procedure was the "checkerboard" tube dilution method of Sabath et al., (Antimicrobial Agents and Chemotherapy, 1956, pp. 149-155) modified for use with microtiter technics. None of the following combinations had a synergistic bacteriostatic effect on the test strains of P. pseudomallei: carbenicillin + gentamicin; ampicillin + either gentamicin, aureomycin, chloramphenicol, kanamycin, erythromycin, neomycin, novobiocin, or streptomycin; novobiocin + either erythromycin, gentamicin, kanamycin or erythromycin.

Summary and Conclusions.

1. Leptospirosis.

Ampicillin had 64 to 128-fold greater antileptospiral activity than penicillin G when tested in a bataviae infectivity system in weanling hamsters for screening drugs. Using delayed treatment regimens at comparable dose levels, the relative therapeutic efficacy of ampicillin, penicillin G, and tylosin was further evaluated in mature

hamsters experimentally infected with a selected concentration of serotype bataviae (1415) which killed animals 8 or 9 days later. Ampicillin afforded greater protection than penicillin G and comparable protection to tylosin. Ampicillin was superior to both tylosin and penicillin G in eradicating leptospiruria of treated hamsters which survived. The potential usefulness of ampicillin in treatment and prophylaxis of leptospirosis merits additional attention.

2. Melioidosis.

a. Approximately 300 cases of melioidosis in U. S. Armed Forces personnel have been affirmed at WRAIR since winter 1965-1966. New tabulations of data from 212 proved cases of melioidosis on correlation of hemagglutination (HA) and complement-fixation (CF) tests and on distribution and range of titers during and after the course of disease were essentially the same as previously reported (WRAIR Annual Report 1968-1969). Variable negative and positive reactions from 8 days to 6 months after disease onset occurred were seen in 8 of 24 patients with localized wound or burn infection primarily with HA test. Transient negative reactions were seen in both CF and HA but rarely with both tests in approximately 11% of the cases with pulmonary and other forms of infection. These findings serve to emphasize the importance of follow-up serological tests in suspected cases of melioidosis. Persistence of HA and CF antibodies was demonstrated from 9 months to 2-1/2 or more years after infection in 31 patients. The duration of positive serological reactions could not be related to type of disease. Persistent high titer reactions were seen in patients with subacute disease of relatively short duration as well as in patients with prolonged complicated infections.

b. The bacteriocin-like inhibitory substance produced by smooth strains of P. pseudomallei has definitely been identified as ammonium. The rough strains produce even larger quantities of ammonium, and in addition, a copious amount of oxalic acid is produced which neutralizes ammonium toxicity.

The addition of glucose, but not glycerol, to smooth strain agar culture neutralizes residual toxicity. Glucose in broth also diminishes the amount of ammonium found in supernatant fluids of smooth strain cultures.

c. Reported high activity of rifampin against Pseudomonas pseudomallei could not be verified by extensive in vitro tests conducted with 31 recently isolated strains. Minimal inhibitory concentration of rifampin was 25 mcg/ml for 3 strains and greater than 25 mcg/ml for 28 strains. Rifampin had relatively poor in vitro activity when compared with tetracycline and chloramphenicol -- drugs now commonly used for treating melioidosis.

A "checkerboard" tube dilution procedure adapted for use with microtiter technics was used to search for synergistic effects of various combinations of antibiotics against P. pseudomallei. No synergistic effect was demonstrated for the combination of carbenicillin and gentamicin, nor of various antibiotic combinations with ampicillin and also with novobiocin.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 305, Military medical research program, SEA, WRAIR - zoonoses

Literature cited.

1. Alexander, A. D. Leptospirosis. In Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections, 5th Edition, 382-421, American Public Health Association, Inc., New York, 1970.

2. Rogul, M., Brendle, J., Haapala, D. K., and Alexander, A. D. Nucleic acid similarities among Pseudomonas pseudomallei, Pseudomonas multivorans, and Actinobacillus mallei. J. Bacteriol. 101(3): 827-835, 1970.

3. Alexander, A. D. Leptospira. ASM Manual of Clinical Microbiology, Chapter 30, 244-250, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6526	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DSEP'N INSTR'N	8B. SPEC. PIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	308
b. CONTRIBUTING				
[redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Prophylactic Use of Gamma Globulin to Prevent Infectious Hepatitis (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 01	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 70	1	40
FISCAL YEAR — CURRENT 71	1	40

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Division of Medicine
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Conrad, COL M. E.

TELEPHONE: 202-576-3365

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Hepatitis; (U) Gamma Globulin; (U) Passive Immunization; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To ascertain if the prophylactic administration of gamma globulin to troops is effective in reducing the incidence of clinical hepatitis, to determine the period of immunity and the dose required.

24. (U) All PCS male U.S. Army personnel assigned to EUSA receive an intramuscular injection at the aerial port of entry and again five months later. These injections contain either 2, 5, or 10 ml. of 16 percent gamma globulin or placebo material. The inoculations are given in a double blinded manner based upon the last digit of the soldier's serial number. Data will be analyzed based upon the incidence of documented viral hepatitis in soldiers receiving each of these materials.

25. (U) 69 07 - 70 06 A WRAIR composite team was assigned to Korea during May 1967. This team has established an inoculation and record center at the aerial port of debarkation for all U.S. Army personnel arriving in Korea. Soldiers assigned to Korea receive biologic materials upon arrival and again five months later. Scrutiny of all admissions to hospitals is maintained to insure proper documentation of hepatitis in each study patient. One hundred and seven thousand soldiers have received initial injections and seventy thousand have received a second injection. Gamma globulin does not protect all soldiers from the occurrence of clinical illness. Whether significant reductions in incidence or morbidity result from gamma globulin administration cannot be ascertained until the study is decoded; this is being examined for both Australia antigen positive and negative hepatitis. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 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.

BLANK PAGE

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00 Tropical and Subtropical Military Medical Research

Work Unit 308, Prophylactic use of gamma globulin to prevent infectious hepatitis

Investigators
Principal: COL Marcel E. Conrad, MC
Associate: Hans F. Smetana, M.D., and Allen A. Young

Description.

Gamma globulin has been used in both civilian and military populations to prevent the occurrence of infectious hepatitis. Scrutiny of the incidence of hepatitis in troops receiving prophylactic injections of gamma globulin has failed to provide convincing evidence that it has reduced the incidence of disease. A double blinded clinical study was initiated to permit evaluation of the effectiveness of U. S. gamma globulin in the prevention of hepatitis in U. S. Forces stationed in Asia.

Progress.

During 1964 a program was initiated by the U. S. Army to immunize all military personnel stationed in the Far East and Southeast Asia with gamma globulin to reduce the incidence of infectious hepatitis. Soldiers were injected with 10 ml of 16 per cent human serum gamma globulin shortly after arrival overseas and again five months later. It was hoped that this would produce passive immunity against hepatitis and that active immunity would develop through a subclinical infection. The escalation of the conflict in Vietnam increased the number of troops stationed in Asia and markedly reduced the amount of gamma globulin available in national stockpiles. During 1965, the recommended dose of gamma globulin was reduced to 5 ml twice during a one year tour in Asia, and during 1966 the recommendation was made that the reduced dose be administered only to soldiers assigned to units with a continued high incidence of hepatitis. Examination of the incidence of hepatitis reported from both Korea and Vietnam showed a marked and sustained reduction in the incidence of hepatitis from 1964-67 when compared to previous intervals. However, this reduction in incidence occurred several months before the initiation of the gamma globulin prophylactic program and could not be attributed to it. In addition, the incidence remained low following reduction in both the dose of gamma globulin and the number of troops included in the immunization program. This information and the relatively limited availability of gamma globulin made it desirable to ascertain the effectiveness of gamma globulin administration in a controlled study.

During May 1967 a field study was initiated in Korea in which soldiers assigned PCS to EUSA were given various doses of gamma globulin or placebo upon arrival and again five months later. Soldiers receive either: (1) 10 ml of 16 per cent human serum gamma globulin (20 per cent); (2) 5 ml of gamma globulin and 5 ml of an albumin-sucrose-potassium glutamate solution (20 per cent); (3) 2 ml of gamma globulin and 8 ml of control material (20 per cent) or (4) 10 ml of the placebo injection (40 per cent). All materials must be characterized for antibodies against known bacteria and viruses and for fragmentation and content of various gamma globulin components. Selection of soldiers for each injection is made based upon the last integer of their military serial number. The various materials used for injection are bottled in 10 ml containers marked with the ten integers, 0 through 9. Two integers were used for each material containing the various quantities of gamma globulin; (1) (2) and (3) above and four integers are utilized for control material. Each soldier received the contents of a bottle labeled with a number matching the last number in his serial number upon arrival in the aerial port of debarkation in Korea and again in medical military dispensaries throughout Korea five months later. All cases of suspected hepatitis are evacuated to one of two military hospitals in Korea where the diagnosis is evaluated by both clinical and laboratory studies. Each documented case of hepatitis is verified as a study patient by maintenance of central immunization files at the aerial port of debarkation.

Results.

During the period 15 May 1967 through 1 August 1969, 107,000 soldiers arriving in Korea received an injection of gamma globulin or placebo material. Sixty five per cent of these soldiers received an injection of the same material five months later. Icteric hepatitis is occurring in soldiers receiving both gamma globulin and placebo materials at all intervals following injection. This project has accumulated a sufficient number of test subjects to evaluate the efficacy or lack of efficacy of gamma globulin in the soldier population and provide additional information about the dose of gamma globulin if efficacy is found. The results of the experiment will be decoded during the oncoming year following the complete retrieval of records and information required for appropriate evaluation and analysis. During the past year acute serum was obtained from each patient developing hepatitis for examination for Australia antigen by both the immunodiffusion and complement fixation mryhofd. It is anticipated that sufficient information will be obtained to ascertain if gamma globulin prophylaxis affects the incidence of either Australia antigen positive or negative endemic hepatitis.

Conclusions and Recommendations.

A double blinded field study was initiated in Korea by a WRAIR team to evaluate the usefulness of gamma globulin in the prevention of infectious hepatitis. Presently, data collection from Korea is being completed and the information is being prepared for appropriate evaluation and analysis. It is anticipated that this study will be completed during FY 71.

Project 3A062110A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 308, Prophylactic use of gamma globulin to prevent infectious hepatitis

Literature Cited.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6463	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. NEW	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A811	00	310
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Etiology of Infectious Hepatitis (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	1.5	35
	CURRENT 71	1.5	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Division of Medicine
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Conrad, COL M. E.

TELEPHONE: 202-576-3365

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Liver Disease; (U) Viral Isolation; (U) Human Volunteers

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Infectious hepatitis has been a major cause of morbidity in soldiers in combat. A collaborative effort was established to attempt to isolate the causative agent(s) of infectious hepatitis with the expectation that this might lead to the development of a protective vaccine.

24. (U) Infectious materials are obtained from carefully documented clinical cases of hepatitis because no specific laboratory test has been developed to document the diagnosis. These materials are shown to be infectious in volunteers by Northwestern University. Aliquots of these materials are distributed to virologists attempting to isolate the causative organism.

25. (U) 69 07 - 70 06 Plasma specimens from patients during the icteric stage of disease have been noninfectious in human volunteer studies. A pool of infectious material was developed from blood obtained during the preicteric stage of illness. Subsequently, this has been shown to be infectious in volunteers. These materials are being distributed to virologists attempting to isolate the etiologic agent of hepatitis and to investigators who are attempting to develop a suitable laboratory animal model for this disease. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A062110A811, MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 310, Etiology of infectious hepatitis

Investigators
Principal: COL Marcel E. Conrad, MC
Associate: CPT Michael A. Davis, MC

Description.

Despite many reports of isolation of a virus from materials obtained from patients with infectious hepatitis, there is no convincing evidence that the causative organism of infectious hepatitis has been cultivated. Studies were initiated to obtain materials from patients with infectious hepatitis and prove that they are infective so that these specimens can be distributed to various laboratories attempting to cultivate and identify the virus. It is postulated that the availability of materials from single sources and their distribution to many laboratories will provide the best opportunity to cultivate the causative organism of disease and hopefully lead to the production of a vaccine for the prevention of hepatitis.

Studies are being performed at the Illinois State Penitentiary in collaboration with Dr. Joseph D. Boggs of Northwestern University. Potentially infectious materials from patients with documented infectious hepatitis are being administered to volunteers. Each patient is hospitalized and carefully controlled clinical and laboratory tests are performed to ascertain if the volunteer develops hepatitis. Blood, urine and feces are collected before the administration of test materials and are stored at -80° C. until the completion of studies. Materials from subjects developing hepatitis are selected and divided into small aliquots for distribution to laboratories attempting to (1) isolate the virus of hepatitis; (2) develop antibody tests to identify the disease and (3) produce the disease in animals to obviate the need for human studies.

Results.

Materials from the MS-1 Willowbrook serum pool was administered orally to volunteers at the Illinois State Penitentiary. Three of ten subjects developed icteric hepatitis documented by examination of liver biopsy specimens between 25 and 40 days after exposure. Serum was obtained from each volunteer at intervals following the oral administration of the infectious material. Serum obtained from one hepatitis patient after the onset of vague clinical symptoms (Day 29) but before an elevation in either the serum bilirubin or transaminase concentration subsequently failed to produce hepatitis in ten volunteers

when administered orally. Serum obtained from another subject after elevation in the serum transaminase concentration but before the onset of icterus caused hepatitis in five of ten volunteers. This infectious serum has been distributed to several virologic laboratories for virologic culture. Presently, two of these laboratories report a capability to produce CPE in Detroit 6 tissue culture material. In addition, serum specimens have been distributed in a coded manner to Presbyterian-St Luke's Hospital for examination in marmosets. In two coded trials hepatitis was detected in marmosets which received infectious materials but not in the control animals. "Hyperimmune" convalescent serum is being obtained from subjects who developed hepatitis to ascertain if it inhibits the development of CPE in tissue culture and prevents hepatitis in the animal model system.

Conclusions and Recommendations.

Serum has been obtained in volunteer experiments which causes hepatitis when administered orally to humans. This infectious serum is Australia antigen negative and causes icteric hepatitis 25 to 40 days after oral ingestion. This serum is being used in both tissue culture systems and in marmosets in an attempt to culture the etiologic agent of hepatitis. Information received from contractors to whom the material was distributed suggests successful experiments in both areas of endeavor.

Project 3A062110A811, MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 310, Etiology of infectious hepatitis

Literature Cited.

None.

PROJECT 3A062110A816
MILITARY MEDICAL MATERIEL

Task 00
Military Medical Materiel

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6463	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	H. Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	622110A	3A062110A816	00	205
b. CONTRIBUTING				
c. [redacted]	CDOG1412B			

11. TITLE (Procede with Security Classification Code)

(U) Military Medical Materiel (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

009800 Medical and Hospital

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 06	Cont	DA	C. In-House

17. CONTRACT/GRANT: NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 69	3	100
CURRENT FISCAL YEAR 70	1	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Biochemistry

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Angel, LTC C. R.

TELEPHONE: 202-576-2211

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Bass, B. G. M.S.

NAME: Lofberg, R. T. Ph.D. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Analytical; (U) Instrument; (U) Information Processing

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to develop and establish analytical systems that will materially reduce the logistical requirements for Medical Department laboratories in the field.

24. (U) The modulization of analytical systems to meet Army Medical Department requirements in the field will be reviewed and a program of development instituted. Development consists of the integration of new technologies into systems that can be standarized for use in the field.

25. (U) 69 06 - 70 06 Considerable effort is being expended to determine the appropriate instrumentation interfacing necessary to couple analytical trains with laboratory based computer systems. Size, configuration and adaptability to field use are being examined. This work unit does not appear in the WRAIR Fiscal Management Document and hence activities under this unit are consolidated under 3A061101A91C, 00, 170. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

Project 3A062110A816 MILITARY MEDICAL MATERIEL

Task 00, Military Medical Materiel

Work Unit 205, Military medical materiel

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: B. G. Bass, B.S.; H. C. Sing, M.S.

Description.

The overall objective of this work unit has been to develop a broad base concept of laboratory analytical chemistry systems that would be applicable for field medical use. Of particular importance is the effort in computer software development and the investigation of applicable interfaces between the instrumentation and the processing unit.

Progress.

This work unit is to be terminated as a separate entity and the efforts are to be consolidated under a work unit entitled biochemical methodology and laboratory automation. The progress herein reported is considered a final report.

During the reporting period, efforts have concentrated on (a) software development; (b) the acquisition of equipment to study time sharing versus dedication; and (c) the interfacing of analytical instrumentation to central processing capability.

a. Software development. Efforts have been confined to the development and extension of a number of statistical methods to include linear regression, curvilinear regression, polynomial least squares curve fitting, multiple analysis of variance and normally distributed population statistics. Each program within this section has been successfully applied to one or more sets of data collected within the Division of Biochemistry over the past several years. This effort has materially hastened the reduction of time necessary to process information into meaningful data arrays suitable for inclusion into publications.

b. Equipment acquisition. The Army Medical Department has not developed and acquired sufficient information on the subject of time sharing versus dedication in relation to computers. The analytical instrumentation industry of today has interfaced each item of their equipment into a family of small unit computers designed to be dedicated to that unit alone. Analysis of this family of instruments suggests that single dedication of an instrument could be extremely wasteful of the total capability of computing unit.

The Army in its efforts towards greater efficiency makes available central processing units that could be potentially used for medical laboratory informational processing requirements. During the reporting period, a field artillery direction and control unit has been acquired. Efforts are under way to examine and evaluate this unit as a possible dedicated device for autoanalyzers, gas chromatography, nuclear magnetic spin resonance and the mass spectrograph. D-17 units will also be evaluated as potential units for medical field teams. These units are a part of the Minuteman Guidance System.

A study has been initiated to evaluate the use of a time shared telephonic terminal that is linked to a large capacity computer. The objective of this study is to determine the level of medical laboratory unit that could satisfy all its data processing requirements by this technique of time sharing.

c. Interfacing. Every analytical chemistry device requires some form of interface to permit its output to be effectively translated to a central processing unit. Interface units have been constructed in cooperation with the U. S. Army Medical Research and Development Command for the unit at William Beaumont Army Hospital. Other interfaces are under development for the FADAC unit and the D-17 unit.

Summary.

This work unit has been involved with development of software program, equipment acquisition and the construction of instrumental interfaces. The unit is to be terminated and the effort consolidated into another work unit.

Project 3A062110A816 MILITARY MEDICAL MATERIEL

Task 00, Military Medical Materiel

Work Unit 205, Military medical materiel

Literature Cited.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6466	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6211 0A	3A062110A821	00	120
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)
(U) Wound healing (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRA T/GRANT Not Applicable
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	3	180
FISCAL YEAR CURRENT 71	3	180

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL, W.H.
TELEPHONE: 202 576 - 3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Division of Surgery
ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Yhap, LTC, E.O.
TELEPHONE: 202 576 - 3791
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Miller, MAJ Joshua
NAME: Rosenthal, MAJ A. Ralph DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Effect of trauma on immune mechanism; (U) Skin grafting; (U) Intraocular foreign body. (U) Host organisms relationship in massive wound sepsis

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) (1) To evaluate first the possibility of altering the body's immune mechanism in prolonging homograft skin coverage and (2) analysis of the effect and detection of intraocular copper foreign bodies.

24. (U) Varying types of antilymphocyte serum are being examined for their effect on prolongation of homograft skin transplants. Multidisciplinary immunologic capability has been established within the Div of Surgery in conjunction with other divisions within WRAIR. Copper foreign bodies are placed in the vitreous of rabbits. Their subsequent effect is determined pathologically as well as by copper content analysis of the vitreous of the eye. Electron probe analysis is being performed. The control group here is an iron foreign body in the opposite eye of the same rabbit.

25. (U) 69 07-70 06 Progress to date indicates that specific types of antilymphocyte serum appear to prolong graft acceptance. Also, the reduction in lymphocyte counts is not as severe as with certain types of antilymphocyte serum which allow the host to sustain other ancillary immune mechanisms. These two findings are of significant potential importance in considering the use of heterologous serum to prolong skin graft survival and possibly permanently cover large areas of skin loss such as occurs in burns in individuals who have been rendered immunologically deficient. Examination of copper foreign bodies has demonstrated a marked purulent inflammatory response compared to the iron foreign body. Furthermore, vitreous copper concentrations in eyes with the copper foreign body are significantly elevated. The earliest that copper can be determined histopathologically is one week. The 60 per cent copper foreign body deposited its copper in macrophages in association with a pseudocapsule, whereas in the case of a 100 per cent copper foreign body one could find copper deposits scattered throughout the posterior segment of the eye. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

Available to contractors upon originator's approval.

DD FORM 1 MAR 66 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

BLANK PAGE

Project 3A062110A821 COMBAT SURGERY

Task 00, Combat Surgery

Work Unit 120 Wound healing

Investigators.
Principal: LTC Edgar O. Yhap, MC
Associate: MAJ Joshua Miller, MC; LTC Merrill C. Johnson, MC*;
LTC Everett Spees, MC**; MAJ A. Ralph Rosenthal, MC;
COL Budd Appleton, MC***

1. Facilitation of Permanent Acceptance of Skin Homografts

a. Statement of the problem: Facilitation of permanent acceptance of skin homografts.

b. Background: Heterologous anti-lymphocyte serum has recently been demonstrated to act on the small lymphocyte population (the immunologically competent cells responsible for graft rejection (without effecting serum antibody responses, when this sera is formed in response to cells of the thymus or thoracic duct drainage.[1-2] We have recently demonstrated that further potential specificity of this sera is possible when the lymph node cells which are used to induce the sera are derived from nodes draining a skin homograft.[3] It is thought that committed lymph node cells may therefore, be antigenically different than uncommitted cells, and that these antigenic differences can induce specific antibody formation in other species. The importance of this finding is the possibility of inducing antisera to lymph node cells that have only the function of graft rejection. This type of heterologous antilymphocyte serum might then cause tolerance induction to donor homograft antigens, while other lymphoid cell functions would be unaltered. Therefore, generalized immunosupression could be avoided.

c. Approach to the problem:

I. To determine if there is strain (H-2) specificity and lymphocyte type specificity of heterologous antilymphocyte serum.

II. To examine the antigenic changes of blast cell cultures in mice, i.e.:

A. Committed cells responding to transplantation antigens

---

* From the Division of Experimental Pathology, WRAIR
** From the Military Medicine and Allied Science Course, WRAIR
*** From the Ophthalmology Service, WRGH

B. Committed cells responding to gram-negative bacterial antigens or sheep red cell antigens, and

C. Non-committed cells

These three groups of cells will be used to induce different heterologous antisera in rabbits. The antisera will then be tested for their specific effects on the capacities of cells of the original immunizing inocula, i.e., skin homograft survival, antibacterial agglutinin production or sheep red cell antibodies.

d. Results and discussion of the results: It was observed that (1) histocompatibility antigens evoked antilymphocyte sera of differing biological specificity, i.e., donor strain specific antisera had a greater effect on skin allograft survival than antisera induced to recipient strains, while recipient strain specific antisera induce a more marked peripheral blood lymphocyte depletion; (2) antisera raised against non-thymic lymphocytes had no significant effect on either skin allograft prolongation or lymphocyte depletion when administered to the recipient specific strain, but there was still some donor strain specific or enhancement-like effect on allograft survival.

e. Conclusions: Further specificity of heterologous antilymphocyte serum has been demonstrated.

f. Recommendations: The project should now continue to the second phase of inducing ALS to lymphoid cells in culture.

2. Studies on Intraocular Copper Foreign Bodies

a. Statement of the problem:

I. To evaluate whether aqueous humor and vitreous humor copper concentrations as measured by atomic absorption spectrophotometry would indicate if a retained intravitreous foreign body contains copper.

II. To examine the pathologic changes which occur when foreign bodies of varying copper compositions are implanted into the vitreous, using new reliable cytochemical methods of detecting copper.

b. Background:

I. It is estimated that about 70% of intraocular foreign bodies resulting from battle casualties are magnetic and 30% non-magnetic.[4] Of the non-magnetic retained metallic foreign bodies, copper and its alloys are the most frequently encountered as well as the most potentially destructive to the eye.[5] With the development of atomic absorption spectrophotometry small amounts of fluid can be analyzed for minute amounts of copper. Runyan and Levi[6] have demonstrated that after

a large copper foreign body is implanted into the vitreous, the vitreous is found to contain up to ten times the amount of copper normally present.

II. Prior to 1969, cytochemical methods demonstrating copper did not exhibit reliability, selectivity, or sensitivity. Linquist[7] has described new reliable, selective cytochemical techniques for the localization of copper.

c. Approach to the problem: Two sets of experiments were performed.

I. Foreign bodies composed either of 60% or 100% copper (obtained from the National Bureau of Standards) ranging in weight from 0.5 - 8.0 mgm were placed into the vitreous of one eye of an albino New Zealand rabbit through a pars plans incision while 100% iron wire of the same surface area was placed into the vitreous of the other eye as a control. The aqueous and vitreous were analyzed by atomic absorption spectrophotometry at 2, 7, 14, and 28 days after insertion of the foreign body. A slightly larger foreign body of 80% copper was placed into the vitreous of one eye of an albino New Zealand rabbit. The other eye was used as a control in the manner mentioned above. Copper analysis of only the aqueous, using atomic absorption spectrophotometry, was performed at 60 and 90 days after insertion of the foreign body.

II. A foreign body composed of 100% copper weighing 1.0 - 5.5 mgm was inserted into the vitreous of one eye of a New Zealand albino rabbit and a foreign body composed of 60% copper of comparable weight and surface area was inserted into the vitreous of the other eye of the same rabbit. The eyes were removed at 1, 2, 7, 14, and 28 days and were examined pathologically with hematoxylin and eosin, rubeanic acid, alizarin blue, and rhodanine stains.

d. Results:

I. When a 60% or 100% copper foreign body was placed into the vitreous no significant increase in copper concentration of the aqueous was found at either 2, 7, 14, or 28 days. However, at 60 and 90 days, using a larger foreign body of 80% copper a significant increase in copper concentration of the aqueous was found.

Vitreous copper analysis yielded a significant elevation in the eye in which either a 60% or 100% copper foreign body was placed in the vitreous. This elevation was detectable at 48 hours after insertion of the foreign body and was consistently elevated at 7, 14, and 28 days.

The normal range for aqueous and vitreous copper concentration in the albino rabbit was found to be 0 - 0.541 ppm and 0 - 0.356 ppm respectively. Four per cent of both aqueous and vitreous determinations were false positives. Thirty per cent and 36% of aqueous and vitreous determinations respectively were false negatives.

These findings indicate the following:

a. Seventy per cent of the time one should be able to detect elevations of aqueous copper levels when a retained copper containing foreign body is present in the vitreous after 60 days but not before 30 days. This should then give the ophthalmologist some proof of the composition of the foreign body and its reactivity. This will be of help in prognosticating if the foreign body will do subsequent damage to the eye.

b. Vitreous analysis should reveal elevation of copper content 64% of the time after 48 hours of the retained intraocular foreign body contains copper. Again this will be of prognostic importance.

c. Pathological studies reveal that both 100% and 60% copper foreign bodies placed into the vitreous can cause acute polymorphonuclear inflammatory changes within 24 hours. Definite abscess formation can be seen by 48 hours with either type of foreign body if the foreign body is adjacent to the wall of the globe. Bacterial stains were all negative. The inflammation is usually more intense with the pure copper foreign body. By one week pseudocapsule formation beings to appear. By one month inflammation around the 100% foreign body can become quite severe with subsequent retinal detachment. In the case of the 60% alloy at one month the acute inflammatory cells are reduced in number and a dense fibrous capsule is present. Large numbers of macrophages are found under the capsule next to the foreign body.

In one case a 60% copper alloy was suspended in the mid-vitreous for one month without any reaction around it. Thus the location of the foreign body surrounded completely by vitreous away from the blood vessels of the uveal tract and retina may explain why in humans one can see an intravitreal copper foreign body without any inflammation around it.

Histochemically copper can be demonstrated at no earlier than one week and both 60% and 100% copper foreign bodies will cause copper deposition at about the same time and in the same frequency. Iron and calcium stains are all negative. When a 60% copper foreign body is implanted into the vitreous, copper can be demonstrated histochemically in macrophages in association with the pseudocapsule. All three stains rubeanic acid, dizarin blue, and rhodanine demonstrate the copper. On the other hand when a 100% copper foreign body is placed into

the vitreous, copper deposition was noted scattered throughout the posterior segment of the eye. Copper was demonstrated (1) in vitreous cells lying free in the vitreous or lying on the retina; (2) in the retina in what appear to be macrophages; (3) in macrophages present in the perivascular space of the central retinal vessels of the optic nerve (4) lying free under the posterior lens capsule.

e. Conclusions:

I. Atomic absorption spectrophotometry was used to determine copper levels of aqueous and vitreous following implantation of copper foreign bodies of different composition into the vitreous. Aqueous levels were elevated at 60 - 90 days, whereas vitreous levels became elevated at 2 days.

II. Pathological studies reveal that both 60% and 100% copper foreign bodies implanted in the vitreous can cause sterile abscess formation. Cu can be demonstrated histochemically at one week with either type of copper foregin body. The 60% copper foreign body deposited its copper in macrophages in association with a pseudo-capsule, whereas in the case of a 100% copper foreign body one could find copper deposits scattered throughout the posterior segment of the eye.

f. Recommendations:

I. It would be important to determine the chemical composition and structural characteristics of copper deposits in the eye resulting from an intravitreal copper foreign body. This could only be done using the Scanning electron probe (Dr. A. J. Tourinis, Biodynamics Research Corporation).

II. A study is in progress to determine whether the location of the copper containing foreign body in the vitreous i.e., suspended in midvitreous vs against the wall of the globe, produces different responses of the eye to the foreign body.

III. An attempt is being made to produce a model of chronic copper posioning to the eye (Chalcosis), to see if the copper chelating agent penicillamine can reduce copper deposition in the eye.

Project 3A062110A821 COMBAT SURGERY

Task 00, Combat Surgery

Work Unit 120 Wound healing

Publications:

Heisterkamp, C. A., Simmons, R. L., Vernick, J., and Matsumoto, T.: An aerosol tissue adhesive. J. Trauma, 9:587-593, (7) 1969.

Matsumoto, T., and Dobek, A. S.: Systemic antibiotic(s) in contaminated crush wound. Without debridement. Arch. Surg., 99:103-106, July.

Berman, I. R., Hamit, H. F., and Clauss, R. H.: A new method for semi-automatic suture plication of the inferior vena cava. Amer. J. Surg., 118:123-125, July 1969.

Matsumoto, T., Wyte, S. R., Moseley, R. V., Hawley, R. J., and Lackey, G. R.: Combat surgery in Communication Zone. I. War wound and bacteriology (preliminary report). Milit. Med., 134:655-665, Sept. 1969.

Simmons, R. L., et al. Post-resuscitative blood volumes in combat casualties. Surg. Gynec. Obstet., 128:1193, June 1969.

Matsumoto, T., Wyte, S. R., Moseley, R. V., Nemhauser, G. M., Henry, J. N., and Aaby, G. V.: Surgical Research in the Communication Zone. II. Enzyme Fluctuations in Wounded Combat Soldiers during the Convalescent Period. Arch. Surg., 99:537-541, October 1969.

McNamara, J. J., Lamborn, P. J., Mills, D., and Aaby, G. V.: Effect of short-term pharmacologic doses of adrenocorticosteroid therapy on wound healing. Ann. Surg., 170:199-202, August 1969.

Sleeman, H. K., Diggs, J. W., Hayes, D. K., and Hamit, H. F.: Value of antibiotics, corticosteroids, and peritoneal lavage in the treatment of experimental peritonitis. Surgery, 66(6):1060-1066, December 1969.

Lamborn, P. B., Jr., Soloway, H. B., Matsumoto, T., and Aaby, G. V.: Comparison of tensile strength of wounds closed by sutures and cyanoacrylates. Am. J. Vet. Res., 31(1):125-130, January 1970.

McNamara, J. J., Mills, D., and Aaby, G. V.: Effect of hypertonic glucose on hemorrhagic shock in rabbits. Ann. Thor. Surg., 19(2):116-121, February 1970.

Project 3A062110A821 COMBAT SURGERY

Task 00, Combat Surgery

Work Unit 120 Wound healing

Literature Cited

References:

1. Lance, E. M.: Advance in Transplantation. Williams & Wilkins, Baltimore, 1967.

2. Levey, R. H., and Medawar, P. R.: Ann. N. Y. Academy of Sciences, 129:164, 1966.

3. Miller, J., and Cohn, R.: A more specific rabbit anti-mouse lymphocyte serum. Surg. Forum 30, 1969.

4. Stallard, H. B.: War surgery of the eye, an analysis of 102 cases of intraocular foreign bodies. Brit. J. Ophth. 28:105-135, 1944.

5. Duke-Elder, S.: Text book of Ophthalmology. Vol. VI, Injuries, London, Henry Kimpton, 1954, pp 6185-6198.

6. Runyon, T. E., Levri, E. A.: Vitreous analysis in eyes containing copper and iron intraocular foreign bodies. Submitted for publication.

7. Linquist, R. R.: Studies on the pathogenesis of hepatolenticular degeneration. II. Cytochemical methods for the localization of copper. Arch. Path. 87:370-379, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6467	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	A062110A821	00	121
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)
(U) Responses to trauma (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 09	CONT	DA	C. In-House

17. CONTRACT/GRANT: Not Applicable
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	5	125
	CURRENT 71	5	125

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W.H.
TELEPHONE: 202 576 - 3551

21. GENERAL USE
Foreign Intelligence Not Consdiered

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Division of Surgery
ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Yhap, LTC E.O.
TELEPHONE: 202 576 - 3791
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Levin, MAJ P.M.; Miller, MAJ Joshua
NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Hemorrhagic shock; (U) Pulmonary microembolization; (U) Alpha adrengeric blockade; (U) Stress ulcer; (U) Posttraumatic pulmonary insufficiency

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 study in experimental animal models the mechanism involved in the production of stress ulcer and posttraumatic pulmonary insufficiency. Other projects relate to the study of a new alpha adrenergic blocking agent on pulmonary vascular resistance and in developing an experimental model to study gastric secretion in liver transport dogs.

24. (U) A good laboratory shock model is now established. The influence of prolonged shock on red cell microaggregation platelet adhesiveness and alterations in red cell oxygen transport are being evaluated. The effect of alpha adrenergic blockade on pulmonary vascular resistance has been studied by chronic implantation of pulmonary artery flow probes in dogs subjected to varying periods of controlled hypoxia. Liver transplantation techniques and contruction of Heidenhain pouches are now perfected.

25. (U) 69 07-70 06. Considerable information has been accumulated supporting the concept that alterations of oxygen transport capability of red cell is a significant contributing factor in the maintenance of prolonged shock. The disulfid fraction of the alpha adrenergic blocker under study has been studied in 12 dogs also subjected to acute hypoxia. Results with the drug indicate a favorable improvement in hemodynamic values of stressed dogs. A model for producing stress ulcer with some consistency has now been established Calculation and tabulation of the results from secretory studies in this protocol in comparison with those observed in the recent Vietnam study will allow some inference as to how applicable this laboratory model is to the clinical situation observed in man. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 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 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121, Responses to trauma

Investigators.
Principal: LTC Edgar O. Yhap, MC
Associate: MAJ Joshua Miller, MC; MAJ Phillip M. Levin, MC; LTC Norman Rich, MC*; MAJ John Hutton, MC*; MAJ Robert T. Solis, MC; MAJ Steven J. Phillips, MC; MAJ J. Judson McNamara, MC; MAJ John F. Stremple, MC; MAJ Mark D. Molot, MC**; 1LT R. A. Dunn, ANC***

1. Lymphocyte Cultures as an Index of Immunity and Responses to Trauma

a. Statement of the problem: Factors in the pre- and post-hepatic venous circulation associated with gastric and duodenal ulcer disease and transplantation immunity are being investigated.

b. Background: Transplantation of the liver has led to both physiologic and immunologic findings previously not observed with other organs.

(1) In "stress" ulcer, diathesis occurs which is unexplained by local ischemic factors which is increased. The diathesis appears to be increased with rejection of the liver.[1]

(2) A tolerant state has been induced in some species, while in others less immunosuppression has been needed than with other organ homografts for prolonged acceptance.[2]

c. Approach to the problem: Ulceration in dogs and humans has been produced by porta-systemic shunting.[3] It is theorized that inactivation by the liver of humeral substances which stimulate hypersecretion cannot occur.

A hypothesis involved in the ease of immunologic acceptance of orthotopic liver homografts is that the graft comes in contact with lymphocytes derived from gut-associated, primordial lymphoid tissue. This tissue is thought to contain a population of immature cells, able still in adult life to become tolerant of foreign antigens rather than immunized. Other organ homografts[4] and purified antigens[5] have also evoked weaker immunity when transplanted into the "privileged site" of the mesenteric circulation. In order to study the immune mechanisms involved in rejection

*Department of Surgery, WRGH
**Division of Experimental Pathology, WRAIR
***US Army Medical Research Team (WRAIR), Vietnam

lymphocyte transformation in vitro is being used. It is the objective of a phase of this protocol to study the ability of dog lymphocytes in the portal and hepatic veins to transform in response to mitogens, such as phytohemagglutinin (PHA), anti-lymphocyte serum (ALS) and to donor cells in mixed leukocyte cultures (MLC) under a variety of experimental conditions.

d. Results: As there had been essentially no previous experience in culturing characteristics of dog lymphocytes in this laboratory, it was deemed necessary to study dog lymphocytes in a number of preliminary experiments. Kidney auto- and allografts to the neck were performed in unrelated adult mongrel dogs. The effects of phytohemagglutinin (PHA-P) and one way mixed lymphocyte cultures (MLC) on proliferation of peripheral blood lymphocytes were studied, comparing the use of fresh with frozen-thawed stimulating cells. There was a loss of response to donor lymphocytes in MLC which may be due to inactivation in vivo by the allograft, or to a shift in the response curve to a peak earlier or later than 7 days in culture. There was a dissociation in response of cells from certain dogs to fresh and frozen-thawed stimulating cells in MLC.

e. Discussion, Conclusion and Recommendations: It is possible that either an unresponsive state developed in in vitro testing of dog lymphocytes at the peak of a massive vascularized allograft reaction. This is being looked into. Since lymphocyte reactivity in vitro is a potentially very important way to measure immunity and in individuals with infection and trauma, this study should be pursued with interest.

2. The Evaluation of Arteriovenous Shunt to Maintain Patency of Venous Autografts[6]

a. Statement of the problem: The evaluation of arteriovenous shunt to maintain patency of venous autografts was carried out.

b. Background: A recent report from the Vietnam Vascular Registry, encompassing over 5,000 patients among whom more than 1,000 venous injuries have been documented, emphasized the importance of repairing the acute venous injury. The clinical experience has shown that: venous repair can be performed with few complications; many extremities have been lost due to venous hypertension despite adequate arterial repair; the majority of lateral venous repairs remain patent; autogenous vein graft in the venous system are prone to thrombosis, but may provide time for collateral development and may undergo recanalization; the potential benefits of venous repair, particularly in the lower extremities, outweigh the risks. Stimulated by these clinical impressions, the patency of autogenous vein grafts in the venous system augmented by a distal arteriovenous shunt was studied in the laboratory.

c. Approach to the problem: Large mongrel dogs were divided into two groups, depending upon the type of distal AV shunt employed.

Group I - side-to-side AV shunt. Five cm. segments of both jugular veins were removed and inserted into the superficial femoral veins bilaterally. A side-to-side anastomosis between the superficial femoral artery and superficial femoral vein was then constructed distal to the vein graft on one side with the other limb serving as a control in both groups.

Group II - "H" type AV shunt. A procedure similar to Group I was performed, except that the AV shunt was constructed using a 1 cm. length segment of vein between the superficial femoral artery and superficial femoral vein distal to the vein graft.

The patency of the venous graft was determined by venography and arteriography immediately following surgery, on postoperative day 3, postoperative week 3, and postoperative week 6. Re-operation was done during the sixth postoperative week at which time the patency of both shunt and graft was confirmed. If venography had demonstrated occlusion of the graft prior to the sixth postoperative week, then re-operation was done earlier and occlusion was confirmed. At the same time, the graft was removed and studied morphologically. In those grafts that remained opened for six weeks only the shunts were closed. Venography was again performed immediately following operation, on the third postoperative day, and during the third postoperative week. A third operation was then done to confirm the patency of the graft three weeks following removal of the shunt. These grafts were then removed and studied morphologically.

Data will be organized in tables for comparison values in control limbs and in limbs with associated arteriovenous shunts. The sequential evolution of the graft patency has been recorded by venography and arteriography.

d. Results: Preliminary results from Group I indicate that 80 per cent of the grafts in control limbs are open immediately following operation; 40 per cent of these grafts are open on the third postoperative day. However, by the third and sixth postoperative weeks 80 per cent of the control grafts are again open, indicating recanalization in a large percentage of these vein grafts. Sixty per cent of the grafts on the shunt side were open on the day of operation. Forty per cent of the grafts on the shunt side were open on the third postoperative day. Only 20 per cent of the grafts on the shunt side were open during either the third or sixth postoperative weeks. The results of Group I tend to indicate that autogenous venous grafts will tend to undergo recanalization in a very large percentage of cases. The use of a side-to-side distal arteriovenous shunt does not increase patency of the proximal vein graft. In fact, this type of shunt is detrimental to the patency of the vein graft.

In Group II or the H-type of distal AV shunt, 100 per cent of the grafts in the control limbs were patent on the day of operation. Fifty per cent of the control grafts were patent on the third postoperative day.

Eighty per cent of the control grafts were patent by the third postoperative week, and this had increased to 100 per cent patency at the end of six weeks. As in Group I this, again, clearly indicates the ability for autogenous vein grafts to undergo recanalization. One hundred per cent of the grafts associated with the distal AV shunt were patent on the day of operation. Eighty per cent of these grafts were patent on the third postoperative day. Eighty per cent of these grafts remained patent from three to six weeks postoperatively. The distal AV shunt has been interrupted in the majority of these animals, and the patency is maintained from 3 days to 3 weeks, postoperatively, in the majority of them. These studies indicate that the use of a distal H-type AV shunt has increased the patency rate of the venous autograft, both immediately postoperatively and over a chronic study period.

e. Conclusions: (1) Autogenous vein grafts in the venous system are prone to early thrombosis but undergo recanalization in a large percentage of cases if surgical technique is meticulous. (2) The patency of autogenous vein grafts in the venous system can be increased by the use of a distal H-type AV shunt. (3) The patency of autogenous vein grafts in the venous system is further inhibited by the use of a distal side-to-side AV shunt.

f. Recommendations: As the result of clinical experience in Vietnam and as a result of these preliminary studies, we feel that a greater effort should be made in the repair of the acutely injured large peripheral vein. This is especially true if reconstruction can be accomplished with a lateral repair. Either lateral repair or autogenous venous graft should be considered if the main venous return of the lower extremities has been injured. The use of a distal H-type arteriovenous shunt is not recommended on a routine basis because of the necessity for an additional operation. However, the use of this adjuvant method should be kept in mind for selective cases.

3. Oxygen Transport in Shock

a. Statement of the problem: The transport of oxygen to the peripheral tissues has been investigated in terms of the alterations in the properties of the blood with storage. The physiologic effects of these parameters are of interest because the toxic effect of massive transfusion is felt to be a major source of morbidity in the seriously wounded combat casualty.

b. Background: Among the major alterations in blood resulting from storage, the increased affinity of hemoglobin for oxygen (Bunn, et al, 1969)[7], the reduced flexibility of the red cell membrane (Sirs 1969)[8], and the augmented microaggregation of platelets, white blood cells and nonspecific debris (Swank 1963)[9] are most likely to be of importance in contributing to the morbidity associated with massive transfusion. Although the clinical conditions which lead to an alteration in the hemoglobin-oxygen dissociation curve of whole blood have been defined, the

physiologic consequences of these changes are uncertain. Thus, although there is a shift of the dissociation curve to the right with hypoxia (Oski et al, 1969)[10], high altitude exposure (Lenfant, et al 1968)[11] and conditions of reduced peripheral blood flow and to the left with storage (Bunn 1969)[7], this change is moderate in that the pO_2 at which one half the hemoglobin is oxygenated (P_{50}) changes only a few mm Hg. If the pO_2 of the tissue is unchanged even this small shift of the curve would greatly alter the amount of oxygen unloaded in the tissues; however, in order for these changes in P_{50} to influence the amount of oxygen delivered, the tissue pO_2 and blood flow would have to remain constant. Because of the known adaptive changes of the microcirculation it remains to be shown whether a change in P_{50} of the blood perfusing a tissue significantly alters the delivery of oxygen in vivo.

c. Approach to the problem: Our approach has been to develop experimental models whereby the effects on peripheral tissue of changing the P_{50} of the blood could be studied. This has required the development of a means of lowering the P_{50} and also the development of models whereby tissue functions could be studied.

d. Results: Rats and dogs were exposed to high concentrations of inspired O_2 and a fall in red cell 2,3 DPG was noted. In dogs who were in profound shock, no change in red cell 2,3 DPG was found.

e. Conclusions: The importance of this finding is that it allows the development of a model whereby tissue function can be evaluated under conditions that exist soon after massive transfusion.

f. Recommendations: Our plans are to study oxygen transport utilizing the rat and dog whose 2,3 DPG has been depleted by oxygen exposure. Emphasis will be placed on (1) measuring tissue pO_2 in the rat, (2) studying the delivery of O_2 to the stomach with the intention of relating changes in oxygen transport to the development of stress ulcers and (3) studying the function of a transplanted puppy heart before and after transfusion with blood that has been depleted of 2,3 DPG.

4. The Improvement of Peripheral Blood Flow by Preventing Flow Reversal with a Vein Valve.[12]

a. Statement of the problem: This study is about the improvement of peripheral blood flow by preventing flow reversal with a vein valve.

b. Background: Original work.

c. Approach to the problem: A jugular vein valve was implanted in the femoral artery of dogs. The valve was placed so that it would open during systole and close during diastole so it would prevent flow reversal.

d. Results and discussion of the results: Eleven dogs had successful vein valves implanted. Mean flow was improved on the average by 40 cc/min, and mean blood pressure by 50 mm Hg.

e. Conclusion: Flow reversal was eliminated by the valve and mean diastolic pressure was favorably increased.

f. Recommendations: Chronic canine and primate studies should be performed with the thought of using this technique in the treatment of human peripheral arterial insufficiency.

5. The Effect of Bee and Snake Venoms on Cardiovascular Hemodynamics[13]

a. Statement of the problem: The effect of bee and snake venoms on cardiovascular hemodynamics was studied in mongrel dogs.

b. Background: Original work.

c. Approach to the problem: Depending on the venom used, varying hemodynamic responses were observed.

d. Results and discussion of the results: Analysis of results revealed all snake and bee venoms to be cardio and/or respiratory toxic except the venoms of the sea snake which was neurotoxic.

e. Conclusions: It is suggested that venom-antivenom studies be performed with the experimental model used in these studies.

f. Recommendation: Venom anti-venom studies should be performed.

6. Intravenous Alimentation: An Important Adjunct in the Treatment of Combat Casualties.[14]

Intravenous alimentation is an important adjunct in the treatment of combat casualties. This was demonstrated in a series of 20 patients, 8 who received intravenous alimentation and 12 did not following combat injury. Patients who received intravenous alimentation had a decrease in body weight loss and a decrease in negative nitrogen and glucose balance. Furthermore, it appeared to be of distinct clinical benefit in at least two cases who were presented individually.

Recommendation: The use of intravenous alimentation after major surgery and major injury is advocated to decrease metabolic wasting and thus by improving post-injury nutrition to hopefully reduce morbidity and mortality from complications relating to sepsis and poor wound healing.

7. Changes in Some Physical Properties of Blood with Storage[15]

Nineteen units of blood were collected from volunteers in Vietnam and studied serially for hematocrit, fibrinogen concentration, screen filtration pressure (SFP), and whole blood work viscosity. Significant elevations in screen filtration pressure and blood viscosity were noted with storage. These were not related to alterations in fibrinogen concentration or hematocrit. The elevation in SFP occurred more rapidly and in greater magnitude than changes in viscosity noted. However, the elevation in blood viscosity with storage was significant. These appeared to represent one further inadequacy of our storage method of whole blood. It appears that this data will provide further impetus for increased effort to investigate methods for handling of storage of blood and blood preservation.

8. Fat Embolism after Combat Injury[16]

Fat embolism is a clinical diagnosis made on the basis of a combination of signs and symptoms that frequently appear together after major trauma. A study was performed in which 15 combat casualties with clinical fat embolism were collected at an evacuation hospital in Vietnam. Cerebral symptoms and some decrease in respiratory insufficiency were present in all. All had long bone fractures. Petechiae and fever were seen in 9 of 10 patients, respectively. Therapy consisted primarily of respiratory support. Adrenocortical storage was used empirically in high dose short term regimen but with inconclusive results. The disease appeared unique in this group of patients because of its unusually virulent nature characterized by (1) onset of 24 hours or less in 8 of 15 patients, (2) rapid progression of symptoms and (3) death of 7 patients. The importance of fat embolism in the pathogenesis of posttraumatic pulmonary insufficiency syndrome remains to be determined.

Considerable experimental work is needed to define the relationship between the pathologic observation of fat embolization to the pulmonary arterioles and the clinical syndrome recognized as fat embolism.

9. A Double Check Valve Device without Moving Parts for Intravenous Fluid Administration[17]

A double check valve in an inexpensive plastic disposable system which can be used for controlled infusion of intravenous fluids has been developed and tested. The system allows for exact monitoring of in-put of a given source of fluid without the necessity of any moving parts to change the direction of flow. Its characteristics, convenience, low cost, ease of operation, disposability make it a useful adjunct in administration of fluids and blood to infants, small children and in the administration of fluid in repeated doses from a constant source either in clinical medicine or in the laboratory.

10. Blood Viscosity in Combat Casualties[18]

The importance of blood viscosity in patients following trauma is difficult to evaluate clinically. A study of blood viscosity in 44 patients following combat injury was undertaken at the 24th Evacuation Hospital in Vietnam. Thirteen were considered as control patients on the basis of relatively minor soft tissue wounds, and the others were all seriously injured patients. No significant abnormalities were noted in blood viscosity in either group either preoperatively or postoperatively or subsequently. Similarly, extrapolated yield shear stress did not show any significant changes. Concomitant study of hematocrit and fibrinogen values showed an inverse relationship between the two during the first three days after injury.

The conclusions are that gross abnormalities in whole blood viscosity recognizable at even high rates of shear are undoubtedly of physiologic significance and may be greatly magnified as shear rate decreases. However, clinical viscosity determinations as obtained in the present study do not reflect the rheologic status of blood in the patients in the microcirculation. The data primarily point out the inadequacy of the present clinical methods used to determine whole blood viscosity and the even more important physiologic determination of yield shear stress.

Recommendations: It is recommended that methods be sought to determine whole blood viscosity at very low shear rates with relative ease and accuracy and that a device to actually measure yield shear stress is of real importance in the study of viscosity following injury.

11. Blood Coagulation after Injury

Coagulation defects were studied in a series of 80 combat casualties without coagulopathy and in a series of 20 combat casualties with coagulopathies. Studies performed included platelet counts, partial thromboplastin time, prothrombin time, thromboelastogram, fibrinolysin titers, fibrinogens, euglobulin clot lysis, hematocrit, ethanol gelation, screen filtration pressure. The data indicate that a number of factors are depressed following massive injury and massive transfusion but that no one abnormality is sufficient to explain the bleeding defect observed. The defect is always corrected by fresh whole blood. It was usually not corrected by fresh frozen plasma. Heparin was never successful in the current experience in reversing the coagulopathy. The data appeared to indicate that disseminated intravascular coagulation, if it does occur, is an immediate event following the injury and that consumption has occurred at that time but is not a continuing problem in the patient's course. Then, the resulting clotting defect is a result of one episode of DIC and subsequent consumption and what is needed is replacement of clotting factors to reverse this process. The other possibility, of course, is that with massive transfusion there is an alteration in the existing platelet function and qualitative platelet abnormalities are responsible for the observed bleeding defect. This is what the author

currently favors as the hypothesis for the pathogenesis of the coagulopathies observed following massive injury and massive transfusion.

Recommendation: More work is definitely needed to define the exact nature of the coagulation defect.

12. Effect of Hypertonic Glucose in Hypovolemic Shock in Man[19]

Glucose in large volumes has been demonstrated to be a utilizable energy substrate in animals in shock. A study was carried out in which combat casualties in shock were given either hypertonic glucose, hypertonic saline, hypertonic mannitol or no adjunctive therapy.

The treatment regimen was determined randomly. All patients received the usual resuscitative measures.

The data demonstrated clearly that hypertonic glucose in similar osmolar doses to other two materials produced a highly significant increase in blood pressure and in pulse pressure. Since the groups were small, no significant difference in survival was noted. It does appear that glucose, however, has a beneficial immediate effect on the cardiovascular system in shock. In animals it has been demonstrated that there is concomitant vasodilation with a marked increase in peripheral blood flow. This is the ultimate aim in restoration of blood volume in shock.

Recommendation: It is recommended that hypertonic glucose is a useful therapeutic adjunct during a period of time that volume is being replaced. Further work to define the actual fate of the exogenously administered glucose in the animal in shock remains to be determined.

13. Hyperglycemic Response to Trauma in Combat Casualties[20]

Hyperglycemia is said to be a common response following trauma. Whether it is etiologically of any significance in the organisms response to trauma is undetermined. A study of 67 battle casualties confirmed the occurrence of hyperglycemia. It is a post-injury physiologic response to trauma. The hyperglycemia was related to the severity of injury and correlated with serum lactate levels but not with arterial pH or pO_2 values. It seems likely that the hyperglycemic response to trauma is of etiologic significance in that it is a readily available source of energy substrate to the body at a time when its needs are presumably increased.

Recommendations: Future efforts should be directed at (1) defining the need for increased energy substrate in an acutely traumatized animal, and (2) determining the rate of the utilization of freshly mobilized glucose stores in the acutely traumatized animal.

14. Screen Filtration Pressure in Combat Casualties[21]

Pulmonary complications are the major cause of death following injury in combat casualties in Vietnam. The two factors that all patients who subsequently develop pulmonary injuries have in common are massive injury and massive blood transfusion. Screen filtration pressure was used to detect particular debris in the blood of combat casualties. The data demonstrated that (1) considerable debris was present in banked blood, (2) that, when banked blood was administered intravenously, debris was filtered primarily by the pulmonary capillary bed and secondarily by the peripheral capillary bed; (3) that postoperative hypoxemia in combat casualties was found to relat. significantly to the volume of transfused blood. The possible relationship between massive pulmonary microemboli resulting from debris in banked blood and the development of the post-traumatic pulmonary insufficiency syndrome was discussed.

Recommendation: Further work is needed to define the relation of banked blood to the development of posttraumatic pulmonary insufficiency.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121, Responses to trauma

Literature cited:

References

1. Stuart, F. P., Torres, E., and Moore, F. D.: The association of upper gastrointestinal ulceration and orthotopic hepatic allotransplantation in the dog. Transplantation 5: 804, 1967.

2. Calne, R. Y., et al.: Induction of immunologic tolerance by porcine liver allografts. Nature 223: 1969.

3. Clarke, J. S., et al.: Peptic ulcer following portacaval shunt. Ann. Surg. 148: 551, 1958.

4. Fukuda, A., et al.: Inhibition of second set renal allograft responses by portal vein drainage. Transplantation Proc., 602, 1969. H. M. Stratton, Inc., Publisher, N. Y.

5. Battisto, J., and Miller, J.: Immunological unresponsiveness produced in adult guinea pigs by parenteral introduction of minute quantities of hapten or protein antigens. Proc. Soc. Exp. Biol. Med 111: 111, 1962.

6. Levin, P. M., Rich, N., and Hutton, J.: The evaluation of arteriovenous shunt to maintain patency of venous autografts. (in press)

7. Bunn, H. F., May, M. H., Kocholaty, W. F., et al.: Hemoglobin function in stored blood. J. Clin. Invest. 48: 311, 1969.

8. Sirs, J. A.: The respiratory efficiency and flexibility of erythrocytes stored in acid-citrate-dextrose solution. J. Physiol., 203: 93, 1969.

9. Swank, R. L.: Alteration of blood on storage: measurement of adhesiveness of "aging" platelets and leukocytes and their removal by filtration. N.E.J.M., 265: 728, Oct. 12, 1961.

10. Oski, F. A., Gottlieb, A. J., Delivoria-Papadopoulos, M., et al.: Red-cell 2,3-diphosphoglycerate levels in subjects with chronic hypoxemia. N.E.J.M., 280: 1165, 1969.

11. Lenfant, G., Torrance, J., English, E., et al.: Effect of altitude on oxygen binding by hemoglobin and on organic phosphate levels. J. Clin. Invest., 47: 2652, 1968.

12. Phillips, S. J.: The augmentation of peripheral forward blood flow by prevention of flow reversal with a vein valve. (in press)

13. Phillips, S. J.: The effect of snake and bee venoms on cardiovascular hemodynamics and function. Russell and Saunders: Animal Toxins, Pergamon Press (chapter in a book), Phila. & London. (in press)

14. McNamara, J. J., Molot, M. D., Wissman, J., Collins, C., and Stremple, J. F.: Intravenous alimentation: important adjunct in the treatment of combat casualties. (in press)

15. McNamara, J. J., Boatright, D. B., Burran, E. L., Summers, E. S., Molot, M. D., and Stremple, J. F.: Changes in some physical properties of blood with storage. (In press)

16. McNamara, J. J., Stremple, J. F., and Molot, M. D.: Fat embolism after combat injury. (In press)

17. McNamara, J. J., and Rosenberg, D. J.: A double check valve device without moving parts for intravenous fluid administration. (In press)

18. McNamara, J. J., Molot, M. D., and Stremple, J. F.: Blood viscosity in combat casualties. (In press)

19. McNamara, J. J., Molot, M. D., Dunn, R. A., and Stremple, J. F.: Effect of hypertonic glucose in hypovolemic shock in man. Amer. J. Cardiol. (In press)

20. McNamara, J. J., Boatright, R. D., Kniesel, M., Molot, M. D., and Stremple, J. F.: Hyperglycemic response to trauma in combat casualties. Arch. Surg. (in press).

21. McNamara, J. J., Molot, M. D., and Stremple, J. F.: Screen filtration pressure in combat casualties. Ann. Surg. (in press).

Publications

Berman, I. R.: Intravascular microaggregation in young men with combat injuries. Surgical Forum XX: 14, 1969.

Doty, D. B., Moseley, R. V., Pruitt, B. A., and Randolph, J. G.: Hemodynamic consequences of respiratory insufficiency following trauma. J. Thor. Cardiovasc. Surg., 58: 374, Sept 1969.

Ducker, T. B., Simmons, R. L., and Martin, A. M.: Pulmonary edema as a complication of intracranial disease. Amer. J. Dis. Child., 118: 638, Oct. 1969.

Moseley, R. V., Doty, D. B., and Pruitt, B. A.: Physiologic changes following chest injury in combat casualties. Surg. Gynec. Obst., 129: 233, Aug. 1969.

Soloway, H. B., Robinson, E. F., Hufnagel, H. V., Huyser, K. L.: Experimental fat embolism. Initial distribution of fat emboli labeled with ^{131}I in normotensive and hypotensive rabbits. Arch. Path., 88: 171, Aug. 1969.

Hewitt, R. L.: Technical considerations in acute military vascular injuries of the extremities. Milit. Med., 134: 617, Aug. 1969.

Simmons, R. L., Heisterkamp, C. A., Collins, J. A., Bredenberg, C. E., and Martin, A. M.: Acute pulmonary edema in battle casualties. J. Trauma 9: 760, Sept. 1969.

Moseley, R. V., and Doty, D. B.: Physiologic changes due to aspiration pneumonitis. Ann. Surg., 171: 73, Jan. 1970.

Berman, I. R., Lemieux, M. D., and Aaby, G. V.: Responses of skeletal muscle pH to injury: a new technique for determination of tissue viability. Surgery 67: 507, March 1970.

Moseley, R. V., and Doty, D. B.: Long-term arterial catheterization for repeated blood sampling. Surgery 67: 455, Mar. 1970.

Berman, I. R., and Rogers, L. A.: Cerebral acidosis following increased intracranial pressure. Surg. Gynec. Obst. 130: 483, Mar. 1970.

Doty, D. B., Kugler, H. W., and Moseley, R. B.: Control of the hepatic parenchyma by direct compression: a new instrument. Surgery 67: 720, Apr. 1970.

Doty, D. B., Hufnagel, H. V, and Moseley, R. V.: The distribution of body fluids following hemorrhage and resuscitation in combat casualties. Surg. Gynec. Obst. 130: 453, Mar. 1970.

Moseley, R. V. and Matsumoto, T.: Demonstration of pulmonary microemboli by a microradiographic technique. Vascular Surgery 4: 63, Mar. 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6468	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6211 0A	3A062110A821	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CDOA 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Anesthesia and pulmonary complications of combat injury (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002400 Bioengineering 012900 Physiology 008800 Life Support

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 05	CONT	DA	C. In-House

17. CONTRACT/GRANT: Not Applicable

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	3	25
FISCAL YEAR CURRENT 71	3	25

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W.H.

TELEPHONE: 202 576 - 3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Surgery

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Yhap, LTC E.O.

TELEPHONE: 202 576 - 3791

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Phillips, MAJ S.J.; Solis, MAJ R.T.;

NAME: Levin, MAJ P.M. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Controlled ventillators (U) Oxygen toxicity (U) Oxygen transportation (U) Distribution of pulmonary artery blood flow (U) Pulmonary insufficiency

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 examine in depth several factors that may contribute to ineffective oxygenation of the body tissues, a major problem following injury in combat casualties. These are (1) the study of oxygen toxicity (2) study of alterations in the capacity of the red blood cell to transport oxygen following exposure to hyperbaric oxygen concentrations, transfusion of banked blood, massive injury and shock (3) study distribution of pulmonary artery blood flow following anoxia, hypoxia, acute and chronic atelectasis of the lung.

24. (U) Experimental models to study oxygen toxicity in the dog and baboon have been established. This clinical model allows for the utilization of a double lumen tube thus permitting the lungs to be independently ventilated. The effect of hyperbaric oxygen concentration and trauma on changes in red blood cell oxygen transport is being studied in rats. In addition, the distribution of pulmonary artery blood flow following anoxia and hypoxia in the lung is being studied in an established experimental model by means of chronic implantation of pulmonary artery flow probes.

25. (U) 69 07-70 06. Clinical models for studying the differential ventilation of the lungs, and distrubtion of pulmonary artery blood flow in dogs have been established. Considerable information has been obtained supporting the concept that oxygen toxicity is due to the direct effect of oxygen on the lung itself. The oxygen ventilated lungs were edematous with the presence of hyaline membrane, destruction of capillary endothelium and alveolar epithelial cells. The air treated lungs were normal. The study of alterations in red cell oxygen transport is fully underway. Abnormalities is red cell affinity correlate with alterations in 2,3 DPG and P50. Direct measurements of pulmonary artery blood flow to both lungs following controlled hypoxia and anoxia is underway.

For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 70 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 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 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 122, Anesthesia and pulmonary complications of combat injury

Investigators.
Principal: LTC Edgar O. Yhap, MC
Associate: MAJ Phillip M. Levin, MC; MAJ Steven J. Phillips, MC; MAJ Robert T. Solis, MC; MAJ Jack A. Zeller, MC*; MAJ J. Judson McNamara, MC; MAJ John K. Messersmith, MC**; MAJ John F. Stremple, MC

1. The Effect of High Inspired Oxygen Tension and Its Relationship to Oxygen Toxicity.

a. Statement of the problem: The effects of high inspired oxygen tension and its relationship to oxygen toxicity were studied.

b. Background: The toxic effects on the lung from breathing pure oxygen have been demonstrated by many investigators.[1,2] The anatomic changes observed at post mortem explain the alterations in blood gas levels. The functional consequences of continuous exposure at 1 atmosphere include decreases in oxygen consumption and partial pressure oxygen.

The role of the respirator in the pathogenesis of the pulmonary lesion has been difficult to evaluate.[3] However, recent studies designed to examine the effect of ventilation with room air and 100% oxygen showed significant damage in the lung of oxygen breathing animals after 36 hours, whereas the lungs of air breathing animals were essentially normal. We have examined the effects of artificial ventilation in dogs with one lung exposed to 100% oxygen and one dog expired to room air.

c. Approach to the problem: Fifteen dogs were studied: 5 control and 10 experimental. Under pentobarbital anesthesia tracheostomy was performed and a double lumen endotracheal tube was inserted into the trachea. In the experimental animals one lung was ventilated with 100% O_2 and the other with room air; pO_2, pCO_2, pH and oxygen consumption determinations were made every six hours. The animal was given antibiotics. These animals were then maintained on Ace promazine and ventilated until death. Another group of animals was ventilated with air.

d. Results and discussion of the results: After 72 hours of continuous ventilation, the lungs that received 100% oxygen were strikingly different from the air respirator lung. The oxygen treated lungs were heavy

*Division of Experimental Pathology, WRAIR
**Department of Surgery, WRGH

and boggy. Microscopically, the oxygen treated lung was edematous with the presence of hyaline membrane, destruction of capillary endothelium and alveolar epithelial cells.

e. Conclusion: The results of this study show clearly that oxygen causes fatal pulmonary damage when it is delivered in high concentrations. We were able to demonstrate that the toxic effects on the lung is local and not a systemic reflex because of elevated Pa_{O2} levels. None of the microscopic findings observed in the oxygen toxic lung were seen in the lung ventilated with room air. It is evident then that the acute lesion seen is the lesion of oxygen toxicity alone and that the respirator does not produce or aggravate the injury.

f. Recommendation: The problem of etiology of lesion seen cannot be resolved by this short term study. We propose to continue this study with baboons with a hope that they can be maintained for longer periods and to study other parameters in order to comprehend some of the physiologic changes we observed.

2. Physiologic Changes Observed in the Anoxic and Hypoxic Lung of the Dog.

a. Statement of the problem: Physiologic changes observed in the anoxic and hypoxic lung of the dog were studied.

b. Background: The lung receives the entire output of the right heart which is distributed to either lung.[4] Normally, blood leaving the lungs is almost fully saturated with oxygen and relatively constant in its carbon dioxide tension, not only at rest but also during exercise. It has been shown in the past that hypoxia of a lung results in an increase in pulmonary vascular resistance, bronchoconstriction, and a decrease in blood flow and ventilation to the hypoxic lung.[5] Euler and Liljestrand proposed that the adaptation of alveolar perfusion to alveolar ventilation was a local phenomenon affected by local sensitivity of pulmonary vascular segments to changing oxygen and carbon dioxide tensions rather than by the autonomic nervous system.

c. Approach to the problem: Under sodium pentothal anesthesia the dogs (Florida Foxhounds) were intubated and then maintained on fluothane, N_2O, O_2 anesthesia on a Harvard animal respirator. A left thoracotomy was performed and two electromagnetic flow probes were chronically implanted, one on the right pulmonary artery and one on the left pulmonary artery.

After the wound was healed the animal was anesthetized with pentobarbital, 30 mg/kg, and intubated with a double lumen canine endotracheal tube in order that each lung could be studied separately. Blood gases, oxygen consumption and carbon output were measured.

d. Results and discussion of the results: Following anoxia to the experimental lung (100% N_2), there was an immediate increase of blood flow to the opposite or normal lung. This increase in blood flow to the normal lung did not occur as a result of a decrease in blood flow to the anoxic lung but as a result of an increase in the cardiac output. The blood flow to the anoxic lung either remained the same or in some cases it actually increased. There was an increase in O_2 consumption in the normal lung. This increase in oxygen consumption did not parallel the increase in blood flow but occurred after a delay of about 10-15 seconds. However, this increase in oxygen consumption continued for about 30 seconds after the peak blood flow to the normal side was reached. This same phenomenon was observed in the normal when air low in oxygen (10%) was used to ventilate the experimental lung, except that the changes were less dramatic, and the decrease in O_2 consumption in the hypoxic lung was small. The blood flow returned to baseline levels within seconds following cessation of the naoxic or hypoxic stimulus.

e. Conclusion: According to the data just presented one must conclude that acute hypoxia or anoxia of one lung does not result in an immediate fall in the volume of blood flow to that lung. There is an increase in oxygen consumption but it followed the increased blood flow by about 10 seconds and continues to increase after the peak blood flow was reached.

f. Recommendations: (1) These studies should be pursued further to determine the mechanism involved; (2) to measure blood flow following acute and chronic atelectasis; (3) to measure blood flow following re-expansion of totally collapsed lung; and (4) to correlate these measurements with measurements of other physiologic parameters.

3. The Effect of a New Alpha Adrenergic Blocker (WR 2823) on the Pulmonary Vascular Resistance.

a. Statement of the problem: The effect of a new alpha adrenergic blocker (WR 2823) on the pulmonary vascular resistance was studied.

b. Background: The pulmonary vascular resistance (PVR) which normally is low, is elevated in certain pathological conditions, such as hypoxia and endotoxin shock. Results of treatment of elevations of the PVR by alpha adrenergic blockade have been equivocal in the past.

c. Approach to the problem: Acute hypoxia was created in approximately 40 open chest mongrel dogs by rebreathing of expired air. Control elevations in the PVR were easily produced. Treatment of acute hypoxia with WR 2823 and its various analogues (WR 2823 AC, WR 2823 AB, Disulfide) did not prevent or reduce the elevated PVR.[6]

Eleven dogs were studied under the conditions of endotoxin shock. The controls showed significant elevations in pulmonary vascular resistance.

d. Results and discussion of the results: Treatment of these dogs with WR 2823 AB (formulated) significantly prevented the elevation of PVR and favorably improved the other hemodynamic parameters studied.

e. Conclusions: WR 2823 favorably improves the PVR under conditions of endotoxin shock. WR 2823 does not affect the elevated PVR in acute hypoxia.

f. Recommendation: Chronic hypoxia studies and the response of the PVR to WR 2823 should be performed.

4. Thoracic Injuries in Combat Casualties[7]

A report was made on this subject on pp.195-196, U. S. Army Medical Research Team (WRAIR) Vietnam and Institute Pasteur of Vietnam, Annual Progress Report, 1 July 1968 - 30 June 1969.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 122, Anesthesia and pulmonary complications of combat injury

Literature cited:

References

1. Balentine, J. D.: Pathologic effects of exposure to high oxygen tension. A review. N.E.J.M. 275: 1038, 1966.

2. Dubois, A. B.: Oxygen toxicity. Anesthesiology 23: 473, 1962.

3. Nash, G.: Blennerhassett, J. B., Pontoppidou, H.: Pulmonary lesions associated with oxygen therapy and artificial ventilation. N.E.J.M. 276: 368, 1967.

4. Dugard, A. and Naimark, A.: Effect of hypoxia on distribution of pulmonary blood flow. J. Appl. Physiol. 23: 613, 1967.

5. Weber, K. C., Engle, J. C., Lyons, G. W., Madsen, A. J., and Fox, I. J.: In Vivo calibration of electromagnetic flowmeter probes on pulmonary artery and aorta. J. Appl. Physiol. 25: 455, 1968.

6. Phillips, S. J. and Vick, J. A.: The pretreatment of E. coli endotoxin shock with WR 2823: a new alpha adrenergic blocking agent. (Submitted for clearance)

7. McNamara, J. J., Messersmith, J. K., and Stremple, J. F.: Thoracic injuries in combat casualties. Presented at the Society of Thoracic Surgeons, Atlanta, Ga., January 1970. Ann. Thor. Surg. (in press).

Publications

Hewitt, R. L., and Matsumoto, T.: Post-systolic myocardial augmentation with the Army artificial heart pump. J. Thorac. Cardiovasc. Surg. 57: 527, Apr. 1969.

Hewitt, R. L., and Matsumoto, T.: Army heart pump for postsystolic augmentation. Arch. Surg. 99: 88, July 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6469	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A822	00	120
b. CONTRIBUTING				
c. ~~SECONDARY~~	CDOG 1412(2)			

11. TITLE (Procede with Security Classification Code)

(U) METABOLIC RESPONSE TO DISEASE AND INJURY (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 PHYSIOLOGY 003500 CLINICAL MEDICINE 002300 BIOCHEMISTRY

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	20	365
FISCAL YEAR CURRENT 71	20	365

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL, W.H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Medicine

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)

NAME: Canfield, LTC, C.J.

TELEPHONE: 202-576-3529

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Earll, LTC, J.M. DA

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Metabolic; (U) Stress; (U) Endocrine; (U) Hormones

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

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.

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 General Hospital.

25. (U) - 69 07 - 70 06. Investigations were carried out on the hormone-energy-fuel relationships in acute starvation, electrolyte depletion, fever, and disease utilizing the techniques of radioimmunoassay, whole body radioactive counting, and metabolic balance study. A dual isotope technique was developed and used to assess rates of degradation and release of thyroid hormones in a variety of disease states, including malaria, and under the influence of some commonly used drugs. Studies were instituted to evaluate methods for combating immobilization osteoporosis and hypercalciuria resulting in prolonged convalescence and renal stones respectively in patients with war wounds and fractures. Comparative evaluation of methods for assessing ACTH reserve were completed. Techniques for sensitive determination of steroid hormones in small amounts of biologic fluids are being developed including competitive protein binding techniques, solid phase radioimmunoassay and gas chromatography. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Investigators.
Principal: LTC Craig J. Canfield, MC
Associates: LTC Jerry M. Earll, MC; Marcus Schaaf, M.D.; MAJ Leonard Wartofsky, MC; MAJ William J. Howard, MC; Elliot Danforth, Jr., M.D.; Joseph Bruton, Ph.D.

Description.

This work unit is concerned with investigations 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. Metabolic balance studies are utilized with precise collections of biologic samples from patients during rigid control of diet, drugs, and activity. In addition, support is afforded Walter Reed General Hospital in training house staff, endocrine patient care, and technical laboratory support to other departments.

Progress.

1. Steroid Metabolism.

Infection with Plasmodium falciparum is characterized by severe weakness, hypotension, vomiting, diarrhea, hyponatremia, and other clinical findings compatible with adrenal insufficiency. Thus it has been suggested that adrenal insufficiency might play a role in pathogenesis of acute pernicious malaria.

Recent publications from this department reported on the use of standard methods for assessing pituitary and adrenal function in 29 nonimmune patients with naturally acquired falciparum malaria (1). Levels of plasma 17-hydroxycorticosteroids (17-OHCS) were increased in all patients, and 12 of 14 patients tested had diurnal variation of abnormalities. In contrast to the increased plasma 17-OHCS, the urinary 17-OHCS and 17-ketosteroids (17-KS) were at the lower limit of normal in all patients. Administration of metapirone to 14 patients elicited a normal response. Thus from this study it was assumed that the observed depression of urinary-steroid excretion in association with increased plasma 17-OHCS levels reflects decreased hepatic conjugation during the acute illness since hepatomegaly and increased serum glutamic oxaloacetic transaminase levels were frequent and creatinine clearance was normal.

Further studies are planned to answer the following: a) the effect of exogenous ACTH during acute falciparum malaria, and b) the effect of malaria drugs (chloroquine and pyrimethamine) on conjugation mechanism

and plasma clearance. In addition, cortisol secretory rates will be determined during acute falciparum malaria in order to explain the differences seen in plasma cortisol levels and urinary 17-OHCS.

One of the most pressing needs in the general field of hormone assay is the development of techniques of sufficient sensitivity to enable quantitative determinations to be performed in biological fluids at frequent intervals on small volumes of materials in order to study acute physiological changes. Also, such techniques will enable a better understanding of the temporal relationship between the pituitary hormones and the feedback effects of hormones produced by the target glands.

In order to meet these needs the steroid laboratory is developing a series of new methods for assaying minute quantities of steroids in small volume of biological fluids. These methods are as follows: a) competitive protein binding (CPB) techniques for cortisol, desoxy-cortisol, testosterone, 17-OH progesterone, and other plasma steroids, b) solid-phase radioimmunoassay techniques for testosterone, estradiol-17B, and other plasma steroids, and c) gas-chromatography using an electron-capture detector for urinary steroids and plasma steroids.

The competitive protein binding technique has allowed us to measure plasma cortisol levels in plasma volumes as low as 50 microliters as compared to a previous requirement of 2-5 milliliters. Also, the assay conditions do not require complete withdrawal of medications from the patient. In addition, the number of samples handled by a single analyst is 40 per day as compared to 40 per week by previous methods. All these advantages have been achieved without any loss of precision, sensitivity, or accuracy.

Solid-phase radioimmunoassay techniques are relatively new for steroids and today show great promises of being even more sensitive and accurate than the CPB techniques with even more advantages. This technique is now being explored for use in our laboratories for the assay of testosterone, dihydrotestosterone, and estradiol-17B.

Continuation in these areas of steroid analysis should allow for better understanding of the temporal relationship between the pituitary hormones and target glands and a saving in manpower, time, and accuracy of steroid analysis.

To assess agreement between several tests of growth hormone and ACTH presently in use clinically, 35 evaluations were carried out in patients suspected of hypopituitarism. Growth hormone reserve was assessed by the insulin, arginine and pyrogen (Piromen) tests while metyrapone, insulin, and Piromen were used to test ACTH reserve. Of 20 instances in which all three growth hormone tests were performed, disparate results were encountered in seven. The overall incidence of disagreement between tests was 31 percent. In the 21 studies in which all three ACTH tests were carried out, divergent results were observed in 15.

In the entire series, 46 percent of ACTH evaluations gave conflicting results. There seemed little difference in sensitivity of the various tests and the results could not be correlated with anatomic lesions. The results indicate the difficulty of arriving at a conclusion on the basis of any single test. Further information on the mechanisms of pituitary hormone release in response to stimuli is necessary for interpretation of divergent test results.

2. Thyroid Metabolism.

A study examining a method to evaluate the latency, potency, and duration of action of the various antithyroid drugs has been completed. This study begun by Dr. Wartofsky prior to coming to the Walter Reed Army Institute of Research was presented at The 6th International Thyroid Congress in Vienna, Austria in June 1970.

The method involves an IV injection of ^{131}I-T_4 which serves via its deiodination as a continuous source of ^{131}I which partitions between thyroid and urine as would a diagnostic oral dose. Hence, urinary I^* excretion (UI^*), determined at frequent intervals, varies inversely with thyroid I^* uptake during the same interval. The ratio, UI^*/plasma PBI^* corrects UI^* for the exponential decline in precursor PBI^*, and should remain constant during the steady state. Inhibition of thyroid I^* uptake should be followed by a rising ratio, the promptness of the rise reflecting latency of onset, and its magnitude and persistence reflecting the potency and duration of drug-induced antithyroid action. A number of thyrotoxic and normal subjects were studied, and after single oral doses of methimazole (MMI; 30 mg), $NaClO_4$ or KSCN (500 mg), ratios rose and remained elevated for 24 to 96 hours. Peak values of the ratio were usually well in excess of those predicted from the previously measured thyroid uptake of ^{131}I. This excessive UI^* could only be I^* generated by intrathyroidal deiodination. Following each agent, peak values of the ratio were not reached for 6 to 24 hours, but occurred promptly (2 to 4 hours) when $NaClO_4$ was given during MMI blockase (30 mg q 6 hr).

Conclusions: These agents block organification of both internally generated iodide and that derived from plasma. Quantitatively, the former action is the more important. Iodide derived from intrathyroidal deiodinations may not be readily exchangeable with that derived from plasma, but may become so during MMI blockade.

Using the principles outlined above, following an injection ^{131}I-T_4, the ratios of $U^{131}I/PB^{131}I$ are examined during 4-5 days of therapy with varying regimens of antithyroid drug, PTU or tapazole generally given as a single daily dose or in divided dosage (q 8 hrs).

Of some seven thyrotoxic subjects studied on Ward 30 to date, six clearly demonstrate, by this objective technique, that they are better controlled by divided dose management. This contrasts with reports by Monte Greer that patients achieved satisfactory clinical control often

with only single dose therapy. This study is a collaborative effort with Dr. Sidney Ingbar of Boston and will probably be completed when a total of 12-15 patients have been studied.

A study of the effect of acute malarial infection on thyroxine kinetics and hormonal secretion was done in cooperation with Dr. John Arnold at Kansas City. ^{125}I was given to label the thyroid and hence endogenous hormone release was labeled, while ^{131}I-T_4 was given to label the blood pool. During induction of acute infection, changes in both parameters were assessed by estimates of T_4 degradation and release rates for the two varieties of hormone.

Studies on four normal volunteers have been completed to date, and indicate that acute malarial infection retards the peripheral disposal of thyroid hormone without there occurring any compensation via enhanced endogenous release. There appears to be rapid recovery during convalescence. We plan further studies in an attempt to duplicate these interesting results.

We have had the opportunity to study a patient recently with the rare entity of hyperthyroidism due to selective hypersecretion of T_3 preferentially over T_4. We examined T_3 and T_4 turnover kinetics before and after thyroid suppression. The patient is to be re-evaluated after treatment and may constitute an interesting case report.

A study of a method to establish a test for TSH reserve was initiated in order to evaluate the validity of the use of the radioiodine uptake test following steroid administration, as an index of thyrotropin reserve. The principle is based on recent studies demonstrating a rebound in TSH levels following steroid withdrawal, and our thought that this could be a means of differentiating between normal and hypopituitary responses. To date only hypopituitary subjects have been studied and have not shown any reserve; what remains however, is to prove that normals can elicit enough of a TSH rise by the method utilized to be clearly different from the patients with pituitary insufficiency.

A study of the effects of phenobarbital on thyroidal function and thyroxine utilizing the double isotope technique described in the malaria study above, is designed to examine the effects of customarily used therapeutic doses of phenobarbital on hormonal release and degradation rates in man. The latter have been shown to be accelerated in rodent studies and the method described represents a valid way to study the problem in humans. Two subjects, one with hyperthyroidism and one with myxedema, have been so studied to date, and the results would indicate little effect on these parameters by ordinary doses of phenobarbital in man. More patients will have to be examined before a definitive conclusion can be reached.

A study of phenobarbital induction of enzymes in rat liver controlling thyroxine degradation was designed to examine the effects of drugs on inducing or stimulating T_4 deiodination. Many enzymes can be so induced.

In some 12-15 experiments with 6-20 rats per experiment, we have looked at the relative degradation rates of rat liver homogenates for T_4-^{131}I in control versus phenobarbital treated rats. These experiments clearly indicate significant stimulation. We are currently engaged in assessing the importance of the animals thyroidal or metabolic status in regard to the capability for induction of this liver deiodinating systems.

3. Calcium Metabolism.

The long periods of immobilization associated with the treatment of fracture, nerve injuries and other serious injuries or illnesses, results in loss of large amounts of calcium in the urine and negative calcium balance. Bones lose their strength, prolonging convalescence, and it is highly questionable whether all the calcium is ever replaced. Recent studies have suggested that the hypercalciuria can be significantly improved with therapies including phosphates, thiazides, and alkali.

Patients requiring prolonged immobilization are being studied on the Metabolic Ward with careful attention to calcium balance. New tests introduced to assist in studies have included ionized calcium determinations and radioactive calcium[47] turnover studies in the whole body counter. Preliminary data suggests that the hypercalciuria is significantly reduced by each of the three forms of therapy. The reduction varies but is usually as much as 50 to 70 percent. Metabolic studies to determine if positive calcium balance can be maintained are being calculated. Patients with other bone diseases and hypercalciuria are being included in this type study. It is established that each of these therapies effectively reduce hypercalciuria and any of the three should improve some of the complications of excess calcium loss in the urine. Comparison of the effectiveness, safety and ease of management of these therapies is being made. Fracture healing notes will also be assessed.

Antibodies to purified calcitonin and parathormone are being prepared in three different animal species in an effort to develop satisfactory radioimmunoassays which would be of great assistance for diagnostic and research studies.

Hydrochlorthiazide, 50 mg b.i.d. orally, has been administered to 12 patients maintained on a constant Ca-P intake. Urinary calcium decreased 20 to 55 percent while urinary phosphate, magnesium, sodium and potassium increased, and creatinine clearance was unchanged. In six patients with idiopathic hypercalciuria mean serum calcium increased slightly, while six patients with borderline hypercalciuria due to hyperparathyroidism (six surgically proven, six awaiting surgery) became frankly hypercalcemic. Ionized calcium showed comparable changes. Serum phosphorus, creatinine, uric acid and proteins also rose, and serum magnesium and potassium declined in some patients. K^{40} whole body counting confirmed potassium loss estimated by urinary changes. Thiazides appear useful in treating and in distinguishing

idiopathic hypercalciuria from suspected hyperparathyroidism. The mode of action of thiazide-induced hypercalcemia in hyperparathyroidism is under continued study.

Severe hypocalcemia is known to impair insulin secretion and carbohydrate tolerance. However, hyperparathyroidism may also be associated with impairment of carbohydrate tolerance, perhaps due to renal potassium loss. To assess this possibility, a standard 8 hour oral GTT with measurement of IRI and HGH has been performed in 8 hyperparathyroid subjects before parathyroid exploration, and correlated with K^{40} counting of whole body potassium; studies will be repeated after cure of hyperparathyroidism. Pre-operatively, 3 patients had frankly diabetic tolerance tests, 1 had possible diabetes, and 4 were normal. One of 3 patients with idiopathic hypercalciuria had chemical diabetes, Insulin and growth hormone determinations are in process.

Recent reports suggest that idiopathic hypercalciuria patients have abnormal parathyroid glands when studied by electron microscopy. An electron microscopy study of normal and adenomatous parathyroid tissue is under way.

4. Carbohydrate Metabolism.

Potassium depletion as a cause of carbohydrate intolerance in normal subjects is poorly documented and unexplained. To document this and to evaluate possible etiologic factors serum glucose, insulin, and growth hormone levels were measured in five normal male subjects during oral glucose (100 gm), IV glucose (0.5 gm/Kg), arginine infusion (0.5 gm/Kg) and glucagon stimulation (1.0 mg) tests before the following potassium depletion. Total body potassium depletion (224 to 375 milliequivalents) was produced using ion exchange resin and formula diet and documented by metabolic balance and whole body counting of K^{40}. Potassium depletion caused increased serum glucose ($p < .05$) and decreased glucose disappearance rates ($p < .05$) with decreased serum IRI ($p < .05$ and $p > .01$) following oral and IV glucose tolerance tests respectively. Serum IRI response to arginine infusion and glucagon stimulation was unchanged after potassium depletion, although arginine infusion resulted in a higher rise and blunted fall in serum glucose. Glucose response to glucagon was unchanged by potassium depletion. Growth hormone response was not significantly different during oral glucose or arginine tests after potassium depletion.

Conclusions: At the levels of potassium depletion obtained 1) there is decreased tolerance to oral and IV glucose, 2) serum IRI levels are decreased per unit of glucose following glycemic stimulus, but unchanged following the nonglycemic stimuli of arginine and glucagon, and 3) growth hormone response is uneffected.

Glucose tolerance to oral and intravenous glucose administration was compared to the tolerance of peritoneal glucose absorption in patients with chronic end-stage renal disease who had undergone peritoneal dialysis

develops following peritoneal glucose absorption than following oral administration of glucose - even at an oral administration rate as much as two times the peritoneal absorption rate in some patients. At comparable serum glucose concentrations the serum insulin to serum glucose concentration ratio tends to be relatively higher than the similar ratio following peritoneal absorption. Slower gastrointestinal glucose absorption rates relative to peritoneal absorption rates and augmented insulin release during gastrointestinal glucose absorption probably both contributed to the above findings. The tendency to hyperglycemia during peritoneal dialysis may thus result from rapid absorption and lack of early augmented insulin release similar to the situation with intravenous glucose administration.

Elevation of growth hormone, epinephrine, norepinephrine, and 17-hydroxycorticosteroids during acute hypoglycemia is well documented, however the responses of these hormones to gradual spontaneous hypoglycemia has not been well studied. They were therefore measured in a group of 33 patients suspected of hypoglycemia during the following tests: 72 hour fast, oral glucose tolerance test, intravenous tolbutamide tolerance test, oral leucine, and intravenous glucagon administration. Four patients with insulinoma and one with hypoglycemia due to gastric adenocarcinoma were discovered. Intravenous tolbutamide tolerance test produced serum growth hormone rises in all insulinoma subjects tested (peak 6.7-24.8 mug/ml), however fasting hypoglycemia failed to elicit consistent growth hormone elevations. No growth hormone response was found to either oral glucose tolerance test or fasting in the one patient with extrapancreatic tumor producing hypoglycemia. Plasma epinephrine and norepinephrine were not elevated on nine occasions during fasting hypoglycemia in two subjects. Urinary epinephrine and norepinephrine were rarely elevated and VMA was consistently normal during days marked by frequent episodes of hypoglycemia. Plasma 17-OHCS were mildly elevated during fasting hypoglycemia with loss of normal circadian pattern, though urinary 17-OHCS remained normal.

Conclusion: Repeated severe episodes of gradual spontaneous hypoglycemia in patients with insulinoma did not produce consistent or impressive elevations in growth hormone, 17-OHCS or epinephrine and norepinephrine, despite uniformly brisk rises of growth hormone and 17-OHCS to acute stimulation, intravenous tolbutamide tolerance test.

The serum growth hormone response to insulin-induced hypoglycemia of 10 thyrotoxic patients was compared with that of 14 normal subjects (3). All thyrotoxic subjects had a distinct rise in growth hormone attaining a value of at least 6.4 mμg per ml. Mean growth hormone levels of thyrotoxic subjects did not differ significantly from those of normals at any time during the insulin tolerance test. These results indicate that growth hormone response to insulin-induced hypoglycemia in thyrotoxicosis is normal.

It has been reported that administration of glucagon stimulates the release of growth hormone. To test this hypothesis 10 male subjects

were given glucagon at two dose levels, 1 mg and 0.1 mg, intravenously, and serum collected for growth hormone and 17-OHCS at intervals for one hour [4]. Results showed no significant change in the level of serum growth hormone to either dose of glucagon. A barely significant increase in 17-OHCS was seen following the high dose of glucagon which can most likely be explained by nausea produced by this dose. It seems unlikely therefore that glucagon plays a direct physiologic role in either the regulation of growth hormone or cortisol and does not seem to be of value as a test of growth hormone or ACTH reserve in adults.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Literature Cited.

1. Brooks, M.H., Barry, K.G., Cirksena, W.J., Malloy, J.P., Bruton, J., and Gilliland, P.F.: Pituitary-adrenal function in acute falciparum malaria. Am. J. Trop. Med. & Hyg. 18:872, 1969.

2. Nolph, K.D., Rosenfeld, P.S., Powell, J.T., and Danforth, E.: Peritoneal glucose transport and glucose tolerance during peritoneal dialysis. Am. J. Med. Sci. 259:272, 1970.

3. Rosenfeld, P.S., Wool, M.S., and Danforth, E.: Growth hormone response to insulin-induced hypoglycemia in thyrotoxicosis. J. Endocr. & Metab. 29:777, 1969.

4. Danforth, E., and Rosenfeld, P.S.: Effect of intravenous glucagon on circulating levels of growth hormone and 17-hydroxycorticosteroids. J. Clin. Endocr. & Metab. 30:117, 1970.

5. Bluemle, M.L. Tracheal bacterial counts of patients following suctioning. Nursing Rsch. 19:116, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6345	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A822	00	121
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)
(U) Pathogenesis of Enteric Disease (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
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: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	4	115
	CURRENT 71	4	115

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D C 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Div of CD and I
ADDRESS: Washington D C 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Formal, S.B.
TELEPHONE: 202-576-3344
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: LaBrec, E.H.
NAME:

21. GENERAL USE
Foreign Intelligence Not Considered

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.)

23 (U) To find improved procedures to control diarrheal disease. Present work involves the preparation and testing of oral vaccines against bacillary dysentery, and the identification and characterization of agents involved in "travelers diarrhea".

24 (U) Attenuated dysentery strains are being developed. They are being evaluated for safety in several systems and are being treated for potency in monkeys and in man.

25 (U) 69 07 - 70 06. Two E. coli serotypes (06:H16: and 0148:H28) which are purported to produce an enterotoxin and an E. coli serotype (0124:K72:H) which produces a dysentery-like disease in humans were compared in several experimental animal models. The 0124 serotype causes keratoconjunctivitis in guinea pig eyes, the 06 and 0148 strains were negative. By the fluorescent antibody technique it was shown 0124 serotype when fed to starved opiated guinea pigs penetrated the intestinal epithelial barrier, the 06 and 0148 serotypes were found only in the lumen. When fed to monkeys the three strains produced diarrhea but only the 0124 strain was found within the intestinal mucosa. Culture supernates of the 06 and 0148 serotypes have a positive enterosorptive effect when inoculated into the isolated rabbit ileal loop. The 0124 strain was negative. Preliminary feeding experiments in human volunteers have shown the 06 and 0148 serotypes capable of producing diarrhea. We have tentatively concluded that strains of these serotypes 06 and 0148 do elaborate a toxin capable of producing a diarrheal disease syndrome without invasion of the intestinal epithelial barrier.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 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 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 121, Pathogenesis of enteric diseases

Investigators.
Principal: Samuel B. Formal, Ph.D.
Associate: Eugene H. LaBrec, Ph.D.

Description.

The pathogenesis of enteric disease is studied to elucidate the mechanisms by which enteric pathogens produce symptoms. By understanding the disease process improved procedures for prevention and treatment of diarrheal diseases will become evident.

Progress.

1. Last year we reported that S. flexneri 2a antigens could be transferred to E. coli using techniques of microbial recombination. Thus E. coli hybrid strains can be constructed with the same antigenic pattern as S. flexneri 2a. Such E. coli hybrids would constitute a safe product for use as a living oral vaccine, but no evidence concerning their ability to confer protection was at hand. Consequently two tests were carried out in monkeys to determine if one selected E. coli hybrid strain would protect the animals against experimental oral challenge with virulent S. flexneri 2a. The results of this study are summarized in Table 1. The vaccine conferred a statistically significant protection against the experimentally induced disease. This was especially noticeable against the severe form (dysentery). Nevertheless this vaccine seemed to offer no particular advantages over the shigella mutant-hybrid type which has been previously described. Therefore, further safety testing in volunteers is not contemplated.

2. During the past year, work has commenced on some aspects of the pathogenesis of "non-specific" or "travelers'" diarrhea. In areas where diarrheal disease is endemic, an etiologic agent can be isolated from only a small proportion of individuals with clinical disease. Indeed Kalas and Bearden found recognized microbial pathogens in only 20 per cent of American soldiers with diarrhea in Viet Nam. Recently Japanese workers have reported that certain serotypes of E. coli are responsible for a dysentery-like illness in children and adults in Japan. Because a significant number of cases of non-specific diarrhea in the Kalas-Bearden study presented a dysentery-like syndrome (defined as diarrhea with an inflamed or hyperemic colon), E. coli strains isolated from them were examined.

None of the strains fell into the serological group which the Japanese workers found causes dysentery-like disease in their country. After preliminary tests in animals, two of these E. coli strains were selected for further study. One of these strains, B2C, was serologically typed as O-group 06:H16; the other strain, B7A, was reported to be untypable, but subsequent work by us showed that it was antigenetically identical with the E. coli 0148:H28 strains (a new serotype) reported to be the cause of "travelers' diarrhea" in British troops in the Middle East. These two strains (B2C and B7A) were compared in various model systems with E. coli O group 124: K72:H- strains 1272/67. This latter strain is one of the group of organisms described by Japanese workers as causing dysentery-like disease in adults. E. coli strain HS, isolated from a normal individual was also included as a negative control.

The O-124 strain caused keratoconjunctivitis, while the O-06, O-148 and HS strains were negative. When fed to starved opiated guinea pigs the O-124 strain was shown by the fluorescent antibody technique to have penetrated the epithelial layer of the intestine and to be present in the epithelial cells and the lamina propria of the intestine. In contrast the O-06 and O-148 strains were observed only in the lumen of the bowel; however there was an increased amount of fluid and mucous in the lumen of the intestines of these animals. The animals did not die as a result of this infection. The four strains were also inoculated into monolayers of HeLa cells. Within 7 hours the O-124 strain was observed within the cells and the monolayer was dead within 24 hrs. We could not observe organisms of the O-group 06 strain inside the HeLa cells, for by 7 hr post-inoculation, large numbers of these bacteria appeared to adhere to the outer surface of the cells and by 24 hours the monolayer was destroyed. Neither the O-group 148 nor the HS strain had any apparent effect on the cell culture. We concluded from these experiments that the O-group 06 and 148 strains were incapable of penetrating epithelial cells and in confirmation of the work of the Japanese workers, that the O-group 124 strain did possess this capacity. In this regard, this strain closely resembled virulent dysentery bacilli.

Each of the four strains were next fed to individual groups of 18 rhesus monkeys. The dose administered was approximately 3×10^{10} cells suspended in brain-heart infusion broth. The HS strain failed to produce diarrhea in any of the monkeys. The strains of O-groups 06, 124, and 148 all evoked a similar pattern. From approximately 18% to 40% of these animals developed diarrhea. In all but 2 cases,

the watery stools ceased 24 to 36 hours post-challenge, and in these 2 monkeys the signs of disease persisted for only 2-1/2 days. In no instance were blood-tinged stools observed. We have had occasion to feed 2 other dysentery-like E. coli strains to monkeys. Strain 1624-56 (0144:K:H-) and strain 4608-58 (0143:K:H-) were obtained from Dr. Ewing at the Communicable Disease Center. Both cultures gave reactions similar to the 0-124 strain in regard to epithelial cell penetrability. Following challenge with these strains the same pattern of diarrhea in monkeys was observed as when the 0-124 culture was fed. Diarrhea was observed in approximately 50% of the animals and subsided 24 to 48 hrs after the bacteria were administered. Blood was not observed in the stools.

Our next study demonstrated that the O-group 06, 124, and 148 strains evoked a positive reaction in the rabbit ileal loop test. The HS strain failed to do so. We then attempted to determine if any of the 4 strains under study elaborated an enterotoxin for the rabbit ileal loop. Sterile filtrates were prepared from 18 hr aerated syncase broth filtrates of the 4 strains and injected in 2 ml amounts into ileal loops of rabbits. Control loops were inoculated with either heated filtrates (100 C for 30 min) or sterile broth. Filtrates from the O-groups 06 and 148 strains produced positive loops. On the other hand the loops injected with the 0-124 strain filtrate, the heated filtrates and the inoculated broth remained negative. Sonic lysates of cells of the O-group 06 and 148 strains also produced positive loops. Approximately 1 mg dry weight of dialysed sonic lysate were required. The results of these animal tests are summarized in Table 2.

Although the O-group 06, and 148 strains were isolated from individuals with acute colitis, there was nothing to indicate that these organisms were the etiologic agent of the disease other than the fact that they provoked a positive ileal loop in the rabbit and produced an enterotoxin by laboratory criteria. Because of their potential medical importance, it was decided to administer them to volunteers. Accordingly 1 X 10^8 cells of either the HS strain and the O-group 06, 124, and 148 strains, suspended in 20 ml milk were fed by Dr. Hornick to groups of 5 well-informed male adults. An individual was considered to have disease if he passed at least 3 watery stools within a 24 hr period. None of the 5 men fed the HS strain became ill. Of the 15 remaining men fed either of the O-groups 06, 124 or 148 strains, a total of 4 men experienced diarrhea (Table 3). The incubation period ranged from 1-5 days; illness lasted from 1 to 6 days; the number of stools per day varied from 3 per day to 6 per day. Neither blood nor mucous was present in the diarrheal stools, and the men did not have elevated body temperatures.

Other groups of men were then fed 1 X 10^{10} organisms of each of the strains. Again none of the men fed the HS strain experienced signs of disease. We were somewhat surprised to observe that none of the 5 men fed the O-124 strain became ill. On the other hand 3 of 5 men fed the O-06 strain and 4 of 5 volunteers fed the O-148 strain developed diarrhea (Table 2). The incubation period, as before ranged from 1 to 5 days. Illness lasted from 1 day to 2-1/2 weeks. The number of stools per day varied from 4 per day to 10 per day. Blood was absent from the stools and fever was not noted. Mucous was not observed in the stools for the first 2 days following challenge, but was present in copious amounts in 3 individuals after this time. The one volunteer whose disease lasted 2-1/2 weeks developed a colitis indistinguishable from shigellosis as viewed by sigmoidoscopy.

TABLE 1

Protection of Monkeys Against Experimental Challenge with S. flexneri using an E. coli - S. flexneri 2a hybrid strain* as an oral vaccine. (Exps. M117 and M121).

Group	No. of Animals	No. with Diarrhea	No. with Dysentery	Total Ill	P
Vaccine	34	11	1	12	
Control	35	13	7	20	.05

*
The hybrid was an E. coli strain which contained the histidine and proline regions of the S. flexneri 2a chromosome.

TABLE 2

REACTIONS OF 4 ESCHERICHIA COLI STRAINS IN VARIOUS LABORATORY MODELS

Strain	Serotype	Sereney Test	Invasion of HeLa Cells	Invasion of bowel mucosa (starved guinea pig)	Rabbit ileal loop	Production of enterotoxin	Diarrh. in monkey
H5	not typed	neg	neg	neg	neg	neg	0/1[illegible]
B2C	0:06:K?:H16	neg	neg**	neg	pos	pos	6/1[illegible]
B7A	0:0148:K?H28	neg	neg	neg	pos	pos	3/1[illegible]
1272-67	0:0124:K72:H-	pos	pos	pos	pos	neg	7/18

* The diarrhea, when observed, lasted 24 to 36 hrs with the exception of 2 animals where it persisted for 2-1/2 days.

** Strain B2C was not observed within the HeLa cell. At 7 hrs post-infection the bacteria were seen to adhere to the outer surface of the cells, and by 24 hrs the cell layer was dead.

TABLE 3

ILLNESS IN ADULT VOLUNTEERS FED FOUR STRAINS OF *ESCHERICHIA COLI*

Strain	Serotype	Dose	No. ill / Total
HS	not typed	$1X10^8$	0/5
		$1X10^{10}$	0/5
B2C	06:K?H16	$1X10^8$	2/5
		$1X10^{10}$	3/5
B7A	0148:K?:H28	$1X10^8$	1/5
		$1X10^{10}$	4/5
1272-67	0124:K72:H	$1X10^8$	1/5
		$1X10^{10}$	0/5

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Un. 121, Pathogenesis of enteric diseases

Literature Cited.

Publications.

1. DuPont, H.L., R.B. Hornick, A.T. Dawkins, M.J. Snyder, and S. B. Formal. The response of man to virulent Shigella flexneri 2a. J. Inf. Dis. 119: 296-299, 1969.

2. DuPont, H.L., R.B. Hornick, J. Libonati, M.J. Snyder and S. B. Formal. Study of shigella vaccines in man. Ninth Interscience Conference on Antimicrobial Agents and Chemotherapy 27-29 Oct. 69, Washington Hilton Hotel.

3. Falkow, S. and S.B. Formal. Restriction in genetic crosses between Escherichia coli and Shigella flexneri. J. Bacteriol., 100: 540-541, 1969.

4. Formal, S.B., P. Gemski, Jr., L.S. Baron and E. H. LaBrec. Genetic transfer of Shigella flexneri antigens to Escherichia coli K-12. Inf. and Immunity 1: 279-287, 1970.

5. Lawrence, T.L. Jr., and Hugh Collins. The effect of parenteral and oral immunization on encephalomyocarditis infection in mice. Proc. Soc. Exp. Biol. Med. 133: 1066-1069, 1970.

6. Formal S. B., E.H. LaBrec, R.B. Hornick, H.L. DuPont and M.J. Snyder. Attenuation of strains of dysentery bacilli. Round Table Conference on Vaccines from Salmonella, Shigella, V. cholerae. Berne, Switzerland 1969.

7. DuPont, H.L., R.B. Hornick, J. Libonati, M.J. Snyder and S.B.Formal. Study of Shigella vaccines in man. Round Table Conference on Vaccines from Salmonella, Shigella, V. cholerae. Berne, Switzerland 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6436	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A822	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with security Classification Code)

(U) Microbial Genetics and Taxonomy (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 70	4	125
FISCAL YEAR — CURRENT 71	4	125

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Communicable Disease and Immunology

ADDRESS: Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Baron, Dr. L.S.

TELEPHONE: 202-576-2230

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Wohlhieter, Dr. J. A. DA

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Microbial Genetics; (U) Taxonomy; (U) Enteric Bacteria; (U) Antigens; (U) Virulence; (U) Salmonella; (U) Drug Resistance

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Definition in genetic and molecular terms of the metabolic, antigenic and pathogenic characteristics of enteric bacteria. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and/or pathogenicity to serve as vaccine strains or as tools to study the infectious process.

24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.

25. (U) 69 07 70 06. Investigations on the molecular and genetic aspects of intergeneric bacterial hybridizations are continuing to provide basic information for use of such hybrids in understanding bacterial virulence and the development of vaccines. Strains enabling the direct hybridization of Salmonella species with chromosomal genes of Shigella flexneri have been developed. These strains have been employed in constructing S. typhimurium hybrids which express, in addition to their native somatic antigenic properties, the S. flexneri 2a group antigenic determinants. Immunization of rabbits with such hybrids induces the formation of antibodies specifically directed against both these medically important microbes. Investigations on the behavior of E. coli phage lambda on S. typhosa and S. flexneri have revealed that the transduction of the gal operon (including the galactose epimerase genes involved in lipopolysaccharide somatic antigen synthesis) can be achieved between different genera of the enterobacteraceae. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 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.

BLANK PAGE

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 122, Microbial genetics and taxonomy

Investigators.

Principal: Louis S. Baron, Ph.D.

Associate: John A. Wohlhieter, Ph.D.; Edward M. Johnson, Ph.D.; Peter Gemski, Jr., Ph.D.; Sheridan Easterling, M.S.; Isaac R. Ryman; Charles Life; SP-4 Ronald W. Leavitt, M.S., PFC Joseph Alexeichik, A.B.; Charlotte Parker, M.S.

Description.

The purpose of these studies is to investigate the genetic characteristics of the metabolic and antigenic changes occurring in the enteric bacteria as a consequence of genetic recombination, episomic transfer and transduction.

1. The ability of certain Salmonella hybrid strains to repress the multiplication of coliphage λ can be overcome by the presence of additional segments of the Escherichia coli genome within the hybrids.

2. A genetic exchange system between Shigella flexneri 2a Hfr donor and Salmonella typhimurium recipients has been developed.

3. Salmonella typhimurium phage 9NA employed in genetic studies on Salmonella lipopolysaccharide structure has been characterized by biological and physico-chemical techniques.

4. A partial diploid hybrid derived from a Salmonella typhosa Hfr strain was shown to conserve and transfer a continuous Escherichia coli chromosomal segment, genetically complete from the pro-A locus to the str locus, as determined by gradient of transmission, interrupted matings and unselected marker analysis.

5. Matings of an Escherichia coli Hfr with a Salmonella typhosa recipient results in a partial diploic which maintains certain regions of the transferred Escherichia coli DNA. When the DNA is extracted from the Salmonella diploid hybrid, the Escherichia coli DNA from the hybrid is found in the form of closed supercoiled circles. In the case of one diploid examined, this circular

E. coli DNA had a molecular weight of between 66 to 70 million daltons.

6. The Vibrio cholerae mating system originally described by Bhaskaran has been re-examined and confirmed as a possible genetic system for this organism. The classical Vibrio cholerae strain NIH41 is lysogenic for at least two different bacteriophages which plate on El Tor indicator strains.

Progress.

1. Salmonella typhosa strains are unable to adsorb bacteriophage λ because they lack the ability to produce functional receptor for this phage. As a consequence, these strains are inert to infection with λ. In order to study the pattern of infection of Salmonella by λ, we have transferred by conjugation with Escherichia coli K-12 Hfr donors, a chromosomal segment containing the genes controlling the λ phage receptor to S. typhosa recipients.

Despite acquisition of the ability to adsorb λ, such S. typhosa hybrids still remain insensitive to lysis by λ though they can be transduced by λdg, the defective phage carrying the galactose genes of E. coli K-12.

Additional segments of the E. coli K-12 genome have transferred to S. typhosa recipients through the use of other E. coli Hfr strains. The S. typhosa hybrid strain containing further K-12 genes is still insensitive to wild-type λ, but is susceptible to rare mutants in the λ phage population which we have isolated and termed λsx. The λsx mutant phage in part owes its ability to plate on particular S. typhosa hybrids to a mutation which maps between genes P and Q. The λsx mutant phage produces plaques at equal efficiency on both E. coli K-12 and S. typhosa hybrid WR4253 but fails to produce discernible plaques on S. typhosa hybrids containing only the λ receptor region. λsx mutants also are unable to produce plaques on the S. typhosa diploid hybrid WR4251 from which the λsx sensitive strain originated as a segregant. S. typhosa hybrid WR4251 gives rise to another segregant type WR4252, also insensitive to λsx, but from which a λsx sensitive derivative was isolated fortuitously. These Salmonella hybrid strains can be recognized on the basis of their colonial morphology. The small dense colony type has been characterized as a S. typhosa diploid which is insensitive to λsx, as is a large crinkled colony type segregated by the diploid hybrid.

Though insensitive to λsx, both of these strains are transduced by λdg at a frequency similar to that found with E. coli K-12. A smaller translucent colony type has been correlated with the λsx sensitive segregant. The basis for this variation in λsx sensitivity in the diploid hybrid and its segregant types has not been established, but may be due to the chromosomal or extra-chromosomal state of the K-12 genes in the hybrids.

We have also tested λ^{i434}, a hybrid between phage λ and 434, as well as λ^{i434}sx mutants isolated when λ^{i434} is plated on S. typhosa hybrid WR4253. The efficiency of plaque formation obtained with these phages on the various S. typhosa hybrids depends upon the extent of E. coli genetic material within the hybrids. The results also established that further mutant phages exist in the population of λ^{i434}sx which can produce plaques on S. typhosa hybrids containing only the λ receptor region of the K-12 chromosome. This is also the case with the diploid hybrid WR4251 and its segregant type WR4252 which are insensitive to λ, λsx, or λ^{i434}sx, but are sensitive to mutant phages in the λ^{i434}sx population. A series of hybrids between phage λ and phage 21 referred to as λ^{i21} hybrids have also been tested and behave much like λsx except that λ^{i21} hybrid 1 and λ^{i21} hybrid 2 have mutants in their population which produce plaques on S. typhosa hybrid segregant WR4252 though not on S. typhosa hybrids with only the λ receptor region.

We have recently prepared diploid hybrids employing an Hfr strain of S. typhosa as the recipient in crosses with a K-12 Hfr as the donor. Such S. typhosa hybrids permit us to determine whether the K-12 diploid material within the S. typhosa donor hybrids is continuous since they can be used to donate the E. coli genes which they contain to F⁻ E. coli recipients. We have found that when S. typhosa hybrid strains have the continuous chromosomal segment extending from the origin of E. coli Hfr WR2004 through the λ receptor locus which is approximately 40% of the E. coli chromosome, they are able to plate wild-type λ and λ^{i434} to titer and conform in all other respects tested to the pattern of interactions observed with λ in E. coli K-12.

At the present time, we are unable to define the specific nature of the gene or genes which permit λ phage to plate as efficiently on certain S. typhosa hybrids as it does on E. coli. It is perhaps likely that the chromosomal segment involved includes various loci specifically related to the expression of λ in Salmonella. It is also conceivable that the genes in this segment are not specifically related to λ expression but may be concerned with the general

functioning of foreign DNA in Salmonella.

2. Genetic exchange between Shigella flexneri and Salmonella typhimurium.

a. Previous investigations by this department have extended the fertility system of Escherichia coli K-12 to other genera classified in the Enterobactereaceae. This has been accomplished by the use of episomal elements such as R-factors (multiple drug resistance factors) as well as by the use of E. coli Hfr donor strains to genetically hybridize species of Salmonella, Shigella and Proteus. A direct application of these intergeneric hybridization techniques has been the construction of avirulent E. coli hybrids which, after conjugation with either Shigella flexneri or S. typhosa Hfr donors, are able to synthesize and express antigenic factors characteristic of these donor strains.

b. Research, currently in progress, has resulted in an extension of the intergeneric mating systems presently available. In this instance, we have circumvented the use of E. coli strains for such studies, having developed a strain of S. typhimurium which is capable of serving as a recipient of S. flexneri chromosomal DNA.

c. In initial experiments, several auxotrophic strains of S. typhosa 643 W^{sr} and S. typhimurium LT2 and LT7 were mated with S. flexneri 2a Hfr 256 to screen for a functional hybridization system. Although most of Salmonella recipients appeared to be sterile in matings with the S. flexneri donor, two derivatives of S. typhimurium LT7 exhibited recipient ability.

d. Subsequent mutagenesis of these strains for additional genetic markers has resulted in two sublines; strain THAXGL, a S. typhimurium LT7 which is genotypically thr^- his^- ara^- xyl^- gal^- deu^- and strain THM rfd, a S. typhimurium LT7 which is thr^- his^- met^- and altered in its lipopolysaccharide O antigen specificity (rfd^-).

e. After mating these strains with the S. flexneri Hfr donor, S. typhimurium hybrids, which have inherited Shigella genes controlling threonine and leucine biosynthesis (i.e. thr^+, leu^+), can be recovered at frequencies of about 10^{-5} per donor cell. Genetic characterization of such hybrids has revealed that they segregate the thr^+ and leu^+ Shigella genes either as a unit or

individually. Thus, as has been reported for other intergeneric hybrids, S. flexneri-S. typhimurium hybrids are partial diploids.

f. Previous investigations in this Division have shown that the genes controlling S. flexneri group antigenic specificity are genetically linked to the histidine operon. Hence, it was of interest to determine whether S. typhimurium could express Shigella antigenic determinants along with its native 4, 5, 12 antigenic characteristics. S. flexneri Hfr 256 was mated with thr^+ S. typhimurium hybrids and selections were made for his^+ hybrids, with the expectation that some of these would inherit S. flexneri group antigen genes. Of 10 such his^+ hybrids studied, all were found to have inherited Shigella antigen genes. These hybrids, in addition to expressing their native Salmonella antigenic factors, also express S. flexneri group B antigenic determinants, as detected by slide agglutinin reactions. Moreover immunization of rabbits with one of these hybrids yielded antisera containing antibodies directed against both the Salmonella and Shigella antigenic characters.

g. Further investigation along these lines are continuing, and it is anticipated that transduction utilizing phage P_1, will be feasible between S. flexneri and S. typhimurium. Such S. typhimurium X S. flexneri hybrids can prove useful in the development of antigenically polyvalent enteric strains for immunization against quite distinct gastrointestinal disease. Likewise, they should prove useful in elucidating the pathogenic characteristics of these organisms.

3. Characterization of S. typhimurium phage 9NA.

a. By means of biological and physical-chemical techniques, we have characterized a new S. typhimurium phage, termed 9NA, which is being employed in this laboratory and others in genetic studies on Salmonella lypopolysaccharide.

b. Phage 9NA is a "tadpole" shaped bacteriophage which produces small plaques with irregular edges on S. typhimurium. These plaques measure 0.5 mm in diameter. This phage is specific for smooth strains of S. typhimurium with somatic antigens 4, 5 and 12 and it cannot establish a lysogenic condition with this host. One-step growth and intracellular growth for phage 9NA on S. typhimurium showed that it has a latent period of 35 minutes and an eclipse period of 15 minutes at 37 C. The burst size is 150 functional phage particles per infected bacterial cell. The adsorption rate constant of this virus is 4.7×10^{-9} ml/min, which falls

within the range of adsorption rate constant for other phages.

c. In a CsCl density gradient, phage 9NA formed a single band. The buoyant density of the band is 1.471 g/cm^3. Using this value, a value of 1.30 g/cm^3 for phage protein and the buoyant density of the DNA of this phage (1.700 g/cm^3), it was estimated that the whole phage is composed of 43% nucleic acid and 57% protein. The ultraviolet absorption spectrum of the DNA of phage 9NA shows a maximum at 260 mμ, a minimum at 230 mμ and a 260/280 ratio of 1.90. The melting curve for phage 9NA DNA is characteristic of double stranded DNA. The melting temperature of 86.2 C corresponds to a guanine plus cytosine content of 41%. The buoyant density of the nucleic acid of phage 9NA, in a cesium chloride density gradient (1.700 g/cm^3) corresponds to a guanine plus cytosine content (G-C) of 41%. The buoyant density of the nucleic acid for phage 9NA confirmed that it is DNA. The sedimentation coefficient for the DNA of phage 9NA, determined in 0.1 M potassium phosphate was 23 S. Using this value, the molecular weight of the phage was calculated to be 20 million daltons.

4. Conservation and transfer of Escherichia coli genetic segments by partial diploid Hfr strains of Salmonella typhosa.

a. We have continued our studies of partial diploid hybrids obtained by using the Salmonella typhosa Hfr strain WR4000, as the recipient in crosses with Escherichia coli Hfr donors (see Annual Report, WRAIR, 1969). Three of these S. typhosa Hfr hybrids were obtained from the cross with the E. coli Hfr WR2004 which transfers its chromosomal markers in the following order: origin- pro$^+$ (proline synthesis), leu$^+$ (leucine synthesis), ara$^+$ (arabinose utilization), thr$^+$ (threonine synthesis), arg$^+$ (arginine synthesis), rha$^+$ (rhamnose utilization), tna$^+$ (production of indol), xyl$^+$ (xylose utilization), str-s (sensitivity to streptomycin, fuc$^+$ (fucose utilization), and his$^+$ (histidine synthesis). One of the S. typhosa Hfr hybrids designated WR4272 contained the E. coli markers ara$^+$, rha$^+$, tna$^+$ and xyl$^+$, the others designated WR4270 and WR4271, contained the E. coli markers ara$^+$, rha$^+$, tna$^+$ xyl$^+$ and fuc$^+$. Previously, these hybrids had been shown to transfer some of these E. coli diploid markers to the E. coli recipient strain WR3051 at frequencies characteristic of the E. coli Hfr from which they were derived. We have subsequently examined the transfer of E. coli markers which are present in these strains but not differentiated by phenotypic expression, i.e., pro$^+$, thr$^+$, leu$^+$, arg$^+$ and str-S. The gradient of transmission of most of these markers from the S. typhosa Hfr hybrids to E. coli WR3051 is shown in Table 1 . The transfer frequencies of these markers from the analogous E. coli Hfr WR2004 are also shown for comparison. With each of the S.

typhosa Hfr hybrids pro$^+$ was transferred at the highest frequency, with thr$^+$-leu$^+$ (and ara$^+$), arg$^+$, xyl$^+$ and (in the case of WR4270 and WR4271) fuc$^+$ transfer frequencies in descending order of magnitude. The transfer of his$^+$ was not detected.

b. Interrupted matings were performed with the S. typhosa Hfr hybrid WR4272, again using E. coli WR3051 as the recipient. The observed entry times of 5 minutes for pro$^+$, 12 minutes for thr$^+$-leu$^+$, and 23 minutes for arg$^+$ were the same as those observed with E. coli Hfr WR2004. No attempt was made to determine the time of entry of the xyl$^+$ marker. However the examination of 100 xyl$^+$ selected hybrids from the S. typhosa Hfr 4272 X E. coli WR3051 cross showed the following unselected marker inheritance percentages: arg$^+$ 46%; thr$^+$-leu$^+$, 44%; pro$^+$, 48%. In addition to exhibiting this proximal unselected marker inheritance pattern typical of E. coli Hfr X F$^-$ crosses 20% of the xyl$^+$ hybrids were found to have become sensitive to streptomycin. This result indicates their receipt of the distal (to xyl$^+$) E. coli str-s allele, thereby revealing its presence in S. typhosa Hfr hybrid WR4272. These findings are consistent with the interpretation that S. typhosa WR4272 contains, in addition to its Salmonella genome, a physically continuous E. coli chromosomal segment which is genetically complete from the pro A locus to at least the str locus. In S. typhosa Hfr hybrids WR4270 and WR4271, the diploid segment appears to be complete and continuous through the fuc locus.

c. S. typhosa Hfr hybrids capable of transferring E. coli genes at high frequencies were constructed also by mating S. typhosa Hfr WR4000 with the E. coli Hfr donor WR2010 (origin... tna$^+$...xyl$^+$). These S. typhosa Hfr hybrids were determined to contain either the linked E. coli xyl$^+$ tna$^+$ markers or the selected xyl$^+$ marker alone. The ability of ten Hfr hybrids containing the xyl$^+$ tna$^+$ markers to transfer xyl$^+$ to E. coli WR3051 was compared with that of ten Hfr hybrids containing only the xyl$^+$ marker. Seven of the ten xyl$^+$ tna$^+$ hybrids transferred xyl$^+$ to E. coli WR3051 at frequencies of 5×10^{-3} to 5×10^{-2} per donor cell. In contrast, the hybrids exhibiting only xyl$^+$ transferred this marker to E. coli WR3051 at frequencies of 10^{-4} to 10^{-6}, and one showed no transfer at all. Since the tna$^+$ allele is close to the origin of E. coli Hfr WR2010 the higher transfer frequencies observed among the hybrids exhibiting this marker may suggest that the presence of the Hfr origin on the dipolid segment is of some importance with regard to transfer efficiency.

d. Because the E. coli gene segments contained in the E. coli WR2010-derived hybrids of S. typhosa WR4000 appeared com-

paratively short, we attempted to determine if their transfer occurred in the manner observed with F- merogenotes. We examined a number of E. coli WR3051 hybrids which had received the xyl+ marker at high frequency from a xyl+ tna+ S. typhosa Hfr hybrid to see whether they, in turn, might transfer this marker to S. typhosa WR4204. Transfer of the xyl+ marker in these crosses was not observed. S. typhosa WR4204 did, of course, accept transfer of the xyl+ marker from the S. typhosa xyl+ tna+ Hfr hybrid but again, the resulting xyl+ hybrids of S. typhosa WR4204 were not observed to transfer xyl+ to E. coli WR3051. Thus, transfer in the manner of F- merogenotes was not detected. It seems likely that the E. coli diploid segments are transferred from these strains as a consequence of their interaction with the chromosomally located sex factor of the S. typhosa Hfr.

5. Isolation of circular DNA from Salmonella typhosa hybrids obtained from matings with Escherichia coli.

a. It is possible to mate a Salmonella typhosa with an E. coli Hfr donor. During the mating a segment of the E. coli chromosome is transferred to the Salmonella typhosa cell making it diploid with respect to those markers carried on the E. coli segment.

b. We have initiated studies designed to determine the physical characteristics of the E. coli exogenote DNA in diploid Salmonella typhosa hybrids. These Salmonella typhosa diploids were made by mating E. coli AB313, an Hfr, with Salmonella typhosa 643. The lead markers of the Hfr AB313 after the origin are tryptophanase, a gene involved in the production of indole, and xylose, which determines the ability to ferment xylose. When this segment of the E. coli chromosome is transferred during mating to the Salmonella cell, the cell becomes diploid for the region it has received from the E. coli Hfr. Although these partial diploids are stable the E. coli characteristics segregate out at a low frequency, resulting in cells which are again haploid Salmonella. This indicates that the E. coli DNA is present and functional in the Salmonella typhosa recipient as an addition to the Salmonella genome. Since these E. coli genes duplicate alleles which are already present on the Salmonella chromosome, they can be lost without effecting the viability of the Salmonella host.

c. The possibility that the E. coli markers might be associated with a functional sex factor was considered since the mating did involve an E. coli Hfr. This idea was rejected, how-

ever, since it was found that the E. coli markers could not be transferred out, as they would be if associated with a functional sex factor F. We were also unable to detect any sensitivity to male phage in these hybrids, and this sensitivity is also determined by the sex factor F.

d. When DNA was extracted from one of these Salmonella typhosa hybrids and examined in a cesium chloride density gradient, only one band of DNA was observed. This is due to the fact that E. coli DNA and Salmonella DNA have the same guanine plus cytosine content. But if ethidium bromide is added to the DNA before it is centrifuged in the cesium chloride density gradient, supercoiled circular DNA is separated from the linear DNA and two bands are observed. Ethidium bromide intercalates better with linear DNA than it does with circular DNA and will cause a decrease in the buoyant density of the linear DNA.

e. Ethidium bromide experiments done on one of the Salmonella typhosa hybrids, 643 xyl$^-$ tna$^-$ and its haploid segregant, 643 xyl$^-$ tna$^-$, show that the partial diploid will give two bands of DNA. The main band corresponds to the highly labeled Salmonella DNA, and the smaller band observed in the 643 xyl$^+$ tna$^+$ represents the more dense, circular E. coli DNA. The per cent of circular DNA is about 1% of the total DNA extracted from the Salmonella partial diploid cells. DNA from the 643 xyl$^-$ tna$^-$ haploid cells has only the large band and does not have any circular DNA. Thus the loss of the genetic markers corresponds to a loss of the band containing circular DNA.

f. The fractions containing the circular DNA were combined and dialyzed to remove the ethidium bromide. A sedimentation velocity run in a 5% - 20% sucrose gradient was done on the dialyzed circular DNA, using a 41 S DNA as a known molecular weight reference. Using the relationship of distance sedimented to molecular weight, the E. coli circular DNA, from the 643 xyl$^+$ tna$^+$ diploid, has a molecular weight of about 70 million daltons. The circular DNA from this same sample was also examined in the electron microscope using the protein monolayer method of Kleinschmidt. Eighteen open circular DNA molecules were measured and give a mean contour length of 33.9 microns. Using a value of 1.94×10^6 daltons per micron, this gives the molecule a molecular weight of 66.4×10^6 daltons.

g. In summary, the mating of an E. coli Hfr, strain AB313, with Salmonella typhosa 643 results in a partial diploid which can express genetic characteristics of the transferred E. coli DNA. When the DNA is extracted from the diploid, the E. coli DNA is found in closed, supercoiled circles. In the case of one diploid examined, this circular E. coli DNA had a molecular weight of between 66 and 70 million daltons.

6. No confirmatory reports of Bhaskaran's earlier observations on the mating system in Vibrio cholerae have been published as yet. We have now confirmed some of Bhaskaran's results using plate mating procedures similar to those he described and by means of broth matings. There remains, however, a dearth of both genetic and biophysical evidence suitable to allow an unequivocal interpretation of the results as being due to conjugal transfer. Both maltose and galactose negative Vibrio cholerae mutants were used as recipients but no linkage of these markers to any of the five auxotrophic markers present in Bhaskaran's strains (pur$^-$, leu$^-$, arg$^-$, his$^-$, ilv$^-$) has been demonstrated in colonies presumed to be recombinants. Attempts to produce a genetic donor of Vibrio cholerae able to yield a higher number of genetic recombinants in mating experiments have been unsuccessful.

Strain NIH41, a classical Vibrio cholerae strain has been shown to be lysogenic for at least two bacteriophages which plate on El Tor strains.MAK757 and a streptomycin resistant variant of Phil 6973. Bacteriophage 41B plates on MAK757 and on H218. All the plating strains are El Tor cholera vibrios. Clean mutants of 41B have been obtained which plate on MAK757/A, but not on NIH41. These two bacteriophages do not plate on VC154, the commonly used indicator strain for classical cholera bacteriophage.

Summary and Conclusions:

1. Salmonella typhosa hybridized by conjugation for the malA region encompassing the λ rcp locus with an Escherichia coli Hfr adsorb λ, but lysis does not occur. In contrast, S. typhosa hybrids having more extensive regions of the E. coli K-12 genome fall into different groups with respect to lysis by wild-type λ, hybrids of λ, and mutants of λ. Thus certain S. typhosa hybrids behave similarly to E. coli K-12 in susceptibility to λ lysis while others are lysed only by a λ mutant we have isolated and termed λ sx. The λ sx mutant in part owes its ability to plate on these particular S. typhosa hybrids to a mutation which maps between λ genes P and Q. Various hybrids between λ and phage 21 (λ^{121}) have also been

tested and those with the N gene of phage 21 behave much like λsx. There appears to be a difference in that λ^{i21} hybrid 1 and λ^{I21}hybrid 2 produce plaques at a reduced efficiency of plating on S. typhosa hybrids which are not lysed by λsx. These results suggest that lysis of S. typhosa hybrids is dependent upon the presence of K-12 genes in addition to the λrcp region.

2. A genetic exchange system between S. flexneri 2a Hfr 256 and S. typhimurium recipients is described. After mating S. typhimurium LT7 strains THAXGL and THM rfd with a S. flexneri Hfr donor, thi$^+$, leu$^+$ and his$^+$ hybrids, which behave as partial diploids, were recovered. The his$^+$ S. typhimurium hybrids also inherited the closely linked group antigen genes of S. flexneri, expressing both their nature 4,5,12 antigenic characters and the group antigenic characters of S. flexneri 2a.

3. S. typhimurium phage 9NA, is a"smooth specific",CNA containing phage (43% DNA, 57% protein) which plates only on S. typhimurium with wild type lipopolysaccharides. It has a latent period of 35 minutes (15 min. eclipse), resulting in bursts of about 150 phages per infected cell. It's DNA is double stranded, with a guanine-cytosine content of 41% as revealed by melting curve and C_sCl gradient analysis. Its molecular weight is about 2×10^7 daltons.

4. Three partial diploid Salmonella typhosa Hfr strains, construced from matings with Escherichia coli Hfr WR2004 (origin... pro A ...ara), transferred the diploid E. coli genes at frequencies characteristic of Hfr WR2004. The gradient of transmission of the diploid markers from these S. typhosa Hfr hybrids was the same as that of E. coli Hfr WR2004. With one of the S. typhosa Hfr hybrids interrupted mating entry times for the pro$^+$, thr$^+$-leu$^+$, and arg$^+$ markers were identical to those of Hfr WR2004, as was the pattern of unselected marker inheritance. The findings indicated that the S. typhosa Hfr hybrids conserved a single, continuous E. coli chormosomal segment, which was in one of these hybrids, genetically complete from the pro A through the str locus. In the other two, the diploid E. coli segment appeared complete from pro A through fuc. Other classes of S. typhosa Hfr hybrids, derived from mating with E. coli Hfr WR2010 (origin. . .tna. . . xyl) were also observed to transfer E. coli genes at high frequency.

5. We have investigated several Salmonella typhosa hybrids obtained from matings with Escherichia coli and found the genetic material of the E. coli parent exists in the hybrids as circular molecules of DNA. The S. typhosa hybrids, from matings with an E. coli Hfr are partial diploids in which the E. coli exogenote is maintained in a relatively stable condition. Such hybrids, examined by the ethidium bromide-CsCl technique for demonstrating circular DNA, were shown to contain a band of circular DNA. This band is correlated with the presence of E. coli DNA since no band is observed with haploid segregants of the S. typhosa hybrids from which the E. coli genetic markers have been lost. It is unlikely that this phenomenon is associated with the presence of a functional sex factor, F, in the hybrids. Neither transfer of F nor the E. coli exogenote occurred nor was sensitivity to male phage detected. The circular DNA molecules from a diploid S. typhosa hybrid containing the xylose fermenting allele of E. coli have been isolated and characterized by sucrose density gradient centrifugation and by electron microscopy. These molecules have a weight of 66 X 10^5 daltons and a length of 34 microns.

6. The mating system of Vibrio cholerae has been investigated using the auxotrophic mutants originally isolated by Bhaskaran and carbohydrate non-fermenting derivatives of his strain. However, linkage of the maltose or galactose markers to five auxotrophic markers previously present was not observed. The classical strain of Vibrio cholerae, NIH41, has been shown to be lysogenic for two bacteriophages, designated 041A and 041B which can be separated by means of different El Tor indicator strains, as well as different plaque morphologies.

TABLE 1. TRANSFER FREQUENCIES OF DIPLOID E. COLI MARKERS FROM S. TYPHOSA HFR HYBRID STRAIN WR 4270, WR 4271, AND WR 4272 TO E. COLI WR 3051

Selected marker	S. typhosa WR 4270	S. typhosa WR 4271	S. typhosa WR 4272	E. coli WR 2004
pro^+	2×10^{-2}	1×10^{-2}	4×10^{-2}	2×10^{-2}
thr^+-leu^+	1×10^{-2}	9×10^{-3}	3×10^{-3}	1×10^{-2}
ara^+	1×10^{-2}	9×10^{-3}	3×10^{-2}	1×10^{-2}
arg^+	1×10^{-3}	8×10^{-4}	2×10^{-3}	2×10^{-3}
kyl^+	4×10^{-4}	3×10^{-4}	9×10^{-4}	5×10^{-4}
fuc^+	4×10^{-5}	3×10^{-5}	2×10^{-5}	6×10^{-5}
his^+	2×10^{-5}	2×10^{-5}	2×10^{-5}	2×10^{-5}

The transfer frequencies of the analogous E. coli Hfr WR2004 are shown for comparison. Transfer frequencies are expressed as the number of recombinants per donor cell.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 122, Microbial genetics and taxonomy

Literature Cited.

Publications.

1. Easterling, S.B., E.M. Johnson, J.A. Wohlhieter and L. S. Baron. Nature of lactose fermenting Salmonella strains obtained from clinical sources. J. Bacteriol. 100: 35-41, 1969

2. Johnson, E.M., and L.S. Baron. Genetic transfer of the Vi antigen from Salmonella typhosa to Escherichia coli. J. Bacteriol. 99: 358-359, 1969.

3. Baron, L.S., E. Penido, I.R. Ryman and S. Falkow. Behavior of coliphage lambda in hybrids between Escherichia coli and Salmonella. J. Bacteriol. 102: 221-223, 1970.

4. Falkow, S. and L. S. Baron. Plasmid formation after lambda phage infection of Escherichia coli-Salmonella typhosa hybrids. J. Bacteriol. 84: 1303-1312, 1970.

5. Formal, S.B., P. Gemski, Jr., L.S. Baron and E. H. LaBrec. Genetic transfer of Shigella flexneri antigens to Escherichia coli K-12. Infection and Immunity 1: 279-287, 1970.

6. Baron, L.S., I.R. Ryman, E.M. Johnson, and S. Falkow. Interaction of lambda and lambda derivatives with Escherichia coli-Salmonella typhosa hybrids. Bacteriol. Proc. p. 158, 1970.

7. Leavitt, R.W., J.A. Wohlhieter, E.M. Johnson, R. L. Ladda and L. S. Baron. Isolation of circular DNA from Salmonella typhosa hybrids obtained from matings with Escherichia coli. Bacteriol. Proc. p. 55, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6446	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A822	00	123
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)*

(U) Histopathologic Manifestations of Diarrheal Diseases (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

00 26 00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:* Not Applicable

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	6	100
	CURRENT 71	6	100

16. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* Walter Reed Army Institute of Research
Division of Experimental Pathology

ADDRESS:* Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Sprinz, COL H.

TELEPHONE: 202-576-2677

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Sheahan, LTC D. DA

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Intestinal Secretion and Absorption;
(U) Enteric Infections; (U) Bacterial and Parasitic; (U) Radiation Biology

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security Classification Code.)

23.(U) Pathology and Pathogenesis of various conditions of the gastrointestinal tract of man and experimental animals are studied by multidisciplinary approaches with emphasis on morphology. These investigations are considered essential parameters for a comprehension and scientifically based therapy of diarrheal diseases, enteric infections and radiation injury to the intestine.

24.(U) Principally morphologic, including light, fluorescent and electron microscopic examinations. Kinetic studies using tritium labelled thymidine and histochemical investigations are also employed.

25.(U) 69 07-70 06 Work on the mucosubstances has been extended into collaborative study on stress ulcer. A manuscript "The Effects of Staphylococcal Enterotoxin on the Epithelial Mucosubstances of the Small Intestine of the Rhesus Monkey" has been accepted for publication by Am. J. Path. A summary of this work will be presented at the Spring meeting of FASEB and at the March meeting of the AFEB. Sequential immunopathologic studies on cholera toxin absorption have been submitted for presentation at the annual meeting of the Am. Gastro. Assoc. The paper "Effects of a Protein-Deficient Diet on the Liver, Pancrease, Stomach and Small Intestine of the Rat" has appeared in Arch. Path., Dec.1969. The manuscript on the effect of neutron irradiation on the gut of germfree and conventionally raised mice referred to in the previous report has been completed. The manuscrip "Idiopathic Recurrent Cholestasis"has been accepted for publication in Pediatrics. The manuscript "Experimental Diarrhea: Salmonella Enterocolitis in the Rat" by Maenza et al. has been accepted for publication by J. Inf. Dis. Work on intestinal spirochetosis, amebiasis, choleraic and non-choleraic diarrhea is continuing. An abstract "Intestinal Spirochetosis, a Unique Host-Parasite Relationship" has been selected for presentation at the 1970 American Association of Pathologists and Bacteriologists. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

(Continued on continuation sheet)

DD FORM 1 MAR 66 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.

BLANK PAGE

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123, Histopathologic manifestations of diarrheal diseases

Investigators.
Principal: COL Helmuth Sprinz, MC
Associate: LTC Daniel Sheahan; Akio Takeuchi, M.D.; Helen R. Jervis, D.Sc.; MAJ Vincent Kao; MAJ Michael Zimmerman

Description

Studies of the pathogenesis and the evolution of the histologic lesions of the various forms of enteritis were continued and elaborated. New approaches to some of these studies have commenced.

Progress

1. LTC Sheahan together with COL Sprinz handles consultations. He collaborates with Dr. S. B. Formal in the investigations of effects of bacterial hybrids on the gastrointestinal tract of guinea pigs and rabbits. Studies on the histochemistry of intestinal mucins have continued. A paper entitled "The Effects of Staphylococcal Enterotoxin on the Epithelial Mucosubstances of the Small Intestine of the Rhesus Monkey" has been published in the American Journal of Pathology. A summary of this work was presented at the Spring Meeting of FASEB and also at the March meeting of the AFEB. Current investigations include the histochemical study of such mucins in various species of mammals, in experimental animal models of cholera and shigellosis and in acute gastroenteritis due to coliform organisms including backerial hybrids. Sequential pathologic studies of staphylococcal enterotoxin absorption in susceptible as well as non-susceptible animals have also commenced. The study of intestinal mucins in "stress ulcers" produced in the guinea pig continues.

2. MAJ Kao has devoted his attention to sequential immunopathologic studies on cholera exotoxin absorption by immunofluorescent means. A summary of this work was presented at the 71st Annual Meeting of the American Gastroenterological Association in May 1970. In collaboration with Dr. Takeuchi the same phenomenon is being studied ultrastructurally using horse radish peroxidase labelled anti cholera toxin antibodies.

3. Dr. Takeuchi continues his studies of bacterial penetration of the intestinal mucosa using multiple morphologic techniques.

These include light microscopy, transmission, scanning and negative staining electron microscopy and freeze etching. He has been invited to submit a monograph in Japanese on the mechanism of penetration of enteric pathogens. This will appear in the Proceedings of the Japanese Society of Microbiology held in Tokyo in 1968. A shorter version will appear in Current Topics in Pathology Series (Springer Verlag, New York) also by invitation. Current emphasis on intestinal spirochetosis has received wide acclaim. Further observations on this work were presented at the American Society of Pathologists and Bacteriologists in St. Louis. A further manuscript describing the relationships of such organisms to the intestinal mucosa of both man and monkey is in preparation. Further investigation of this phenomenon includes studies of experimental amebiasis in guinea pigs in collaboration with Dr. Phillips of the National Institute of Infectious Disease. National recognition was given to Dr. Takeuchi's investigative efforts when a scientific exhibit "Penetration of the intestinal epithelium by various microorganisms" by A. Takeuchi and H. Sprinz received a certificate of merit at the annual convention of the American Medical Association in New York in July 1969, first prize at the American Academy of Gastroenterology at Houston in October 1969 and second prize at the New York State Medical Society Meeting in February 1970.

4. Dr. Jervis has been the principal associate collaborating with members of the Department of Radiobiology, Division of Nuclear Medicine. A manuscript comparing the small bowel histology in germ-free and conventional mice following neutron irradiation has been submitted. Preliminary results of this work were presented at the 8th Annual Meeting of the Association for Gnotobiotics at Oak Ridge, Tennessee in June 1969. A paper describing the effects of protein depletion in the rat which was started by Dr. K. Madi, a Brazilian citizen, was completed by Dr. Jervis and published in Archives of Pathology. She is currently investigating the pathological responses to malarial infection in Aotus monkeys and the histochemical properties of the Clara cell in collaboration with Dr. Takeuchi.

Summary and Conclusion

In our research effort we have been trying, to the permitted extent or that material is made available to us, to study human conditions or experimental models applicable to man. Great emphasis was placed on multidisciplinary and collaborative research.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123, Histopathologic manifestations of diarrheal diseases

Literature Cited

1. Sprinz, H.: Pathogenesis of infections. Arch Path. 87: 556, 1969.

2. Ruymann, F.B., Takeuchi, A., and Boyce H.W.: Idiopathic recurrent cholestasis. Pediatrics 45: 812, 1970.

3. Takeuchi, A. and Sprinz, H.: Intestinal spirochetosis: a unique host-parasite relationship. Am. J. Path. 59:46a, 1970.

4. Sheahan, D.G. , Jervis, H.R., Takeuchi, A. and Sprinz, H.: The effect of staphylococcal entertoxin on the epithelial mucosubstances of the small intestine of the rhesus monkey. Am. J. Path. 60:1, 1970.

5. Madi, K., Jervis, H.R., Anderson, P.R., Zimmerman, M.R.: A protein deficient diet: effect of the liver, pancreas, stomach and small intestine of the rat. Arch. Path. 89: 38, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6485	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A822	00	125
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Hematology of Nutritional Deficiencies of Military Importance (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING	70	2	45
FISCAL YEAR — CURRENT	71	2	45

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Division of Medicine
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Conrad, COL M. E.

TELEPHONE: 202-576-3365

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Diet; (U) Intestine; (U) Iron; (U) Protein; (U) Hemoglobin

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number, procede text of each with Security Classification Code.)

23. (U) The nutritional anemias are correctible diseases which are commonplace in geographic areas of military importance. These diseases markedly reduce the capability of affected populations to perform work or sustain a military effort and remain self supporting.

24. (U) Establishment of standards and standard methods for detection and quantification of these diseases. Studies of the nutrient content of various foodstuffs and the effects of commonplace diets and chronic infection upon the absorption of essential nutrients.

25. (U) 69 07 - 70 06 International standards for hemoglobinometry were developed in this laboratory and are maintained for worldwide distribution. Iron standardized methods are being developed in collaboration with ICSH and WHO. Basic studies of the mechanisms of iron absorption by the gut have shown the importance of the composition of the diet because of the capability of commonplace ingredients in foodstuffs to chelate iron and make it more acceptable or unacceptable for absorption. Ferritin in the small intestinal mucosal cell was shown to be an excretory and storage protein and not a regulator of absorption. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

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

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 125, Hematology and nutritional deficiencies of military importance

Investigators.
Principal: COL Marcel E. Conrad, MC
Associate: LTC George M. Bernier, MC, MAJ Stanley G. Schade, MC
Mr. Harold L. Williams

Description.

Studies of the mechanisms regulating the body content of trace metals by investigation of both absorption and excretion of radioactive labeled compounds.

Progress and Results.

Investigation into the metabolism of iron continues in this laboratory because iron deficiency is a major nutritional problem and a source of debility to many underprivileged persons in diverse geographic areas of the world. Progress would be furthered by rapid, reliable, preferably simple and widely accepted methods of measurement of serum iron and total iron binding capacity. For several years now, cooperative work to develop and standardize such methods has been in progress under the auspices of the International Committee for Standardization in Hematology and in collaboration with the World Health Organization. This laboratory has been a focal point of this cooperative effort. It is anticipated that this endeavor will permit quantification of the nutritional problem and improve laboratory techniques in both military and civilian laboratories.

The ultimate regulator of iron absorption in the body is not known. Many factors - intraluminal, mucosal, corporeal - demonstrably influence absorption, but the primary regulator has yet to be discovered (1-4).

Studies showed that intestinal motility affects the absorption of iron. Both atropine and phenoxybenzamine slowed intestinal motility when injected into rats, and these rats absorbed more inorganic iron than control animals. Isolated loops of intestine from animals treated with phenoxybenzamine absorbed the same amount of iron as controls. Thus the drug effect did not appear to be on the mucosal cells. This contrasts with isolated loops from animals treated with endotoxin, which absorbed less iron than controls (3). This suggests that the frequency of diarrhea among underprivileged peoples in many geographic areas may contribute significantly to iron deficiency.

The role of transferrin in iron absorption was studied both immediately and 3 days after infusion of human transferrin. Iron absorption was also measured in normal and iron-loaded rats over a 5 day period after infusion of transferrin. The absorption of iron was unaffected by the administration of transferrin (4).

Cobalt and iron have similar structures and seem to share a common absorptive intestinal pathway. Sequimolar amounts of cobalt or iron are equally effective in decreasing the absorption of the other metal. Further, iron deficiency and enhanced erythropoiesis increase absorption of both cobalt and iron. On the other hand, cobalt absorption is affected less in conditions which diminish iron absorption. Using rabbit antibodies to rat ferritin, it was possible to demonstrate that whereas orally administered radioactive iron is incorporated into ferritin in the mucosal cells of rat small intestine, cobalt is not. The capability of ferritin to hold iron in intestinal cells and prevent its transfer, when not needed, into the body serves as a mechanism for diminishing iron absorption which is not available to cobalt. The data support the hypothesis that ferritin serves a storage function in the intestine and not as the primary regulator of iron absorption.

It has been demonstrated that iron administration stimulates the synthesis of ferritin in the gut and apparently also in liver and spleen. This synthesis in the intestinal mucosa was shown here to be dependent on dose (linear within a certain range) and upon route of administration. Cobalt does not have such activity. When iron was administered parenterally to rats, significant ferritin synthesis occurred in the distal ileum. Following oral administration most of the synthesis was in the proximal ileum. These results suggest that intestinal ferritin plays an important role in the elimination of iron from the body, as well as its incorporation.

Basic studies of the regulation of iron absorption provide insight into both the etiology of this commonplace nutritional deficiency and the problems encountered in the appropriate prevention and therapy of this disorder on a large scale basis.

Conclusions.

International cooperative effort between 8 laboratories is succeeding in developing rapid, reliable, simple and widely acceptable methods for measuring serum iron and total iron binding capacity. Other work in this laboratory is cited which provides the following evidence:

a. Slowed intestinal motility enhances iron absorption.

b. Transferrin administration does not influence iron absorption.

c. Orally administered iron is incorporated into ferritin in the intestinal mucosa; cobalt is not. The data indicate that ferritin serves a storage function but is not the prime regulator of iron absorption.

d. Iron administration induces synthesis of ferritin in the intestinal mucosa. If given intravenously, most ferritin is formed in the distal ileum; if given orally, most is formed in the proximal ileum. These observations are believed to be evidence for a function of ferritin in the excretion of iron from the body, as well as its incorporation.

Recommendations.

Standardized methods to quantify the magnitude of the problem of iron deficiency on a worldwide basis are required. Effective nutritional recommendations which would permit correction of this widespread nutritional problem would enhance the capability of peoples in underprivileged geographic areas to become more self supporting.

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 125, Hematology and nutritional deficiencies of military importance

Literature cited.

1. Conrad, M. E.: The role of protein in iron absorption. Presented to Pan American Health Organization, June 9, 1969.

2. Conrad, M. E.: Humoral regulation of iron absorption. Gastroenterology 57:225, 1969.

3. Conrad, M. E.: Pathophysiology of malaria: Hematologic observations in human and animal studies. Ann. Intern. Med. 70:134, 1969.

4. Schade, S. G., Felsher, B. F., and Conrad, M. E.: An effect of motility on iron absorption. Proc. Soc. Expl. Biol. Med. 130:757, 1969.

5. Schade, S. G., Bernier, G. M., and Conrad, M. E.: Normal iron absorption in hypertransferrinaemic rats. Brit. J. Haematol. 17:187, 1969.

6. Schade, S. G., Bernier, G. M., Felsher, B. F., and Conrad, M. E.: Competition of iron and cobalt for absorption. Amer. Soc. Hematology, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6470	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A823	00	030
b. CONTRIBUTING				
c. [illegible]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)*
(U) Military Psychiatry (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
002500 Clinical Medicine 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT: Not Applicable
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	8	220
FISCAL YEAR CURRENT 71	8	220

19. RESPONSIBLE DOD ORGANIZATION
NAME:* Walter Reed Army Institute of Research
ADDRESS:* Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME:* Walter Reed Army Institute of Research
Division of Neuropsychiatry
ADDRESS:* Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Rose, R. M., M.D.
TELEPHONE: 202-576-5210
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Kreuz, MAJ L.
NAME: Borus, MAJ J. DA

21. GENERAL USE
FOREIGN INTELLIGENCE NOT CONSIDERED

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Stress Performance; (U) AWOL Behavior; (U) Military Adjustment; (U) Psychiatric Treatment; (U) Endocrine Response; (U) Aggressive Behavior

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 psychologic, organizational and physiologic factors which predispose the soldier to perform ineffectively or develop psychiatric or psychosomatic disease, and to develop appropriate preventive and treatment techniques.

24. (U) The methods of experimental psychology, sociology, clinical psychiatry, and biochemistry are used to identify and modify factors that contribute to ineffective military performance.

25. (U) 69 07-70 06 A systematic comparison of AWOL offenders and patients diagnosed at WRGH as character-behavior disorders continues. Studies are in progress on the factors contributing to difficulty of Vietnam returnees in readjustment to garrison life. Analysis of data collected on 700 men (350 MHCD patients and 350 controls) demonstrates that separation during certain critical periods in childhood is associated with future psychological difficulties as indicated by standard test measures. Plasma testosterone was found to correlate significantly with aggressive and dominance behavior in a well established, closely observed group of male rhesus monkeys living in semi-natural conditions. Standardization of short-term psychotherapy along more operational lines and its application to MHCD practices is in progress. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00, Military Psychiatry

Work Unit 030, Military Psychiatry

Investigators
Principal: Robert M. Rose, M.D.
Associate: MAJ Leo E. Kreuz, MC; MAJ Jonathan F. Borus, MC;
MAJ Jay P. Mohr, MC; MAJ Gene R. Moss, MC;
MAJ Alan D. Sklar, MC; CPT Timothy F. Hartnagel, MSC;
CPT John W. Holaday, MSC; David H. Marlowe, Ph.D.;
SP 5 Gary Sax; SP 4 H. Lester Karns, SP 4 Joyce Pittman;
SP 4 Kenneth J. Sulak

I. INTRODUCTION

Work in the Department of Psychiatry in the past year has continued in three broad areas. Greater emphasis has been placed on the field and preventive aspects of military psychiatry. Studies of the effects of social and psychological stress on various hormones have continued, using animal models as well as human studies. The third area involves the application of behavior principles to specific clinical problems, e.g., accurate assessment of sensory and cognitive loss following brain injury.

II. FIELD AND PREVENTIVE STUDIES

With the arrival of LTC Holloway, MC and MAJ Borus, MC ongoing work in the department has been increasingly focused on the plan for establishment of an expanded MHCD facility at Ft. Meade. This involves not only provision of consultation to individuals and command, about specific problems, but a study of group interactions along a systems model to gain better understanding of the origins of ineffective and maladaptive behavior.

CPT Hartnagel, MSC, is completing the follow-up of 250 men sent to the stockade at Ft. Meade after AWOL offenses along with 100 patients admitted to the NP Wards, WRGH with a diagnosis of character and behavior disorder. The coding of interviews and records is in progress. This study will provide reliable information as to similarities and differences between these two groups with respect to a number of relevant variables, such as performance in the Army, incidence of previous military offenses, and past personal history. Serious difficulty has been experienced in arranging for the use of computer facilities for data analysis. This appears to be related primarily to a lack of adequate programming help, despite the general availability of a number of canned programs for data analysis. Arrangements have been made with COMPSY program at WRGH for computer assistance in comparing the MMPI of AWOL prisioners with that of character disorder patients.

The department is collaborating with CPT Stanton, MSC, at the MHCD at Ft. Meade on the analysis of data he has collected on the incidence of use of marijuana and other drugs in Army personnel. However, similiar to the follow-up data on AWOL prisioners and character disorder patients, this analysis will be severely hampered unless adequate computer help can be arranged.

MAJ Sklar, MC has completed a large study of over 700 men, comprised of 350 patients seen at the MHCD as well as 350 controls selected from a platoon at Ft. Meade. He studied the relationship between separation from parents during childhood and increased susceptibility to various behavioral difficulties as estimated by significantly altered scores on a variety of psychological tests. These data gathered over a wide population base, provide more reliable guidelines for the counseling of dependents during separation from active duty personnel.

MAJ Borus, MC has set up a study this spring in collaboration with the Sixth Armored Calvary Regiment at Ft. Meade, to examine the adjustment of Vietnam returnees upon arrival from Vietnam and assignment to a garrison post. The prospective study will assess difficulties in readjustment of the next 200 men to enter the Sixth Calvary from Vietnam.

A second project is planned for the design and testing of an instrument to measure racial perceptions in the Army. Appropriate clearance has been obtained from the DA for the use of various records specifying race. It is hoped that this Racial Perceptions Inventory will help evaluate the effectiveness of various interventions proposed to diminish interracial disorders.

Dr. Marlowe continues his analysis of the 4 years of data collected on social groups in Thailand. His work clarifies the various ways groups attempt to cope with crises and how the group effects the form of interaction between patient and health practitioner. These analyses are relevant to the plans for the expansion of the MHCD planned at Ft. Meade in FY 71.

MAJ Moss, MC in collaboration with Dr. John Boren, Department of Psychology, American University, has been studying the application of operant techniques to outpatient psychiatric treatment. The purpose of the study is to develope explicit psychiatric procedures that can be made public for utilization in counseling at the MHCD. MAJ Moss has written a comprehensive outline of the behavior therapies, which recently appeared in the International Journal of Psychiatry.

III. HORMONES & BEHAVIOR

A. Animal Studies

The first major study of the interaction between social behavior, including aggression and dominance rank, and testosterone in rhesus monkeys was completed this past year. In collaboration with Dr. Bernstein of the Yerkes Regional Primate Center, a group of 34 adult male animals was formed, followed by observation and recording for three months of all behavioral interactions between animals. These data were analyzed by computer for the frequency and duration of all interactions, including a fine grain analysis of all agonistic behaviors. Plasma testosterone was measured in all animals after a period of adapting to them to capture and venipuncture.

Testosterone was found to correlate significantly with dominance rank as well as overall aggressive behavior, which consisted primarily of non-contact aggression (threats to subordinate animals). Threats and submissive gestures are the primary means of maintaining the dominance hierarchy in the rhesus, rather than actual physical attack. It was also found that animals who most frequently exhibit aggressive behavior have the highest testosterone even though they were not necessarily the highest in dominance rank.

The performance of high and low dominance animals will be studied this year in standard stress paradigms. Previous work has suggested that animals who appeared in the laboratory to be most aggressive (to humans) showed more intense endocrine responses to stress than more passive or submissive animals. This relationship will be more rigorously tested utilizing this group of 34 animals.

As an adjunct to these studies of aggression, performance and testosterone, MAJ Kreuz, MC, is collaborating with Dr. Perachio of the Neurophysiology Laboratory at Yerkes in a study of endocrine correlates to brain stimulation which evokes aggressive and sexual behavior. This will help evaluate the pathways responsible for the control of testosterone secretion. In addition, MAJ Kreuz is studying the relationship between prolonged fighting behavior in rats and levels of plasma testosterone.

B. Human Studies

Clearance has been requested from the Office of the Provost Marshal General and USAMRDC for a study of hormone and XYY chromosomes in men sent to the Correctional Training Facility, Ft. Riley, Kansas.

Men six feet in height and over will have their karyotype analyzed for the presence of an extra Y chromosome. They will be matched with controls selected for body weight and military offense and will be interviewed by two psychiatrists blind to their identity to test concepts of increased aggression or impulsiveness presumably associated with XYY males. In addition, performance at the CTF and future Army career of the XYY men will be compared to the controls. The identity of XYY men will remain confidential from the CTF staff in order to prevent biased assessment of their performance. In addition, selective controls and XYY males will have more comprehensive studies of their testosterone metabolism using labelled hormones, performed on a volunteer basis as per AR 70-25.

Clearance is being obtained for the long term study of candidates at the Officers Candidate School, Ft. Belvoir. Performance of individual men will be correlated with levels of 17-OHCS excretion and testosterone. Measurement of individual coping styles as well as rate of adaptation to the group will be correlated with stress measures and performance at OCS.

The prison inmate group at Patuxent Institute for Defective Delinquents is being studied longitudinally in order to determine possible relationships between behavior and endocrine functioning. Blood and testosterone levels are being followed over a two week period. Behavior measures include clinical interviews and psychological tests designed to study aggression and anxiety, along with behavior rating scales filled out by the prison guards. Preliminary data suggested that this population includes an interesting subgroup of men characterized by high activity and low anxiety who may show elevated testosterone levels.

IV. CLINICAL STUDIES

MAJ Mohr, MC had a protocol recently approved for the study of traumatic brain injury utilizing behavioral testing of various defects. This approach derived from operant psychology provides a reliable and accurate method for estimation of deficits in perceptual and cognitive functioning, the lack of which prevents adequate clinical follow-up as well as the estimation of appropriate compensation. In addition, MAJ Mohr has been working in collaboration with the Department of Neuropathology, University of Maryland to clarify the pathological and clinical correlates of hypotensive cerebral infarcts.

MAJ Moss has also been working on the development of electronic instrumentation to conduct a study of the application of operant conditioning techniques to the treatment of primary hypertension. Mr. Pearlman in the Department of Experimental Psychophysiology has been in charge of instrument design and construction. Preliminary tests with normal volunteer subjects are underway and work with patients at WRGH is planned to begin by autumn 1970.

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00, Military Psychiatry

Work Unit 030, Military Psychiatry

Literature Cited.

1. Rose, R.M., Mason, J.W., and Brady, J.V. Endocrine Responses to Maternal Separation and Chair Adaptation in Experimentally Raised Rhesus Monkeys. In: Proceedings of 2nd International Congress of Primatology, New York, Karger, Basel, 1969, Ch. VII, pp. 211-218

2. Levine, M.D., Gordon, T.P., and Rose, R.M. Behavioral and Endocrine Correlates of Adaptation to Chronic Shock Avoidance. In: Proceedings of 2nd International Congress of Primatology, New York, Karger, Basel, 1969, Ch. VII, pp. 204-210.

3. Rose, R.M., Androgen Responses to Stress: I. Psychoendocrine Relationships and Assessment of Androgen Activity. Psychosom Medicine 31, 405, 1969.

4. Rose, R.M., Bourne, P.G., Poe, R.O., Mougey, E.H., Collins, D.R. and Mason, J.W. Androgen Responses to Stress: II. Excretion of Testosterone, Epitestosterone, Androsterone and Etiocholanolone During Basic Combat Training and Under Threat of Attack. Psychosom Medicine 31, 418, 1969.

5. Rose, R.M. Androgen Excretion in Stress. In: The Psychology and Physiology of Stress, New York, Academic Press Inc., 1969, Ch. 6, pp. 117-147, P. G. Bourne, Ed.

6. Marlowe, D. Upland-Lowland Relationships: The Case of the S'kaw Karen of Western Central Chiang Mai. In: Tribesmen and Peasants, Hill Tribes Research Center, Bangkok, 1969, ed P. Hinton.

7. Moss, G.R. An outline of the behavior therapies. Int J Psychiat 8, 883, 1969

8. Mohr, J.P., Leicester, J., Stoddard, L.T., Sidman, M. Some Aspects of Visual Neglect. J Neurol Neurosurg Psychiat 32, 580, 1969.

9. Mohr, J.P. and Adams, R.D. Affections of Speech. In: Principles of Internal Medicine, Ch. 29, pp. 172, 1970, ed Harrison.

10. Mohr, J.P., Fisher, C.M., and Adams, R.D. Cerebrovascular Disorders. In: Principles of Internal Medicine, Ch. 357, pp. 1727, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6456	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A823	00	031
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code): (U) Military Performance and Stress; Factors Leading to Decrements of Performance and Disease. (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:

016200 Stress Physiology 009400 Man-Machine Relat 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 07	CONT	DA	C. In-House

17. CONTRACT/GRANT: NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 69	10	280
CURRENT FISCAL YEAR 70	14	370

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Mercney, COL W.H

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Division of Neuropsychiatry
Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Frazier, T.W., Ph.D.

TELEPHONE: 202-576-5257

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Ernest, J.T., LTC

NAME: Williamson, P.D., MAJ DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Electrophysiology; (U) Brain Injury; (U) Psychophysiology; (U) Stress; (U) Performance; (U) Learning; (U) Memory; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Drugs, stressful environments, and performance demands likely to produce a breakdown in a soldier's performance, alertness or thought processes are studied. The psychologic and physiologic functions correlated with ineffective performance are identified and strategies for preventing or treating such performance are established.

24. (U) Using psychophysical methods, operant conditioning, computer processing, and electrophysiologic techniques, behavioral and psychologic events are isolated and correlated. Sensory inputs are correlated with behavior and neurophysiologic events; work-rest schedules and the effects of bodily physiologic rhythms are studied under specified normal and stressful conditions to characterize altered patterns of performance.

25. (U) 69 07 - 70 06 Research performed during this reporting period includes a study comparing time-on-vigil effects with day-night effects on efficiency in panel monitoring performance. Results indicated distinctly different power spectrum patterns from one condition to another. A drug testing model is now undergoing testing to permit quantitative analysis of a drug's time course on given life systems response without interference by other sources of variability associated with environmental or physiological influences. Further electroencephalographic and visual studies have been performed to study relationships between brain activity and ocular standing potentials, intellectual test performance, and clinically significant variables including developmental changes, hearing loss, brain damage, and hormonal anomalies. A hypoxia study was performed to study its effects on CFF, dark adaptation, in both rod and cone ranges. Studies of the brachial biceps response to isometric loading have indicated phase relationships between electrical and mechanical activity which can be expressed by a log function relating mechanical lag to frequency of tracking a sinusoidal varying load. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 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

BLANK PAGE

Project 3A062110A823, MILITARY PSYCHIATRY

Task 00, Military Psychiatry

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

Investigators.
Principal: Thomas W. Frazier, Ph.D.
Associate: Ann Barnet, M.D.; Robert Chapman, Ph.D.;
CPT H. Peter Clamann, MSC; LTC J. Terry Ernest, MC;
CPT J. Ronald Gentile, MSC; CPT John R. Jennings, MSC;
Abner B. Lall, M.S.; Harold F. Lawson, M.A.; MAJ Jay P. Mohr, MC;
CPT William C. Orr, MSC, William Pearlman, EE;
Guy C. Sheatz, Ph.D.; MAJ Peter D. Williamson, MC.

Description.

This work unit studies influences of biological and environmental variables upon human performance. It includes psychophysiological measurements to determine life systems relationships underlying the decrement in performance associated with stressful situations. Research content includes: vigilance performance decrement associated with sleep deprivation, tranquilization, different work-rest cycles; studies of brain-behavior relationships during problem solving as a function of age, mental deficiency and normal development; study of muscle function during different kinds of isometric loading; studies of perception during hypoxia and following brain injuries, as well as various animal studies designed to study interpretability of the electroretinogram, visual spectral mechanisms, and microelectrode recording techniques.

Progress.

1. Spectral characteristics of operant behavior in humans.

Our ongoing research program concerning the study of human performance decrement has led to the development of an approach to measurement which promises to be highly useful for a variety of applications for which more traditional approaches are of limited value. This approach was first taken in a study of performance variability. The first experiment was performed to study variability in well stabilized performance of a complex signal detection task. It was hypothesized that much of this variability was not random, but that it represented basic features of behavioral processes arising from biological rhythms as well as results of certain environmental manipulations. The approach which was taken consisted of: (A) training subjects up to asymptotic levels in performance at the task, which involved placing monitoring behavior under the control of three concurrent reinforcement schedules; (B) preprocessing or transforming the resulting data from the time domain to the frequency domain via power spectrum and coherence analyses; and (C) subjecting the transformed data to parametric

statistical analyses to test hypotheses concerning spectral curve characteristics. This approach can be characterized as one which first takes advantage of high degrees of experimental control and then utilizes statistical control techniques to isolate regularly occurring variability from error. The results indicated that most of the variability remaining after behavior had been placed under the control of reinforcement schedules could be associated with regularly occurring variation and statistically isolated from error. This approach was considered to be potentially useful for: reducing the amount of treatment effect needed to demonstrate statistical significance; increasing the sensitivity of the analysis in detecting how the behavior was changed by the treatment; and studying behavioral dimensions which could not be analyzed with more conventional approaches to performance measurement.

2. Effects of chlordiazepoxide administration on human data processing performance.

Following the refinement of methodology for studying regularly occurring variability in baseline performance via spectrum analysis preprocessing and subsequent use of parametric statistics for analyses of spectrum curve information, it became evident that such an approach might deliver much additional information which could be used to identify how performance is degraded by agents or conditions known to reduce performance levels. That is, these analyses seemed potentially valuable for specifying much more about performance decrement than the simple finding of reduced performance levels over some given period of time. Consequently, additional studies were conducted to study how a widely used tranquilizing agent (Chlordiazepoxide) might affect complex data processing performance, and how brief periods of sleep deprivation might degrade data processing abilities. The tranquilizer study was a double-blind study involving testing of three independent groups of 12 subjects each: control, low drug, and high drug. Each group was given a capsule every eight hours, which contained chlordiazepoxide (CDP) for the high and low drug groups, and aspirin for the control group. All subjects were given placebos every one-half hour as well, so that subjects could determine neither whether they were actually given a drug nor when the actual drug was administered. All subjects were trained to asymptotic levels, followed by subsequent testing over a 22-hour experimental session. Results of analysis of variance performed on the raw data indicated an increase in rate of observing panel meters over control group levels for the two drug groups, with the higher rate associated with the high drug dosage for the variable-interval signal presentation schedule. The same analysis revealed increases in signal detection percentages for the fixed-interval and variable-interval schedules and in heart rate over control group levels. Analyses of variance performed on the preprocessed spectrum data revealed two major findings: Coherence was clearly reduced in comparisons of probability

of signal detection across channels, at each of the frequency bands in which substantial coherence values are normally found. This reduction in synchronization was dose-related, with higher degrees of desynchronization in the high drug group. The effects of CDP on power spectrum measures of observing rate revealed increased power in the higher frequency range, only for the variable interval schedule. Several other findings were obtained which provided rationales leading to the execution of two subsequent studies: Comparisons of results obtained during the first 11 hours' performance with results from the second 11 hours' performance revealed significant alterations in spectrum shape. The second 11 hours' performance showed a power shift to the left, indicating a relative increase in the power at the lower frequencies of the ultradian spectrum and a corresponding decrease in the power of higher frequencies in the ultradian range. The other finding was that dispensing placebos at one-half hour intervals induced a clear spectral intensity at 48 cycles/day, for observing rate and signal detection measures, indicating that the procedure induced performance oscillations at this frequency. This spectral intensity was especially marked in coherence measurements, which reflected synchronized oscillation across all performance measures in the 48 cycles/day band.

3. Effects of short-term sleep deprivation on human data processing performance.

An independent study was conducted to study characteristics of decrement associated with sleep deprivation. This investigation was designed to compare performance of two groups of 15 subjects each, under two testing orders. The first group was given a 22-hour test session, then sleep deprived until the same time on the next day and tested again for the same period of time. The second group was deprived of sleep for one day without working, then tested for the same duration as the other group. Several days later, members of the second group were brought back to the laboratory to obtain baseline data. Significant findings resulting from one day's deprivation from sleep were obtained both in power spectrum and coherence analyses. The group which had been given work prior to sleep deprivation showed statistically significant differences from the group rested prior to sleep deprivation. These differences were observed in heart rate and temperature results, and in panel meter observing rates. Coherence differences between the two groups were obtained for observing rate measures.

The shape of the power spectrum was altered by sleep deprivation for both groups both in the case of observing rate measures, and probability of signal detection measures. This change appeared to consist of a redistribution of energy toward greater energy at the lower frequencies in the ultradian range, with a corresponding

reduction of energy at the higher frequencies. The spectrum shift during sleep deprivation bore resemblences to the previously observed change in spectrum shape from daytime to nighttime performance in nondeprived subjects. It seemed noteworthy that this shift-to-the-left phenomenon associated with sleep deprived performance has a biological parallel in the shift of electroencephalographic frequency distributions to lower frequencies as sleep stage depth is increased. It is therefore of interest to inquire whether or not comparisons between frequency shifts in data processing performance and frequency shifts in the surface electroencephalogram are more than parallel phenomena. Perhaps these frequency shifts are synchronized between brain and behavior within given areas of the frequency spectrum, in individuals who are deprived of sleep over successively longer periods of time.

4. An oscillation induction model for studying the time course of drug effects on electrophysiological functioning.

Experimentation is now under way to study a drug effect test model which can be used to characterize the physiological effects of certain commonly prescribed medicinal agents. This model is one in which a fast-acting drug (glyceryl-tri-nitrite) is administered periodically at exact time intervals. The physiological system of interest is monitored continuously over some relatively long number of hours. The time series data resulting from this measurement are subjected to power spectrum analysis to detect whether a spectral intensity can be found which corresponds to the rate of administering the drug. It is predicted that the amplitude and dispersion of such a spectral intensity will be found to be a function of the total proportion of the administration rate cycle that the drug is active, and if the proportion of total cycle time the drug is active is low, the drug effect will be manifested by dispersal of energy at frequencies which are harmonics of the fundamental frequency of administration. The instrumentation is complete for this study and pilot experimentation is beginning. This testing model is viewed as potentially useful for patient monitoring in coronary care and intensive care settings, as a technique for isolating the effective time course of a medicinal agent's effect on the patient. Other potential benefits include assessing the amount of treatment effect more adequately through isolating the drug effect from other sources of variability affecting the same life system(s).

5. Effects of work-rest cycles on human data processing capacity.

In previous studies of this research program it was repeatedly observed that giving subjects brief rest breaks at timed intervals to interrupt long periods of panel monitoring seemed to induce a power spectral intensity corresponding to the timing of the rest breaks. This observation suggested that power spectrum

analysis of timed rest breaks might provide a much more specific index which could be used for the study of work-rest schedule effects than the kinds of analysis used previously in these kinds of studies. Consequently, a study was performed to test specifically whether the observed spectral intensity was actually induced by timing of rest breaks or whether this intensity simply coincided in time with the rest break cycle. One group of 15 male subjects were given 11-hour monitoring vigils on the same panel monitoring task used in earlier studies following stabilization of performance, with a 10-minute rest break scheduled every hour. The second group of 15 subjects were tested over the same duration following training up to asymptotic levels, but these subjects were given a 20-minute rest break at two-hour intervals. This procedure should induce work-rest frequencies in the power spectrum at 24 cycles/day and 48 cycles/day respectively. Data acquisition is now complete, but the results have not yet been subjected to computer analysis.

6. Test of hypothesis that alpha EEG activity originates in the eyes.

The EEG is a universal method of assessing brain function in both psychiatric and neurologic cases. The use of this information has recently been jeopardized by evidence that its site of origin is not the brain, but the eyes. Lippold suggested that "the source of current for generating the waveform of alpha rhythm is the standing potential across the eye." We found in our research that the eye explanation is untenable by recording the alpha EEG rhythm from a person without ocular globes. Bipolar recordings were made from (a) the midline over the parietal and occipital regions for alpha activity, (b) from left and right temporal areas for kappa activity, and (c) from electrodes mounted near the absent right eye for periorbital electrical activity. The two EEG signals were then entered into a two-channel automatic scoring apparatus developed in this laboratory for quantitative determination of alpha and kappa EEG activity. Clear examples of alpha and kappa EEG activity were obtained from this subject and they were not correlated with the periorbitally recorded muscle twitches. A standard set of tasks previously used in a population survey were administered while the EEG was being recorded. The alpha and kappa EEG activity automatically scored for this subject were modulated by the tasks in the same way shown by the majority of normal subjects in the previous study. The amount of alpha activity was less during the "hard" tasks, whereas the amount of kappa activity was more during the "ha tasks. Furthermore, the amount of time that alpha and kappa activity were present simultaneously was extremely low, which is consistent with the earlier findings supporting their independence. The data from this subject with both eyes enucleated demonstrate that neither alpha nor kappa EEG activity originate from the standing potential across the eye.

7. Brain responses, alertness and problem solving.

Our brain research has been largely directed toward the objective of understanding performance demands related to performance, vision, alertness and thought processes and their intimate relationship to underlying physiologic functions which are associated with ineffective performance. The use of computer techniques has made it possible to detect discrete brain responses to stimuli which bear information to an individual actively engaged in problem solving. Research in this laboratory has established that brain responses derived from scalp electrodes as averaged evoked potentials carry more than simple, gated, binary information. The complexities of waveform contain components which are differentially related to various aspects of performance. Thus, the possibility exists of extracting objective information about brain processing through computer analysis; information which otherwise could only be inferred in terms of intervening variables or theoretical constructs. The research designs have employed problems where the stimuli, behavioral answers, and brain responses could be readily manipulated and measured. Usually four stimuli, two numbers and two letters, were presented at 0.75 sec. intervals on each trial. The particular numbers and letters were selected at random. The order of stimulus class was also randomized. The subjects were given simple problem solving tasks involving these stimuli, e.g. which of two numbers on a trial was numerically smaller or what is the alphabetical order of the two letters. The simultaneously recorded brain responses were sorted and averaged separately to the numbers and to the letters for each of the trial positions. It was concluded that even when the stimuli are randomized, the AEP depends upon whether those stimuli are relevant to the task. In the case of the problem involving numerical or alphabetical order of the two relevant imuli, the mental processing of the two stimuli may be different. For the first relevant stimulus, the subject must perceive it and remember it, i.e., store it in memory. When the second relevant stimulus occurs the person must perceive it, compare it with the memory of the first, and solve the problem. Thus, to simplify, we can refer to the first relevant stimulus as a storage stimulus and the second relevant stimulus as a problem-solving stimulus. Thus the poststimulus processing of the information in these two stimuli are different. Are these differences reflected as differences in the recorded brain responses? Our data show that these differences exist. Differences in averaged evoked potentials therefore may be associated with differences in the mental processing requirements of the task. At the same time we studied these poststimulus effects, the experimental constraints of the design imposed sequential conditional probabilities which led to prestimulus effects. For example, the subject knows prior to the presentation of the last stimulus on each trial whether it

will be a letter or a number, although he does not know what letter or number it will be. This fore-knowledge led to a prestimulus effect which affected the recorded responses. This distinction between events that occur before the stimulus is presented and events occurring after the stimulus is presented seems to be important because it relates to the general question of the specificity of brain response effects. Can the averaged evoked potential tell us about the particular poststimulus brain processing or only about the excitability of the brain in being prepared or ready for the stimulus? These problems and data are presented in one of the publications listed (Chapman, 1969, NASA SP-191). Using information theory as a starting point for analyzing psychological effects on the AEP emphasizes operations occurring before the stimulus, such as the relative frequency of stimulus occurrence in the past, and the subject's expressed expectation of a future stimulus. In one sense, at least, the information available is identical for both relevant and irrelevant stimuli since there were six possible numbers and six possible letters that could have appeared in any of the trial positions. Thus, it appears that information theory is not helpful in accounting for the different brain responses recorded. It appears that some of the differences in recorded brain responses may be related to those processes occurring after the stimulus is delivered. This finding is being pursued. One of the ramifications is establishing what the components of the complex brain responses are. This is a multidimensional problem and multivariate statistical techniques are therefore being applied. Measuring these responses at various fixed latencies has shown that these poststimulus effects can occur at latencies as short as 105 msec. This kind of approach leads to the possibility of determining minimum brain processing times required for various kinds of tasks.

8. Brain activity and assessment of intelligence.

This research is directed to the objective of identifying physiologic functions correlated with ineffective performance. The approach has employed electrophysiologic techniques and computer data processing. The possibility of assessing mental ability with an objective brain measure has far-reaching implications. The possibility for such an application comes partly from research showing that sensory evoked brain responses depend in detail on the processing given those stimuli by the individual and partly from research showing correlations between certain aspects of averaged evoked potentials and I.Q. test scores in children or other special populations. The present research has been designed to test this possibility in a sample of Army personnel. The test situation involves applying standard EEG electrodes to the individual's scalp and using standard EEG for initial

stages of amplification. These signals are then led to special filters and averaging equipment for extracting the small evoked responses out of the larger amplitude background EEG. The stimulus is an extremely brief strobe flash with either a uniform or simple geometrical visual pattern. The simultaneously recorded brain responses from several sites are plotted on an X-Y plotter as a permanent record and for subsequent analysis. For the initial criterion measures each individual's AGCT scores were obtained. Previously we had transferred Whittaker's technique from institutionalized children to a sample of Army personnel. In an initial group the correlations were extremely low and further analysis indicated that reliability coefficients were poor. However, we developed and tested a large number of additional measures, some of which yielded significant correlations. The latest group of subjects have been run on an enlarged set of tests which included Whittaker's technique, Rhodes thesis, Ertl's conditions, and the correlates we retained out of 120 less favorable ones. We have processed 127 subjects, but due to failures to recover sufficient AGCT scores, we are adding at least 20 more subjects to our sample before embarking on a full scale statistical analysis. Preliminary analysis of the new data confirm the finding of significant correlations with certain of the averaged evoked potential measures. Thus, the possibility is sustained for a relatively culture-free, non-verbal, non-motor test of intelligence in terms of objective brain activity.

9. Clinical and developmental brain research.

Another, more clinical, direction of our brain research is to examine and evaluate electrophysiological measures which are related to the assessment of neurological development and cognitive processes of infants and young children. The general method is that of recording of computer averaged evoked EEG responses to visual and auditory stimuli in normal children and in children with abnormalities of sensory and mental development. The relationship between sensory evoked potentials, electroencephalographic, behavioral and biochemical parameters of development are being examined. The applications of evoked response recording to clinical diagnosis, i.e., in patients with sensory, mental, and perceptual abnormalities, are being investigated and evaluated. The research will provide basic data on the maturation of the EEG and sensory evoked potentials of human children during the course of development and will attempt to relate data obtained from psychological and neurological testing with the neurophysiological data. It will provide normative data for comparison with that obtained from patients with sensory and neurologic handicaps.

Several investigations are in progress. They include:

a. Studies of the EEG evoked responses of normal children to visual and auditory stimuli.

(1) logitudinal and cross-sectional studies of the relations between evoked responses and age, behavioral state, EEG background tracing, stimulus intensity, modality and order.

(2) correlations between evoked response measures and scores on the Bayley Scales of Infant Development and other psychological tests.

(3) studies of response decrement with repetitive stimulation in normal versus developmentally retarded infants.

(4) studies of evoked responses to paired stimuli as the significance or task relatedness of the stimuli are varied; these studies will be done in normal children and those with learning problems.

b. Auditory evoked responses in patients with suspected hearing loss; EEG evoked response audiometry.

c. Serial evoked response studies in patients with Down's syndrome, some of whom are receiving the serotonin precursor 5-HTP.

d. EEG sleep and evoked response studies in patients with disorders of serotonin and catecholamine metabolism.

e. Evoked responses in patients with visual impairment.

f. Evoked responses in mentally retarded and brain damaged children.

In the normative studies the emphasis in the past year has included completion of data gathering in several age groups and preparation of data on evoked response characteristics and EEG for computer statistical analysis. Dr. Elisabeth Ohlrich is completing a trend analysis of auditory evoked response amplitude and latency for infants from birth to 1 year of age.

We have continued to investigate possible relationships between mental defects and evoked response characteristics. In a study of auditory evoked response decrement with repetitive stimulation with clicks, the findings in normal infants and infants with 21-Trisomy differ significantly. Normal infants (N = 61) 6 and 12 months old show smaller amplitude averaged responses

(p = .001) to the last 25 of a series of 100 clicks than they do to the first 25 of the series. Mongoloid infants (N = 42) 6 and 12 months old do not show significant response decrement. Neither normal 1 month old infants (N = 22) nor 1 month old mongoloids (N = 27) show response decrement. In addition, the form of the response of normal and mongoloids differs. Normal infants show prominent late positive waves while in the trisomics this activity is ill-defined or absent. Further studies were done to find out whether the response decrement is related to habituation or the recovery cycle characteristics of the auditory system. These findings were presented to the Spring Meetings of the American Academy of Pediatrics and the implications of abnormal sensory processes for cognitive development were discussed. A report is being readied for publication.

We have summarized and submitted for publication our experience with the first 100 children under 3 years of age referred for evaluation of possible hearing loss using the method of computer average evoked EEG audiometry. Comparison with the results of clinical audiometric methods, follow up data, and a discussion of the uses and limitations of the method were included in this paper. A report of this work was read to the 1st International ERA Symposium in Freiburg, Germany in April, 1970 and the text will be published in the Archiv fur Hals-Nasen-und Ohrenkrankheiten.

Data collection for a double blind study of evoked response characteristics of infants with 21-Trisomy who are receiving the serotonin precursor 5-hydroxytryptophan or a placebo is continuing. The children are being tested within 4-5 days of birth and at 6 months, 1, 2, and 3 years.

The visual occipital evoked response is potentially useful for testing the presence or absence of vision. The extreme difficulties of stimulus control in young subjects may limit the method for clinical diagnosis, but even at present certain inferences can be drawn from the presence or absence of responses or gross asymmetries of response.

We have had the opportunity to observe 6 patients at the Children's Hospital of the District of Columbia with prolonged acute cortical blindness who showed partial or full recovery of vision. We recorded visual evoked potentials during the course of recovery in an attempt to elucidate the pathologic physiology of this relatively rare condition. A report has been accepted for publication in Neurology. This work was done in collaboration with Drs. James Manson and Elliot Wilner of the Children's Hospital, Dept. of Neurology.

There are a number of mental retardation syndromes that are associated with low blood serotonin (5-hydroxytryptamine, 5HT) levels, e.g.,

Down's Syndrome. We are in collaboration with Dr. Mary Coleman, Children's Hospital, Dept. of Neurology, studying a patient with profound psychomotor retardation and an abnormally high whole blood level of 5HT consistently recorded between the ages of 2 1/2 and 5 1/2 years.

The patient, who has refused all food containing 5HT since infancy, is a microcephalic, negro female with an scaphocephalic configuration to the cranium. She began to walk and talk at age 5. Abnormalities on general examination included a high arched palate, a hint of epicanthic folds, flat nasal bridge, odd dermatoglyphics on the left hand but not true 4 finger line, overriding of the second and fourth toes bilaterally, pronated feet, and moderate hirsutism of the face and legs. Neurological examination disclosed bilateral mild alternating, non-paralytic internal strabismus, buccolingual dyskinesia and increased muscular tonus in all extremities. Reflexes were slightly increased, but there was no clonus, Hoffmann or Babinski. Detailed retardation chemistry work-up was within normal limits except for both whole blood and platelet 5HT levels between 3 to 8 times normal, slightly increased platelet counts, and increased ATP (the binding factor for 5HT in the subcellular organelles) in the platelets. EEG sleep studies have revealed abnormal patterns (decreased stage 3 and 4; fewer REMs).

Oral administration of parachlorophenylalanine (an inhibitor of the tryptophan hydroxylase, the rate-limiting step of 5HT metabolism) resulted in lowering of blood and urinary 5HIAA levels and temporary return of normal muscular tonus. Fourteen nights of EEG sleep recording have been done thus far to study the acute and long term effects on the sleep cycle of PCPA administration.

10. Medical electronics engineering development.

A device has been constructed to display signals recorded on a small segment of magnetic tape. Involving a rotating drum on which tape heads are mounted, it allows steady viewing of a non-recurring signal on magnetic tape by repeated sweeps of the tape, permitting accurate localization and viewing of single nerve action potentials or measurements of interspike intervals of action potential trains. The device is now undergoing tests.

a. Recording and Playback Instrumentation.

A sub-audio frequency time mark was recorded on one channel of a two channel tape which had audio information recorded on the other channel. Upon playback, the time signal from the recorder was amplified and used to actuate an event recorder. The event recorder was provided with four additional channels which were to be actuated by subject operated push buttons. Also furnished were a suitable tape recorder, power supplies and associated hardware.

b. Pulse Polarity Selector.

This device provided either a positive 0.8 volt or a negative 0.8 volt square wave at the output terminal depending on which one of two input terminals received a 12 volt positive square wave.

c. Random Pulse Generator.

A pseudo-random pulse generator module was constructed and checked. Several more are being fabricated. These will be contained in a system to (optionally) increase the probability that there is no orderly sequence of time intervals between pulses during the experiment test period.

d. Programmed Sphygmomanometric Blood Pressure Monitoring System and Dependent Stimulus Controller.

Following are some of the component parts and modules which were designed and incorporated into this system:

(1) Air pressure transducer: (1) an LDVT and suitably designed amplifier. (2) a reluctance transducer with digital readout and an analogue output.

(2) Stimulator: An auditory stimulator was designed and fabricated. The stimulus starts on loginal command and continues for the entire interval.

(3) Blood Pressure Pulse Transducer: (a) a simple, effective amplifier for the ceramic cuff transducer was incorporated. (b) a new type of transducer used external to the cuff was interfaced with the system for continuous detection of the pressure or pulse. Its use permits the effective use of a special filter design.

(4) Artifact Filter: By eliminating base-line shift, this filter design obviates a major difficulty in discriminating against spurious responses.

(5) Air Pressure Control Solenoids: They function as controllable valves for inflation of the cuff. A.C. solenoids previously used have been incorporated into a "zero-crossing" controller; it is being designed into a system for automatic programming of pressure cycle. D.C. solenoids, which produce a reduced number of transients, are now being used. Grounding system has also been improved to reduce transients and their effects.

(6) Electronic circuitry functionally integrating the component parts.

e. Carousel Controller.

A random delay interval is provided after the subject depresses a selector key which is identified on a second channel of a two channel chart recorder. Construction and design is approximately 33% completed.

11. Traumatic brain injury.

The purpose of this research is the application of behavioral and electrophysiological techniques to clarify cerebral deficits due to war injury. Rapid evacuation methods and prompt specialized neurosurgical care have dramatically increased the liklihood of survival following brain injury. One major consequence has been the estimated four-fold increase in cases of total disability following wounds in the Vietnam War compared with World War II. The problems created by these cases have changed both quantitatively and qualitatively. Developments in behavioral science and neurophysiology have provided methods not available previously to evaluate these residual functions. Patients selected for this project undergo special electrophysiological evaluation. This concerns primarily the study of cerebral evoked potentials. Its advantage over routine EEG is that it enables one to examine the cerebral responses to a single sensory modality. That is, by providing visual, auditory, or somatosensory stimuli, a different cerebral evoked response is produced for each modality. The use of this technique with brain damaged patients offers two advantages: first, using simple auditory, visual, or somatosensory stimuli, it should be possible to determine whether or not these different signals are reaching the primary receiving areas of the brain. This is particularly important in severely brain damaged patients with whom little, if any, communication through the behavioral tests is possible. Secondly, the longer latency evoked responses could provide some information regarding the potential for cerebral integration. In conjunction with the behavioral studies, this should further our understanding of residual function in the brain injured patient.

The equipment necessary for this project is currently being constructed. The auditory stimulation apparatus consists of a combination click and tone generater capable of presenting clicks, tones or words to either or both ears at varying amplitudes and inter stimulus intervals. This has been completed and is functioning properly. The somatosensory stimulation device is a current regulated shock stimulater that will deliver low

amplitude shocks to either median or peroneal nerves. Assembly of this has begun but completion awaits the arrival of several necessary components.

The most complicated apparatus is the system for presenting visual stimuli, and recording responses in a coded form that can be accepted by the averaging computer. Stimulus presentation is accomplished by using a standard Kodak Carousel projecter which has been adapted to accept a strobe light in place of the regular light source. The coding system consists of specially designed 35 mm slide holders. That, in conjunction with photodiodes allows digital coding for up to 15 possible combinations. This part of the visual stimulating equipment has been constructed, tested and is working satisfactorily. Once the coded signal has been established, it then must be properly interfaced on magnetic tape. The interfacing involves proper sequencing of EEG signals, stimulus synchronization pulses, and coding pulses in a pattern acceptable for computer analysis. The circuitry for the sequencing has been designed but actual testing awaits the arrival of various equipment.

As the equipment arrives, it is being assembled and tested at Forest Glen. The equipment has been placed in portable racks to enable easy transfer to Walter Reed Medical Center upon completion. It is anticipated that actual testing of subjects can begin this summer.

12. Microelectrode construction and marking techniques.

In conjunction with the microelectrode studies of the visual system on animals, two techniques involving the use of microelectrodes are being developed. The first involves the construction of uniform metal electrodes of desired impedance. This technique produces up to 17 metal electrodes at one time, all of uniform taper. After insulation, impedance testing of these electrodes presents a unique problem. Impedances range up to 109 ohms. Direct current cannot be used for testing because it produces almost immediate polorization of the electrode. A method has been developed using alternating current in the physiological range (1000 cycles per second) which takes into account both the capacitve reactance and resistance of the electrode. This has provided an accurate method of measuring the overall impedance of metal microelectrodes.

In addition to accurately measuring metal electrodes, it is also desirable to be able to produce impedances within a desirable range. In the past, this has been done by producing a large number of such electrodes measuring all the impedances and then

selecting those in the desired range. By using a current limiting circuit, and producing electrodes with impedances above the desired we have been able to etch the insulation from the electrode tips and produce uniform microelectrodes with impedances to within ±5% of a predetermined desired impedance.

The second technique which is currently under development is a method of microelectrode tip position marking. Since most microelectrode investigation involves recording from below the surface of the brain, it is important to know exactly where the recording tip is located. The method being developed involves the use of a highly ionotrophic dye (alcian blue) that is electrically extruded from the tip of a micro pipette after nerve potentials have been recorded. This dye, once deposited, binds with the surrounding nerve tissue and is not diluted by the usual histological fixing and staining techniques.

Summary and Conclusions:

The work performed during this reporting period has continued to embrace a wide scope of interdisciplinary research projects concerning performance decrement and its correlates at various life systems levels. Our applied studies of various conditions leading to performance decrement have led us to certain issues concerning definition of normal behavior and specifications of the mechanisms underlying the body's support of normal behavior. New analytic approaches have been developed which have permitted much more precise specification of normal states and how these normal states are altered by exposure to stressful situations, tranquilizing drugs and disease processes. Thus, the status of measurement has been sophisticated to the point that investigation can reveal not only the presence of performance decrement, but also how performance decrement induced by one agent differs from performance decrement induced by another agent, relative to differentially affecting various processes associated with baseline behaviors. This attention to methodological considerations has allowed us to differentiate very different patterns of effects from one agent or condition to another which could not be discriminated using more traditional approaches to measurement. The work on chlordiazepoxide and short-term deprivation have revealed consistent patterns of effect which have not been reported in the previous literature. The oscillation induction drug effect testing model promises to provide a clinically useful technique for quantitative analysis of drug effects in patients for whom relatively long periods of measurement are available. The continuation of systematic research on relationships between brain responses, alertness, and problem solving is delivering new and productive information concerning discriminating the different roles of the various components of the complex brain response during perceptual processes. The possibility of relating these electrophysiological

processes to intellectual variables have proven to be a difficult task, but correlational results in some cases have been statistically significant. This approach to the study of brain-behavior relationships has provided the methodological foundation for a number of clinical studies now being pursued. Several of the biomedical engineering development projects have yielded a capability for precision in biomedical measurement much more adequate than commercially available instrumentation. The indirect blood pressure measurement system is anticipated to be especially useful for future studies in which direct measurement is contraindicated and should reduce the necessity for requiring direct measures simply to attain improved precision in measurement.

Project 3A062110A823, MILITARY PSYCHIATRY

Task 00, Military Psychiatry

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

Literature Cited.

1. Chapman, Robert M. The definition and measurement of "psychological" independent variables in an average evoked potential experiment. In Donchin, E. and Lindsley, D.B. eds. (1970) Averaged Evoked Potentials: Methods, Results, Evaluations. Washington, D.C. NASA SP-191, 262-275.

2. Chapman, Robert M. Very slow brain potentials relating to expectancy: the CNV. In Donchin, E. and Lindsley, D.B. eds. (1970) Averaged Evoked Potentials: Methods, Results, Evaluations. Washington, D.C. NASA SP-191, 177-180.

3. Coleman, Mary and Barnet, Ann. L-days reversal of muscular spasm, vomiting and insomnia in a patient with an atypical form of familial dystonia. Transactions of the ANA, June 1970.

4. Frazier, T.W. and Bitetto, V. Control of human vigilance by concurrent schedules. J. Exp. Anal. Behav., 1969, 12, 591-600.

5. Frazier, T.W., Weil-Malherbe, H., and Lipscomb, H.W. Psychophysiology of conditioned emotional disturbances in humans. Psychophysiol., 1969, 5, 478-503.

6. Gentile, J.R. "Learning Environment." Science, 1969, 163, 18.

7. Gentile, J.R., Kessler, D.K., and Gentile, P.K. The process of solving analogy items. J. Ed. Psychology., 1969, 60, 494-502.

8. Gentile, J.R. and Seibel, R. A rating scale measure of word relatedness. J. verb. Learn. & verb. Behav., 1969, 8, 252-256.

9. Lodge, A., Armington, J., Barnet, A., Shanks, B., and Newcomb, C. Newborn infants' electroretinograms and evoked electroencephalographic responses to orange and white light. Child Development, 1969, 40, 267-291.

10. Sheatz, G.C., and Chapman, R.M. Task relevance and auditory evoked responses. Electroenceph. clin. Neurophysiol., 1969, 26, 468-475.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
				DA 0A 6471	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A824	00	055
b. CONTRIBUTING				
c. XXXXXXXXCDOG	1212B(21)			

11. TITLE (Precede with Security Classification Code)*

(U) Chemical Protection Against Irradiation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

014000 Radio and Radiation

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
59 05	CONT	DA	C. In-HOUSE

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	6	150
	CURRENT 71	6	150

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research
Washington, D. C. 20012

ADDRESS:*

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* Walter Reed Army Institute of Research
Division of Medicinal Chemistry

ADDRESS:* Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. academic institution)

NAME:* Sweeney, T. R., Ph.D.

TELEPHONE: 202-576-3731

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Rothe, COL W. R.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Activity; (U) Chemical; (U) Compound; (U) Dose; (U) Protection; (U) Radiation Injury; (U) Human Volunteers

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The objective of this research is to develop a militarily useful pill to protect personnel against lethal effects of ionizing radiation. Such a drug would reduce the initial effect of nuclear radiation as well as provide a margin of safety for personnel operating in a contaminated terrain. An efficient antiradiation compound would also be useful to the Army from the clinical standpoint.

24 (U) Approach to the objectives is through accepted drug development protocols. Synthesis and testing of potential agents is being carried out. Test results are analyzed for structure activity relationships and fed back into the synthesis program. Promising compounds are carried forward to testing in large animals and the pharmacology of these compounds investigated. In addition, chronic toxicity studies, dose reduction factor studies and drug antagonism studies are being carried out. Development of efficient methods of handling chemical and biological information are being developed.

25 (U) 69 07 - 70 06 Continued emphasis was laid on latentiation of the thiol group in order to try to increase absorption and effectiveness. In addition, latentiation of the aminoethanethiol moiety through formation of a thiazolidine constituted a major effort. This emphasis during the past year has been on the sulfur rather than the nitrogen function of the aminothiols. Major investigation of the amidines and N-pyridinyloxy alkyl type compounds has continued. Aminothiosulfates have been deemphasized because of poor absorption. Dogs were protected with WR77913 and with a combination of WR638, 1607, and 302. Monkeys were protected by WR2721. Clinical studies on WR2721, 2529, and 2823 are scheduled for the clinic. Compound WR2823 has been successfully formulated for IV Infusion. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 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 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 055, Chemical protection against irradiation

Investigators:
Principal: COL William E. Rothe, VC
Assistant: Thomas R. Sweeney, Ph.D.
Associate: LTC Edgar H. Eckermann, VC
LTC Ray A. Olsson, MC
Melvin H. Heiffer, Ph.D.
Edgar A. Steck, Ph.D.
1LT Joseph E. Tomaszewski, CmlC
MAJ Pasqual Perrino, MC
CPT Gene Withrow, MSC
CPT Richard C. Davis, Jr., MSC
Miss M. M. Grenan
LTC Gale E. Demaree, MSC
Robert S. Rozman, Ph.D.
MAJ Alan S. Nies, MC
MAJ James A. Vick, MSC
CPT Robert W. Caldwell, MSC
Daniel L. Klayman, Ph.D.

Progress

I. General

The program to develop a chemical prophylactic to offset the effect of ionizing radiation on exposed troops has been carried forward on several fronts. The major technical problem confronting the study continues to be the separation of the antiradiation activity of the drugs from their toxicity. Animal pharmacological response to the potential agents often varies widely with species, which makes more difficult the development of an agent. Nevertheless, several agents have successfully traversed the distance to the clinic.

Chemistry

II. Contract Synthesis Program

As of the end of FY-70 there are six active synthesis contracts and one preparations laboratory contract. During the year one preparations laboratory contract with the Aerojet General Corporation was terminated. The contract with New England Nuclear Corporation for synthesis of radiolabeled compounds continues.

During FY-70 there were 232 target compounds submitted that were synthesized in the contract program. This is, coincidentally, the same number that was obtained in FY-69. Twelve new compounds were requested from the preparations laboratory, and five compounds delivered during the year.

Emphasis in the synthesis program during the year has been laid on the latentiation of the thiols. Some twenty new type sulfur-covering groups have been synthesized and have shown various activities and toxicities. Although the antiradiation activity of the compounds containing the new sulfur-covering group is not superior to earlier compounds containing better known sulfur-covering groups, much new and novel chemistry has, of necessity, been studied. This will be reported in chemical journals.

The two most interesting and promising classes of compounds which were studied during the year are the N-pyridinyloxyalkyl type and the amidines. Excellent oral activity in certain compounds of the former class in mice has been obtained when the aminoethanethiol moiety has been latentiated as the thiazolidine. Other sulfur-covering groups have also been run and a fairly comprehensive structure/activity study, as a function of the route of administration, has been completed. Excellent oral activity has also been found in certain of the latter class in mice when the sulfur is in the form of the thiol or disulfide.

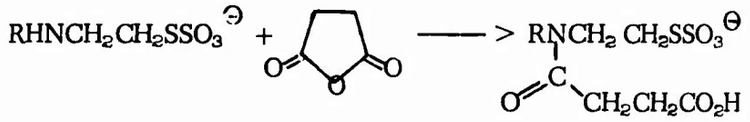
III. Organic Chemistry Laboratory

The series of N-monoalkyl-substituted-2-aminoethanethiosulfuric acids (I), prepared by the alkylation of 2-aminoethanethiosulfuric acid or by the reaction of sodium thiosulfate with N-alkylaminoethyl halides, was completed.

$$RHNCH_2CH_2SSO_3H$$

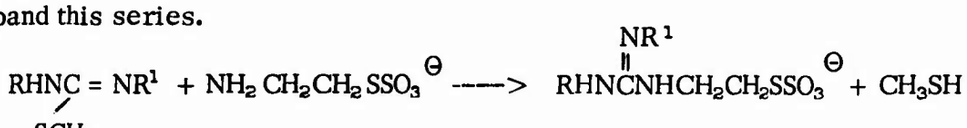
(I)

A new series of aminoethanethiosulfuric acid derivatives, viz., N-(β-carboxy-propionyl) aminoethanethiosulfuric acids (II) from the reaction of the Bunte salt with succinic anhydride was started. The parent compound (R = H) has been difficult to purify. The n-decyl derivative (R = n-$C_{10}H_{21}$) is presently being tested as an antiradiation drug.



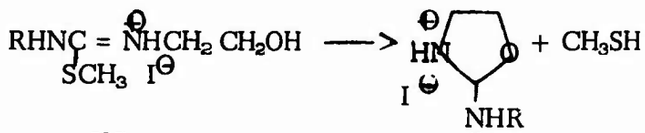
(II)

The reaction of 2-aminoethanethiosulfuric acid with S-methyl derivatives of thiourea to synthesize guanidinoethylthiosulfuric acids (III) was reactivated in order to expand this series.



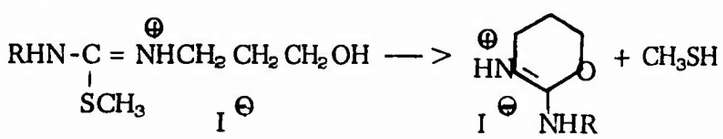
(III)

The utilization of this same reaction type, i.e., the reaction of a nucleophile with S-methyl derivatives of thiourea, was the basis for the investigation of a series of cyclization reactions of 1-(hydroxyethyl)-2-methyl-2-thiopseudoureas (IV) to 2-amino2-oxazolines (V).



IV) (V)

Likewise, the six-membered heterocycle, i.e., 2-amino-5,6-dihydro-4H-1,3-oxazine (VII) could be prepared by starting with 1-(3-hydroxypropyl)-2-methyl-2-thiopseudourea (VI).



(VI) (VII)

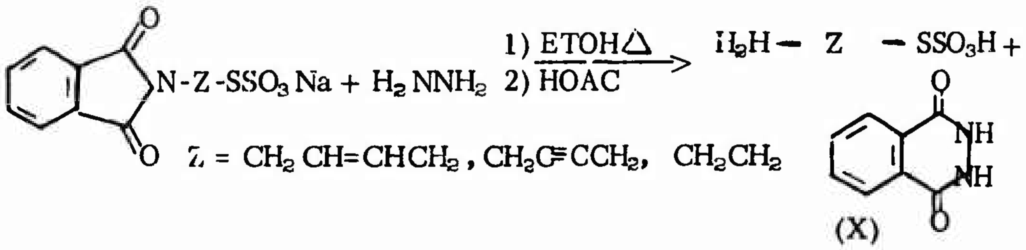
The spontaneous decomposition of 1-hydroxy-3-phenyl-2-thiourea (VIII) was briefly looked into, and the products of this degeneration were found to be elemental sulfur and phenylurea (IX).

$$C_6H_5NHC(=S)NHOH \longrightarrow S^0 + C_6H_5NHC(=O)NH_2$$

(VIII) (IX)

This may be an intramolecular displacement reaction (via an intermediate oxazirane ring) giving the products directly, or an elimination reaction yielding elemental sulfur, phenyl cyanamide, and water, the latter two combining to give the phenylurea.

A new and convenient method of preparing aminoalkyl Bunte salts by the reduction of the corresponding phthalimidoalkyl Bunte salt with hydrazine was developed. The thiosulfate functionality was found to survive the hydrazinolysis, which yielded, besides the desired thiosulfuric acid, phthalhydrazide (X).



This synthetic method would be most useful in the preparation of Bunte salts which could not be made by the classic reaction of aminoalkyl halide with sodium thiosulfate because of cyclization problems. The phthalimidoalkyl Bunte salts are synthesized from the corresponding dihalide by nucleophilic displacement of one halide ion by potassium phthalimide and the other successively by sodium thiosulfate. It is interesting to note that when the alkyl chain (three or four carbon atoms have been studied) of the dihalide is substituted with hydroxyl or keto groups, the initial reaction produces only bis phthalimide, the result of a displacement of both halide ions by two phthalimide ions.

A study of the conversion of aminoalkanethiosulfuric acids to the corresponding disulfides was undertaken. A series of thiosulfuric acids treated with hydrogen sulfide in aqueous solution gave the corresponding disulfide as the thiosulfuric acid salt (XI) in nearly quantitative yields.

$$2H_2NCH_2CH_2SSO_3H + 3H_2S \longrightarrow (H_2NCH_2CH_2S)_2 \cdot H_2S_2O_3 + 2H_2O + 3S^0$$

(XI)

When the aminoalkanethiosulfuric acid was used as the starting material, elemental sulfur precipitated shortly after introduction of the hydrogen sulfide. If the sodium salt was utilized (for solubility reasons), the by-product was instead sodium thiosulfate.

$$2H_2NCH_2CH_2SSO_3Na + H_2S \longrightarrow (H_3\overset{\oplus}{N}CH_2CH_2S)_2 \cdot SS\overset{\ominus}{O}_3 + Na_2SSO_3$$

The exceptions to this technique occur when the amine function is substituted by two large groups. In these cases (e.g., dibenzyl, diheptyl, etc.) the tertiary aminoalkane trisulfide (XII) was isolated.

$$2R_2\overset{\oplus}{N}HCH_2\ CH_2SSO_3^{\ominus} + H_2S \longrightarrow R_2\overset{\oplus}{N}HCH_2CH_2SSSCH_2CH_2\overset{\oplus}{N}HR_2 + 2HSO_3^{\ominus}$$

(XII)

A class of compounds which has avoided much scientific attention are the hydrodisulfides, represented by RSSH. Our interest in studying a new class of potential antiradiation agents prompted the investigation of this functionality. The only hydrodisulfides which have been investigated to any extent in the literature are the benzyl and benzhydryl derivatives, both prepared by tedious routes. Two new routes to benzylhydrodisulfide are being studied in this laboratory as models for the synthesis of aminoalkane hydrodisulfides. By comparison with an IR of authentic benzyl hydrodisulfide, the product obtained from the following schemes appears to be the desired hydrodisulfide:

A) $$C_6H_5CH_2SH + S{=}C(NH_2)_2 \xrightarrow[H_2O_2]{HCl} C_6H_5CH_2SS\text{-}C(NH_2){=}NH \cdot HCl$$

$$C_6H_5CH_2SSC(NH_2){=}NH \cdot HCl + H_2S \xrightarrow[\text{ether}]{H_2O} C_6H_5CH_2SSH + S{=}C(NH_2)_2$$

B) $$C_6H_5CH_2SSO_3Na + H_2S \xrightarrow[H_2O/CHCl_3]{\text{pH-4-5}} C_6H_5CH_2SSH + NaHSO_3$$

IV. Rodent Testing Program

Chemical agents synthesized under government contract for the Army's Antiradiation Drug Development Program are evaluated for radioprotective activity by the Roadent Testing Section of the Division of Medicinal Chemistry. Additional screening of compounds for radioprotective activity is performed by the Woodard Research Corporation, Herndon, Virginia, under the direction of Dr. Henry Horn. This laboratory tests off-the-shelf compounds primarily, and compounds active in the screen are confirmed and further characterized at Walter Reed.

Radioprotective activity is evaluated based on the survival of treated mice to thirty days after lethal irradiation. Treated and control mice are simultaneously exposed to 1000 R of Cobalt-60 gamma radiation or 825 R of KVP x-radiation. All irradiated control mice die within thirty days. In initial tests, compounds are administered fifteen to thirty minutes before irradiation at two dose levels - the maximum tolerated dose and one-half that dose. Female mice eight to nine weeks of age from the WRAIR ICR colony are used for radiation studies. Male mice are used for preliminary toxicity studies. Woodard Laboratories uses ICR "Astro" mice obtained from Charles River Mouse Farms, Inc.

Additional studies are performed on active compounds to determine the duration of action, optimum route of administration, and the radiation dose reduction factor. As much data as possible regarding the acute toxic and pharmacologic effects of each compound is recorded at the time of the primary test.

The table that appears on the following page summarizes the testing activities of the WRAIR and Woodard Laboratories during the past fiscal year. The total number of tests performed include primary tests, confirmatory tests and secondary tests. The number of active compounds includes new compounds with activity, as well as tests of new batches of compounds previously known to have activity.

Summary of Antiradiation Testing

Rodent Testing Section, WRAIR

Test	# of Tests	Good Act.[1]	Slight Act.[2]
Primary - Intraperitoneal	417	85	165
Primary - Oral	512	61	107
Duration of Action	293	--	--
Other	34	--	--
Total	1256	146	272

Woodward Laboratories

Primary - Intraperitoneal	1137	92 Active
Total	2393 Tests	510 Active

[1] Greater than 50% survival after lethal irradiation.

[2] Less than 50% survival after lethal irradiation.

The chemical agents exhibiting the best radioprotective activity in rodents, of those tested during the past year, are tabulated in the following pages. All compounds listed have produced survival of at least fifty percent of the lethally irradiated mice after either oral or intraperitoneal administration. For each compound the approximate LD_{50} in mg/kg, the route and time of administration, the doses of compound administered, and the percent survival are indicated. For each compound dose, if the percent survival is greater than twenty percent, a "Protective Index" has been calculated by the following formula:

$$P.I. = \frac{LD_{50}\ (mg/kg)}{\text{Test Dose (mg/kg)}} \times 1 + \frac{\text{Percent Survival}}{100}$$

A large protective index indicates both protective efficacy and a high therapeutic index. The protective indices are large when good protection is obtained at drug doses well below the toxic LD_{50}.

The protective activity and toxicity of aminothiols is greatly modified both by the addition of substituents to the nitrogen function, and by covering the sulfur function.

Covering of the sulfur by forming the thiazolidine has produced a series of compounds with both interesting protective activity and advantageous therapeutic indices. It is of particular importance that these thiazolidines, when the nitrogen is appropriately substituted, have good protective activity when administered to mice by the oral route. Some of these thiazolidines are even more effective orally than intraperitoneally. Thiazolidines with alicyclic or heterocyclic substituents on this nitrogen have oral and intraperitoneal activity, provided the ring is separated from the nitrogen by an alkyl insulating chain of suitable length. The optimal length of the alkyl chain varies slightly depending on the nature of the cyclic substituent, but is usually four to six carbon atoms. The ring may be directly attached through an ether or thioether link. The compounds with the thioether linkage appear to have exceptionally good protective activity when administered orally. There are several particularly interesting thiazolidines in the series with alkyl thioether substitutions on the nitrogen. The best compounds in this series are those in which the two alkyl fragments are of similar length, i.e. WR 157311 and WR 134783.

The amidine series is also interesting from the standpoint of protection by oral administration and protection at low doses of compound. In this series the sulfur may be uncovered, or may be in the form of a Bunte salt, thiophosphate or disulfide. In a few cases, alkyl substituents on this nitrogen are allowable,

but to date, none have conferred protective activity which offers any marked advantage over the unsubstituted parent thiol (WR 76842). Alicyclic or heterocyclic substituents on the nitrogen of the amidines have, in some cases, improved tolerance without destroying protective activity. In this series, the best activity is obtained when the insulating alkyl chain is short, i.e. one to three carbons. Most of the interesting members of this series are Bunte salts, but there are a few interesting thiols (WR 145720, WR 109342, WR 157078) and disulfides (WR 151331). Some of the most interesting members of the amidine series are those with adamantine substituents on the nitrogen. The most active adamantines are WR 109342 and WR 157078.

Aminoalkylaminoethane thiophosphates are of interest because of their potent radioprotective activity, and because of the beneficial effect of WR 2721 (aminopropylaminoethylthiophosphate) in traumatic and hemorrhagic shock. The degree of protection after oral administration is modest, and the intraperitoneal doses required for protection are large compared to the most active thiazolidines and amidines. None of the new aminoalkylamino compounds examined this year appear to offer advantages in protection over the parent compound, WR 2721.

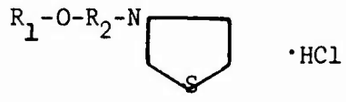
Good protection and a high protective index was obtained with the aminopropylthiophosphate, WR 142489. This compound was not effective orally. Additional members of this series are being studied.

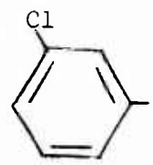
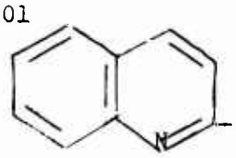
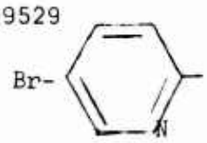
A large variety of Bunte salts and thiophosphates of mercaptoethylamine have been studied this year. Many new compounds in this series have potective activity, and some offer the advantages of large therapeutic indices, and small effective dose. Attempts to protect mice by administration of these compounds orally continue to be disappointing. A large disparity between oral and intraperitoneal LD_{50} in most cases suggests that the compounds are poorly absorbed from the gastrointestinal tract. Some oral protection was obtained with WR 142079 and WR 75232. The best intraperitoneal protection was obtained with WR 138412, WR 138415, WR 122960, WR 122970, WR 134790 and WR 132919.

We continue to explore novel and advantageous sulfur covering functions to improve protective activity and reduce toxicity. WR 76843 is an example of a new covering function which improves the therapeutic index dramatically and also provides some oral protection.

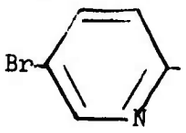
A few novel structures, unrelated to mercaptoethylamine, but possessing interesting protective activity, are in this miscellaneous category.

Thiazolidines A. Ethers

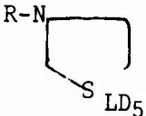


WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
138410		$-(CH_2)_4-$	IP	200	90	15	67	3.71
						30	73	3.84
						60	27	2.82
					45	15	13	
			Oral	650	300	15	27	2.75
						30	47	3.18
						60	7	
138419		$-(CH_2)^*_5-$	IP	160	70	15	93	4.42
						30	73	3.96
						60	20	
					35	15	0	
(*attachment to C_2 of the thiazolidine ring)			Oral	600+	200	15	27	3.81+
						30	20	
						60	0	
108501		$-(CH_2)_5-$	IP	225	100	15	93	4.34
						30	80	4.05
						60	100	4.50
					50	15	0	
			Oral	400	200	15	40	2.80
						30	73	3.46
					100	15	7	
						30	7	
149529		$-(CH_2)_5-$	IP	300	100	15	87	3.62
					50	15	33	7.98
			Oral	400+	400	15	67	1.67+
					400	30	60	1.60+
					200	15	27	2.54+
					100	15	7	

Thiazoldines A. Ethers (continued)

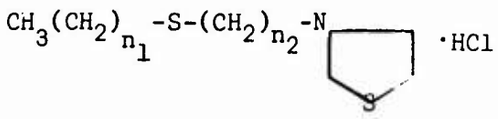
WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
91496		$-(CH_2)_6-$	IP	200	100	15	80	3.60
					50	15	50	6.00
			Oral	525+	300	15	67	2.92+
						30	93	3.38+
						60	87	3.27+
						90	13	

Thiazolidines B. Non-Ethers



WR	R_1	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
2949	$CH_3(CH_2)_7$-	IP	125	50	15	100	5.00
					30	93	4.82
					45	87	4.67
					60	73	4.32
				25	30	53	7.65
		Oral	750+	350	15	67	3.57+
					30	67	3.57+
38490	CH_3O-  -$(CH_2)_4$-	IP	210	80	15	100	5.25
					30	93	5.07
					60	7	
				40	30	60	8.40
				20	30	0	
		Oral	400	200	15	33	2.66
					30	7	
				100	15	27	5.08
					30	7	
36163	 - $(CH_2)_6$-	IP	80	25	15	73	5.53
					30	100	6.40
					60	8	
				12.5	30	0	
		Oral	200	80	30	60	4.00
					60	27	3.17
					15	7	
				40	30	27	6.35

Thiazolidines C. Alkyl Thioethers

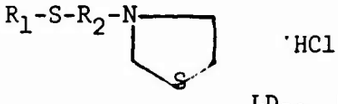


WR	n_1	n_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
157076	8	3	IP	250	100	30	47	3.68
					50	30	13	
			Oral	600+	600	30	73	1.73+
					300	30	20	
157073	6	4	IP	175	80	15	67	3.66
					40	15	20	
			Oral	600	300	15	40	2.80
					150	15	0	
132727	3	5	IP	160	60	15	73	6.92
						30	60	6.40
						60	27	5.08
					30	15	7	
			Oral	900	500	15	26	2.25
						30	40	2.52
					250	30	14	
						60	0	
157311	4	5	IP	125	100	15	60 (toxicity)	2.00
					50	15	67	4.18
					25	15	0	
			Oral	650	250	15	67	4.34
						30	86	4.84
					125	30	92	9.98
					62.5	30	40	14.56
134783	5	5	IP	75	30	30	73	4.32
						60	67	4.18
						90	13	
					15	30	0	
			Oral	500	250	30	87	3.74
						60	80	3.60
					125	15	47	5.88
						30	87	7.48
					62.5	15	20	
						30	20	

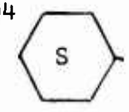
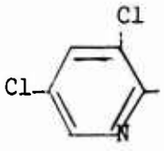
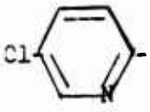
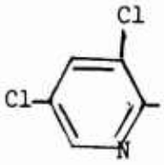
Thiazolidines C. Alkyl Thioethers (continued)

WR	n_1	n_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
157072	4	6	IP	150	80	15	60	3.00
					40	15	38	5.18
			Oral	500	200	15	60	4.00
						30	67	4.17
157312	3	7	IP	175	80	15	75	3.79
					40	15	46	6.40
					20	15	0	
			Oral	650	300	30	67	3.62
					150	30	7	

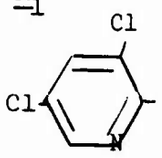
Thiazolidines D. Other Thioethers



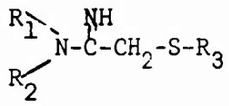
·HCl

WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
146104		$-(CH_2)_5-$	IP	125	60	15	93	4.02
						30	100	4.17
						60	53	3.19
					30	15	13	
			Oral	650	300	15	71	3.71
						30	94	4.20
138417		$-(CH_2)_5-$	IP	300+	100	30	67	5.01+
						60	33	4.00+
					50	30	0	
			Oral	1000+	500	15	0	
						30	27	2.54
132194		$-(CH_2)_6-$	IP	225	100	15	100	4.50
						30	93	4.35
						60	80	4.05
						90	60	3.60
						120	20	
					50	15	0	
			Oral	700	300	15	73	4.04
						30	67	3.90
					150	15	50	7.00
						30	80	8.40
					100	15	26	8.82
						30	13	
152925		$-(CH_2)_6-$	IP	225	125	15	53	2.75
					62.5	15	0	
			Oral	450+	250	30	80	3.24+
					125	30	27	4.56+
138769		$-(CH_2)_6-$	IP	275	140	30	87	3.68
						60	93	3.80
						90	67	3.28
					70	30	40	5.50

Thiazolidines D. Other Thioethers (continued)

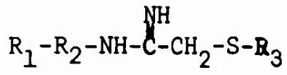
WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
138769		$-(CH_2)_6-$	Oral	1000	500	15	13	
						30	47	2.94
						60	60	3.20
					400	30	33	3.33
						60	80	4.50
					200	15	13	
						30	13	

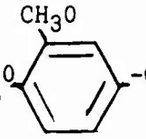
Amidines A. Alkyl Substituted



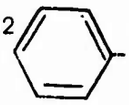
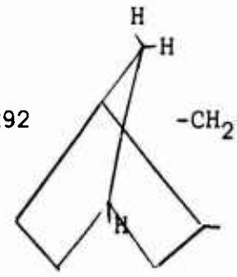
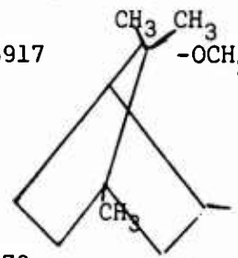
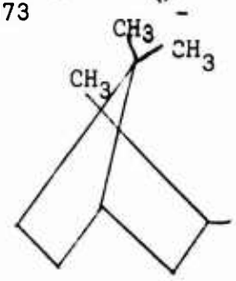
WR	R_1	R_2	R_3	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
76842	H	H	H	IP	50	24	30	100	4.17
						12	30	73	7.20
				Oral	150	40	30	87	7.00
						20	15	67	12.53
							30	40	10.50
						16	15	33	12.48
							30	40	13.12
						8	15	20	
76841	CH_3	CH_3	H	IP	190	100	15	53	2.91
						50	15	20	
				Oral	900+	200	30	67	7.52+
						100	30	54	13.86+
1551	H	H	$-PO_3H_2$	IP	85	50	15	53	2.60
							30	100	3.40
						25	15	7	
							30	53	5.20
						12.5	30	7	
				Oral	300	150	15	67 (toxicity)	3.34
							30	60 (toxicity)	3.20
						100	15	67	5.02
							30	73	5.20
						75	15	33	5.32
							30	27	5.08
108250	H	H	$-PO_3H_2$	IP	180	75	30	53 (toxicity)	3.67
						37.5	30	93	9.26
				Oral	550	350	30	93	3.03
							60	73	2.72

Amidines B. Cyclic Substituents

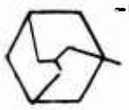


WR	R_1	R_2	R_3	Route	LD_{50} mg/kg	Dose mg/kg	Min.	Percent Survival	Index
8161	CH_3O- 	-(CH_2)-	-SO_3H	IP	85	65	15	73	2.26
						32.5	15	67	4.36
				Oral	600+	100	15	0	
							30	0	
145720	CH_3O  CH_3O	-CH_2CH_2-	H	IP	125	50	15	53 (toxicity)	3.82
						25	15	27 (toxicity)	6.34
				Oral	600+	200	30	27	3.81+
						100	30	0	
151331		-CH_2-	Symmetrical Disulfide	IP	22	10	15	67 (toxicity)	3.67
						5	15	80	7.92
						2.5	15	0	
				Oral	190	50	30	73	6.58
						25	30	40	10.64
						25	15	73	13.16
						12.5	15	13	
150637		-CH_2-	-SO_3H	IP	30	15	15	93	3.86
						7.5	15	67	6.68
						3.8	15	0	
				Oral	150	75	30	47	2.94
						75	60	27	2.54
						50	15	53	4.59
						50	30	80	5.40
						25	15	13	
						12.5	15	0	

Amidines B. Cyclic Substituents (continued)

WR	R_1	R_2	R_3	Route	LD_{50} mg/kg	Dose mg/kg	Min.	Percent Survival	Index
150633		$-SCH_2CH_2-$	$-SO_3H$	IP	40	15	15	87	4.98
						7.5	15	13	
				Oral	300	175	30	0 (toxicity)	
						87.5	30	0	
146092		$-O-(CH_2)_3-$	$-SO_3H$	IP	75	30	15	100	5.00
						15	15	93	9.64
						7.5	15	0	
				Oral	650	250	15	7	
							30	0	
94292		$-CH_2-$	$-SO_3H$	IP	65	20	15	67	5.42
						10	15	67	10.84
						5	15	0	
				Oral	140	60	30	40	3.27
						60	60	40	3.27
156917		$-OCH_2CH_2CH_2$	$-SO_3H$	IP	20	12.5	30	60	2.56
						6.25	30	20	
				Oral	600+	300	30	0 (toxicity)	
						200	30	0	
92973		-	$-SO_3H$	IP	30	15	15	93	3.86
						7.5	15	60	6.40
						3.8	15	7	
				Oral	225	100	15	47 (toxicity)	3.31
							30	80 (toxicity)	4.05
						75	15	36	4.08
							30	47	4.41
						50	30	87	8.42
						25	30	27	11.43

Amidines B. Cyclic Substituents (continued)

WR	R_1	R_2	R_3	Route	LD_{50} mg/kg	Dose mg/kg	Min	Percent Survival	Index
109342		$-CH_2-$	H	IP	22	12	15	93	3.54
							30	83	3.86
							60	93	3.54
						6	15	67	6.13
						3	15	33	9.76
						1.5	15	0	
				Oral	65	30	15	93	4.18
							30	93	4.18
							60	67	3.62
							90	33	2.88
						15	15	75	7.58
							30	92	8.32
157078		$-CH_2-$	H	IP	10	5	15	64	3.28
						2.5	15	33	5.32
				Oral	175	100	15	60 (toxicity)	2.80
						50	15	93	6.56
						25	15	80	12.60
						12.5	15	7	
157798			$-SO_3H$	IP	55	25	15	80	3.96
						12.5	15	0	
				Oral	600+	600	30	13 (toxicity)	
						300	30	0	
						200	30	7	
94140		$-CH_2-$	$-SO_3H-$	IP	25	10	30	93	4.82
						5	30	47	7.35
						2.5	30	0	
				Oral	300+	300	30	0	
							60	13	
						200	30	0	
							60	0	

Amidines B. Cyclic Substituents (continued)

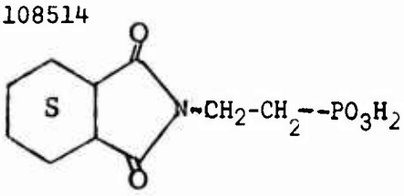
WR	R_1	R_2	R_3	Route	LD_{50} mg/kg	Dose mg/kg	Min.	Percent Survival	Index
145040		$-OCH_2CH_2-$	$-SO_3H$	IP	75	30	15	80	3.60
						15	15	20	
				Oral	600+	400	30	0	
							60	33	8.90+
							120	13	
							190	13	
149750		$-OCHCH_2-$ (CH_3)	$-SO_3H$	IP	60	30	15	67 (toxicity)	3.34
						15	15	73	6.92
						7.5	15	20	
				Oral	300+	300	30	7	
						150	30	0	
157799		$-CH_2CH_2CH_2-$	$-SO_3H$	IP	8	5	30	7 (toxicity)	
						2.5	30	40	4.48
						1.25	30	0	
				Oral	600+	600	30	13	
145378		$-OCH_2CH_2CH_2-$	$-SO_3H$	IP	35	15	15	73	4.04
						7.5	15	0	
				Oral	300+	300	15	0	
							30	0	

Aminoalkylamino Compounds

$R_1\text{-}NH\text{-}CH_2CH_2\text{-}S\text{-}R_2$

WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
1065	$NH_2\text{-}(CH_2)_3\text{-}$	-H	IP	300	150	15	100	4.00
					75	15	73	6.92
					38	15	33	10.64
					19	15	7	
			Oral	500	250	15	27	2.54
						30	67	3.34
					125	15	13	
						30	7	
2721	$NH_2\text{-}(CH_2)_3\text{-}$	$-PO_3H_2$	IP	900	600	15	100	3.00
					300	15	100	6.00
					150	15	100	12.00
					75	15	80	21.60
			Oral	1100	600	15	40	2.57
						30	80	3.30
					300	15	20	
						30	47	5.40
151325	$NH_2\text{-}CH_2\underset{OH}{CH}\text{-}CH_2$	$-PO_3H_2$	IP	825	600	30	40 (toxicity)	1.92
					300	30	93	5.31
					150	30	87	10.28
					75	30	20	
126158	$NH_2CH_2\underset{CH_3}{\overset{CH_3}{C}}CH_2\text{-}$	$-PO_3H_2$	IP	600	300	15	87 (toxicity)	3.74
						30	67	3.34
						60	80	3.60
					150	15	73	6.92
					75	15	0	
125050	$(CH_3)_3C\text{-}NH\text{-}(CH_2)_3\text{-}$	$-PO_3H_2$	IP	450	250	15	93	3.48
						30	80	3.24
						60	73	3.12
					125	30	56	5.61
					62.5	30	0	
			Oral	800+	500	15	0	
					500	30	27	2.03+
					500	60	0	

Aminoalkylamino Compounds (continued)

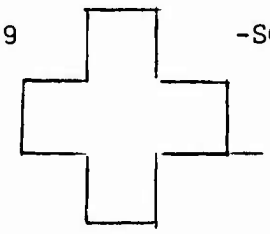
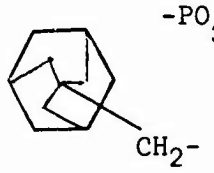
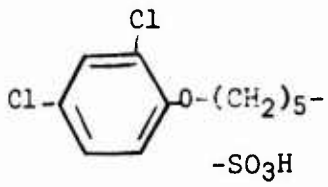
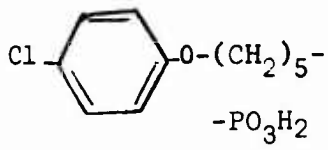
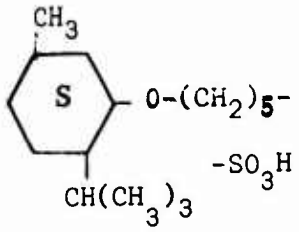
WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
1729	$NH_2(CH_2)_5$-	-H	IP	190	100	15	73	3.29
					50	15	40	5.32
108514			IP	390+	200	15	53	2.98+
						30	87	3.65+
						60	60	3.12+
						90	13	
					100	30	20	
			Oral	900+	600	30	47	2.20+
					300	15	40	4.20+
						30	20	
						60	7	
2529	$NH_2\overset{O}{\overset{\|}{C}}-CH_2CH_2$	H	IP	875	500	15	100	3.50
					250	15	93	6.76
					112	15	54	12.02
					56	15	7	
139245	$NH_2\overset{O}{\overset{\|}{C}}-CH_2CH_2$-		IP	625	500	15	73	2.16
					250	15	13	
		Symmetrical Disulfide						

Aminopropylthiophosphates

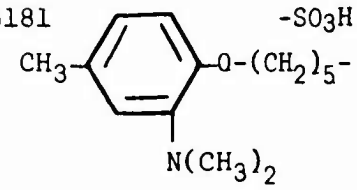
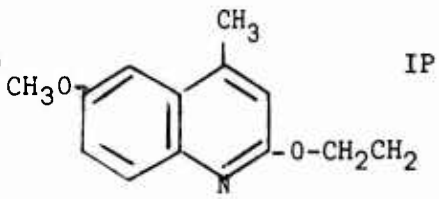
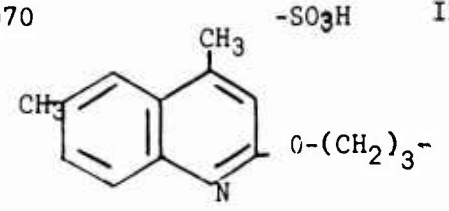
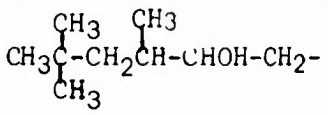
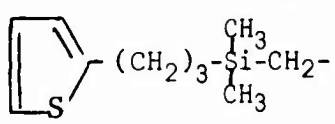
WR	Structure	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
114391	$NH_2CH_2\overset{NH_2}{\overset{\vert}{C}}HCH_2SPO_3H_2$	IP	800+	400	30	80	3.60+
					60	53	3.06+
					90	0	
				200	30	20	
				100	30	7	
				50	30	7	
		Oral	2600	800	30	0	
					60	0	
142489	$NH_2(CH_2)_3NHCH_2\overset{OH}{\overset{\vert}{C}}HCH_2SPO_3H_2$	IP	875	400	15	100	4.38
				200	15	100	8.75
				100	15	53	13.39
				50	15	0	
		Oral	600+	600	30	0	

Bunte Salts and Thiophosphates

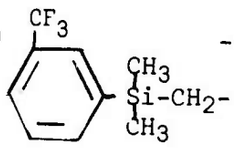
R_1-NH-CH_2CH_2S-R_2

WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
133569		$-SO_3H$	IP	100	50	30	47 (toxicity)	2.94
					25	30	33	5.32
					35	15	13	
					35	60	60	4.58
					35	120	7	
142079		$-PO_3HNa$	IP	150	60	15	60	4.00
						30	100	5.00
						60	33	3.33
					30	15	7	
			Oral	450	200	15	67	3.76
						30	20	
					100	15	0	
						30	0	
138412		$-SO_3H$	IP	175	50	30	87 (toxicity)	6.55
					25	30	80	12.60
					12.5	30	93	27.02
					6.25	30	13	
			Oral	900+	600	30	0	
						60	0	
143996		$-PO_3H_2$	IP	250	100	15	80	4.50
						30	87	4.68
						60	13	
					50	15	7	
			Oral	900	300	15	20	
						30	13	
138415		$-SO_3H$	IP	60	30	15	73	3.46
						30	87	3.74
						60	27	2.54
						90	7	
					15	30	100	8.00
					7.5	30	67	13.37
					3.8	30	0	
			Oral	900+	600	30	0	
						60	0	

Bunte Salts and Thiophosphates (continued)

WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
136181		$-SO_3H$	IP	135	90	15	33	2.00
					45	15	100	6.00
						30	93	5.79
						60	53	4.59
						90	7	
					22.5	15	38	8.28
					11.2	15	7	
122960			IP	100	50	30	80	3.60
					25	30	80	7.20
					12.5	30	67	13.36
					6.25	30	7	
122970		$-SO_3H$	IP	125	70	30	93	3.45
						60	100	3.58
						90	73	3.10
						120	40	2.50
					35	30	87	5.70
					17.5	30	67	11.92
					8.8	30	13	
			Oral	900+	600	30	0	
						60	7	
36978		$-PO_3H$	IP	65	30	15	80	3.90
						30	40	3.03
						60	27	2.75
					15	15	60	6.94
					8	15	0	
					4	15	7	
75232	$CH_3(CH_2)_7-$	$-PO_3H_2$	IP	125	50	15	87	4.68
					25	15	93	9.64
					12.5	15	0	
					6.3	15	13	
			Oral	315	200	30	60 (toxicity)	2.52
					100	30	20	
					50	30	7	
134790		$-SO_3H$	IP	250	50	15	80	9.00
						30	93	9.65
						60	93	9.65
					25	15	67	16.70

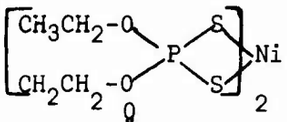
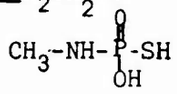
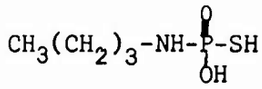
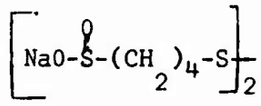
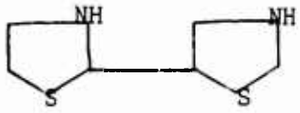
Bunte Salts and Thiophosphates (continued)

WR	R_1	R_2	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
132919		$-SO_3H$	IP	300	50	30	86	11.16
						60	67	10.03
						90	27	7.62
					25	30	40	16.80
			Oral	900+	600	30	0	
						60	0	

Sulfur Covering Functions

WR	Structure	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
146074	$NH_2CH_2CH_2SSCCH_3$ (O)	IP	350	150	15	67	3.90
				75	15	7	
76843	$CH_3CNHCH_2CH_2SS(CH_2)_4SO_2Na$ (O)						
		IP	700	370	15	33	2.52
				185	15	40	5.30
				92.5	15	67	12.63
				46.2	15	53	23.36
				23.1	15	7	
		Oral	1050	600	15	79	3.13+
					30	40	2.45+
139241	$[CH_3(CH_2)_9NHCH_2CH_2S\text{-}S]_2$	IP	40	15	15	73 (toxicity)	4.62
				7.5	15	13	
		Oral	400+	400	30	13	
				200	30	7	
50909	$\left[\begin{matrix}NH \\ \| \\ C\text{-}NHCH_2CH_2S\text{-}S \\ NH_2\end{matrix}\right]_2$	IP	125	40	15	67	4.45
					30	7	
					60	0	
				20	15	7	
		Oral	535	400	30	53	2.43
					60	47	2.34
					90	67	2.65
					120	47	2.34
139240	$[NH_2CH_2CH_2NHCH_2CH_2]_2S_{2.5}$	IP	200	100	15	80	3.60
				50	15	53	6.12
				25	15	0	

Miscellaneous

WR	Structure	Route	LD_{50} mg/kg	Dose mg/kg	Minutes	Percent Survival	Index
33754		IP	43	32	30	60	2.15
					60	40	1.88
127016		IP	50	25	15	93	3.86
				12.5	15	67	6.68
				6.25	15	40	11.20
				3.12	15	7	
		Oral	400	200	30	33	2.66
					60	27	2.54
127017		IP	45	25	15	67 (toxicity)	3.01
				12.5	15	100	7.20
				6.25	15	27	9.15
				3.12	15	13	
		Oral	400	200	30	47 (toxicity)	2.94
					60	13 (toxicity)	
149997		IP	800	400	15	70 (toxicity)	3.40
				200	15	73	6.92
145725		IP	800	300	30	60 (toxicity)	4.26
				150	30	60	8.52
				75	30	13	
		Oral	875+	600	30	47	2.14
					60	86	2.72
				300	30	20	

V. Radiation Protection in Dogs

Chemical compounds that have shown significant radioprotection in mice at Walter Reed were selected for further testing in dogs. An acute toxicity study was performed with each compound prior to use in radiation testing. Radioprotection studies were generally conducted with the maximum tlerated dose of each compound.

Healthy, immature beagle dogs weighing approximately nine to twelve kilograms were exposed to a lethal dose of approximately 650 Rads of ionizing irradiation. Exposures were conducted at the Harry Diamond Ordance Reactor facility under the direction of Mr. Walter Giesler. Animals were exposed in the wood-lined exposure room adjacent to the reactor tank. Dogs were confined during irradiation periods in rectangular lucite cages arranged in a four cage array parallel to the gamma isodose curve produced by the reactor flux. The mid-line of the cages was 130 centimeters distant from the tank wall. With the reactor operating at 250 kilowatts power steady state, gamma dose rate was from 100 to 108 R per minute measured at the midline in air with a tissue equivalent ionization chamber. Neutron contribution to total dose was selectively reduced to 2% by maintaining a thickness of 70 centimeters of tank water between the core of the reactor and the exposure room. Of the 2% neutrons, energies were predominantly thermal.

Under the above conditions the radiation $LD_{50}/30$ was 432 R (Figure 1). Of twenty control dogs irradiated at 650 R during this fiscal year, none survived. The death peak (Figure 2) occurred at 10.0 to 11.0 days with a mean of 11.45 days.

The results of all the compounds tested in dogs in the fiscal year of 1970 are listed in Table I. A comparison to the results in mice is also listed. One combination study was conducted in fiscal year 1970. Results are shown in Table II.

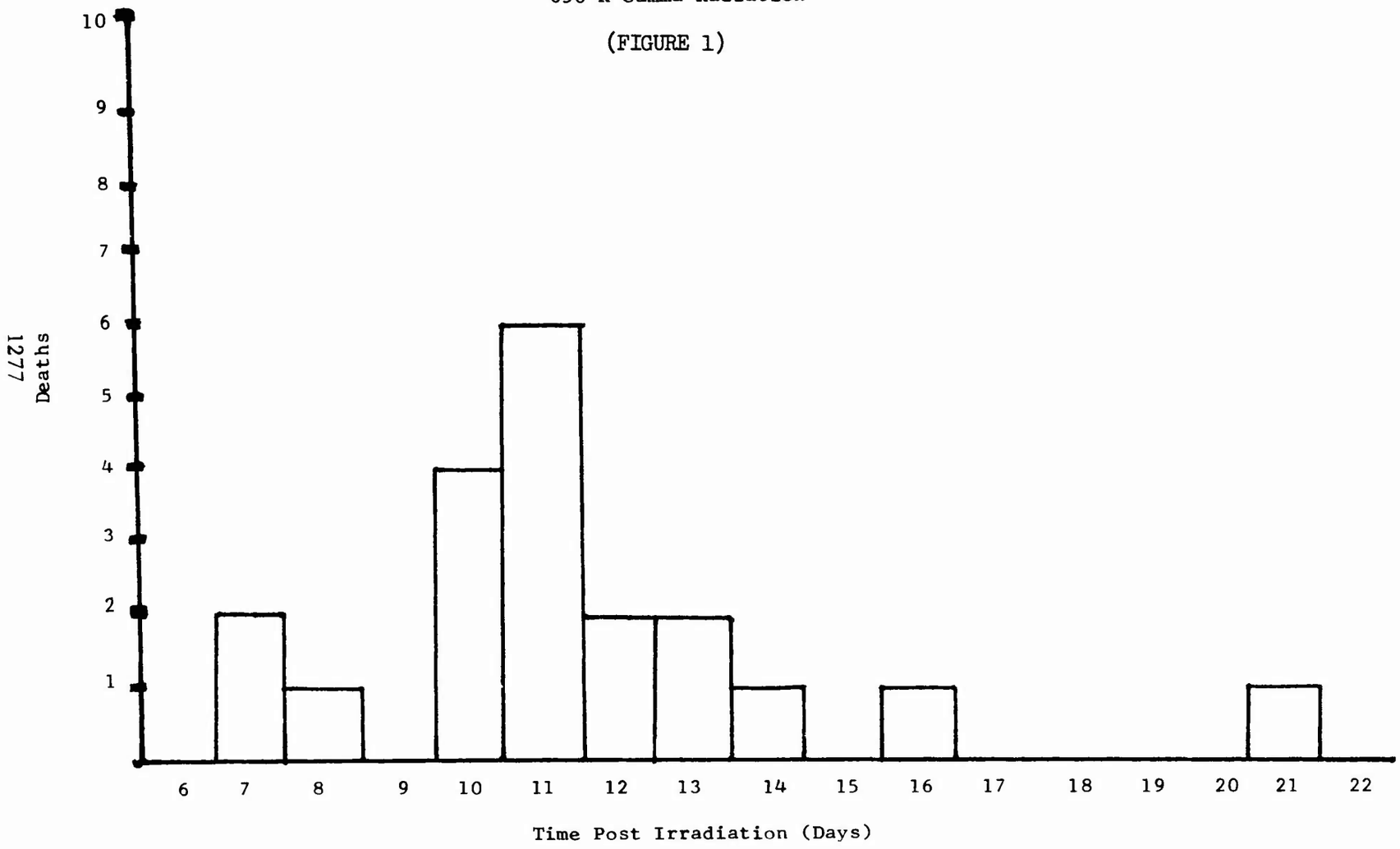
VI. Radiation Protection in Monkeys

Healthy Macaca mulatta (Rhesus) monkeys weighing from four to seven pounds were exposed to a lethal dose of approximately 800 Rads of ionizing radiation at the Harry Diamond Ordnance Laboratory Facility. At 250 kilowatts of steady state power with this particular experimental design, the total dose of radiation is delivered at about 95 R/min. Animals were exposed in leather and "Valcrow" harnesses secured to upright plexiglass boards. These harness allow for less handling and greater ease of drug administration. Since the monkey screen is relatively new, only two compounds ere tested in fiscal year 1970. Results are shown in Table III.

Control Mortality
Dogs

650 R Gamma Radiation

(FIGURE 1)



RADIATION LETHALITY STUDY
DORF REACTOR-GAMMA
DOGS

Dose Rate (Rads)	Animals Exposed	Animals Dead	% Mortality
292	12	2	17
356	12	4	33
432	12	6	50
518	8	6	75
650	20	20	100

(FIGURE 2)

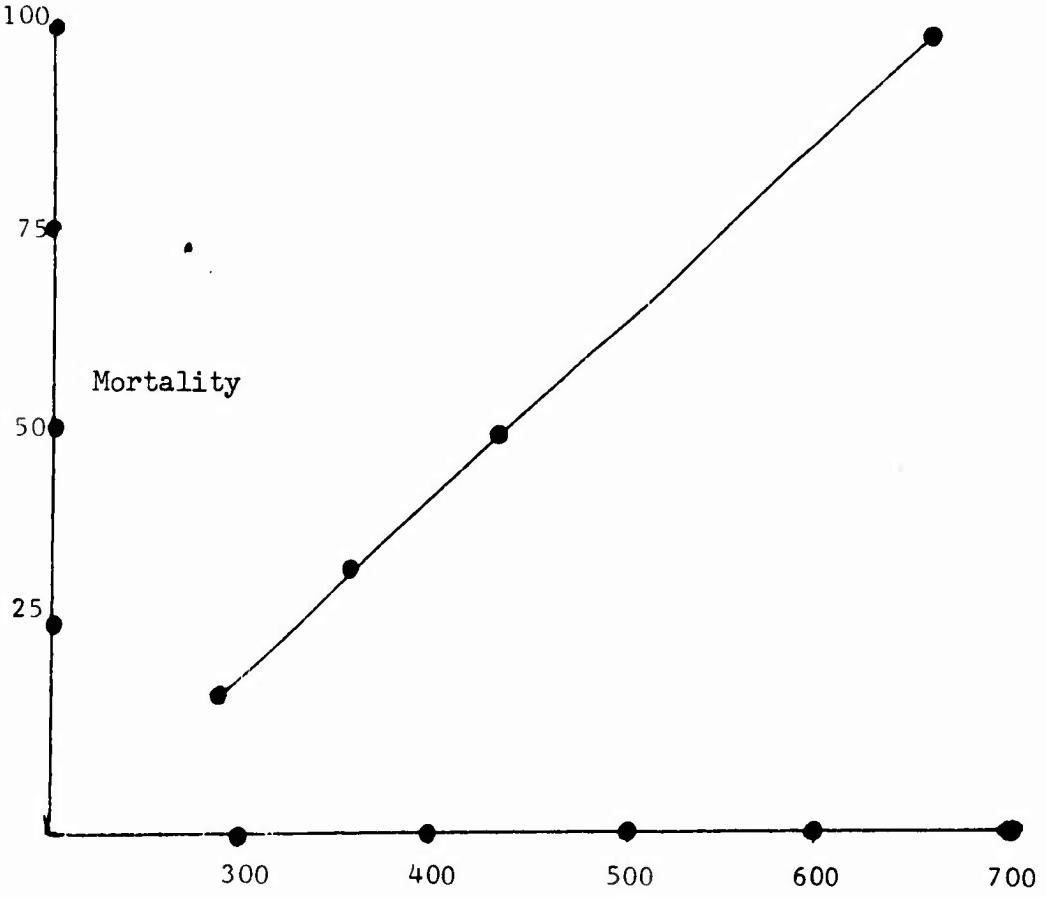


Table I

Dogs vs Mice Irradiation

Structure	WR No.	Test Animal	Test Dose mg/kg	Route	Minutes Pre-Irradiation	Percent Survival
NaO_3V	126	Dogs	5	IV	30	0% (0/9)
		Mice	12.5	IP	15	83%
			6.25	IP	15	67%
$H_2NCH_2CH_2CH_2NHCH_2CH_2SH$	1065	Dogs	60	IV	30	0% (0/9)
		Mice	370	IP	15	33%
			185	IP	15	40%
			92.5	IP	15	47%
			46	IP	15	33%
			23	IP	15	7%
			600	PO	15	79%
			600	PO	30	40%
$NH_2CH_2\underset{\displaystyle OH}{\underset{\vert}{C}}H-CH_2-SPO_3H_2$	77913	Dogs	680	IV	30	88% (6/7)
			720	IV	30	88% (8/9)
			720	IV	30	0% (0/3)
			720*	IV	30	75% (6/8)
		Mice	1000	IP	30	100%
			800	IP	30	100%
			600	IP	30	8%
			400	IP	30	100%
			300	IP	30	100%
			250	IP	30	93%
			150	IP	30	13%
			1500	PO	60	60%
			1500	PO	90	67%

*Only test in fiscal year 1970.

Table I (Continued)

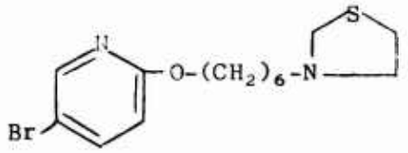
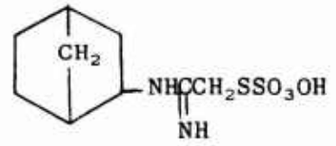
Structure	WR No.	Test Animal	Test Dose mg/kg	Route	Minutes Pre-Irradiation	Percent Survival
	91496	Dogs	35	IV	30	0% (0/9)
		Mice	150	IP	30	47%
			150	IP	60	13%
			100	IP	15	80%
			50	IP	15	0%
			300	PO	15	47%
			300	PO	30	67%
			150	PO	15	47%
			150	PO	30	60%
			75	PO	15	20%
			75	PO	30	27%
	113191	Dogs	25	IV	30	0%
		Mice	30	IP	30	80%
			30	IP	60	67%
			30	IP	90	33%
			15	IP	30	0%

Table II

Dog Irradiation - Combination Study

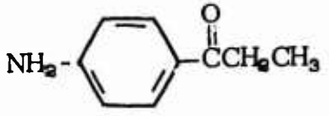
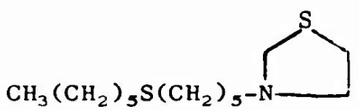
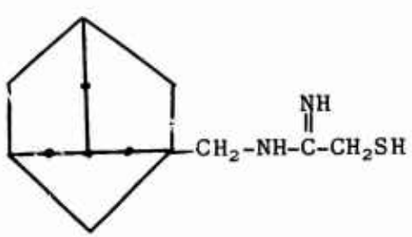
Structure	WR No.	Test Dose mg/kg	Route	Minutes Pre-Irradiation	Percent Survival
$H_2NCH_2CH_2SPO_3H_2$	638	300			
$CH_3(CH_2)_9NHCH_2CH_2SSO_3H$	1607	7.5	IV	30	78% (7/9)
 (PAPP)	302	5			

Table III

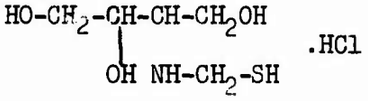
Monkeys vs Mice Irradiation

Structure	WR No.	Test Animal	Test Dose mg/kg	Route	Minutes Pre-Irradiation	Percent Survival
	134783	Monkeys	500	PO	60	
		Mice	250	PO	30	87%
			250	PO	60	80%
			125	PO	15	47%
			125	PO	30	87%
			63	PO	15	20%
			63	PO	30	20%
			30	IP	30	73%
			30	IP	60	53%
			30	IP	90	13%
			15	IP	30	0%
	109342	Monkeys	15	IV	30	17% (1/6)
		Mice	12	IP	15	93%
			12	IP	30	83%
			12	IP	60	73%
			12	IP	90	27%
			12	IP	120	0%
			6	IP	15	67%
			6	IP	90	0%
			3	IP	15	33%
			1.5	IP	15	0%
			30	PO	15	93%
			30	PO	30	93%
			30	PO	60	67%
			30	PO	90	33%
			15	PO	15	75%
			15	PO	30	92%
			7.5	PO	15	70%
			7.5	PO	30	0%

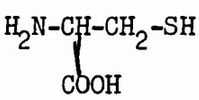
VII. Liver Perfusions

Hepatic neoplasia is generally a condition which is not amenable to resection. Breedis and Young (Am. J. Path. 30: 969-977, 1954) have demonstrated that malignant hepatic neoplasms derive their blood supply almost exclusively from the arterial side. The normal parenchyma, however, receives its blood supply primarily from the portal vein. The dichotomous circulation in the neoplastic liver should allow the selective perfusion of the liver. Nitrogen mustard introduced through the hepatic artery should affect primarily the neoplastic tissue. Administration of a nitrogen mustard antagonist through the portal vein should protect the normal liver. This study was conducted to test whether normal liver parenchyma could be protected from a supralethal dose of nitrogen mustard.

Nitrogen mustard in a dose of 2mg/Kg of body weight is an LD_{99}^{+}. This dose of nitrogen mustard was used in this study. A combination of two drugs were employed as a nitrogen mustard antagonist. This combination consisted of D,L-threo-3-2(2 mercaptoethyl) amino-1,2,4-butanetriol hydrochloride (WR 2347) at 300 mg/Kg and cysteine (WR 348) at 400 mg/Kg. This combination will be referred to subsequently as the WR drugs. The structures appear below.



D,L-threo-3-(2-mercaptoethyl) amino-1,2,4-butanetriol hydrochloride



The experimental subjects were mature, healthy, purebred Beagle dogs in the nine to twelve kilogram weight range. A series of ten dogs was employed. Following anesthesia, entry was made into the abdominal cavity and the portal vein, hepatic artery, gastroduodenal artery and vein were isolated. The gastroduodenal artery and vein were cannulated, the latter cannula extending into the portal vein. Umbilical tape was placed around the portal vein, caudal to the point where the venous cannula entered the portal, to facilitate future blockade. The femoral artery was isolated and a balloon catheter inserted to a point just anterior to the coeliac artery. Surgical preparation for the perfusion was complete at this point, with two patent catheters, one each in the gastroduodenal artery and the gastroducdenal vein; ligatures or clamps ready to provide temporary blockade of the hepatic artery, bile duct, and portal vein; and a balloon catheter ready to provide blockade anterior to the coeliac artery.

Following inflation of the femoral balloon catheter, blockade of the portal vein and the bile duct, perfusion of the liver was started with the WR drug combination via the gastroduodenal-portal vein route. One minute later the nitrogen mustard was started via the gastroduodenal hepatic artery route. Mustard administration was completed in ten to fifteen seconds. The WR drugs required about 5 minutes for complete administration. The liver was kept isolated for 10 minutes following the administration of nitrogen mustard to assure complete neutralization of the mustard before release to the general circulation. Following the perfusion, removal of catheters, restoration of the afferent hepatic circulation, and removal of the aortic block, routine closure was performed.

All ten dogs had an uneventful post operative recovery. The animals were sacrificed at 45, 60 and 90 days post surgery by random selection. Microscopic examination revealed the following results.

The primary insult appears to affect the gallbladder. The result was hemorrhage followed by fibrosis and eventual atrophy. Bladders in some cases were not apparent. Lobes of the liver adjacent to the bladder fossa were fibrotic with adhesions to stomach, intestines and adjacent lobes of the liver. Portal fibrosis and sclerosis in adjacent parenchyma was extensive by forty-five days. Bile accumulated in the area and there was mild proliferation of reticulo-endothelial cells in the portal triads. A definitive difference in hepatic change was difficult to ascertain, but there appears to be more bile in the tissues and more R.E. cell proliferation than normal. There were biliary duplication, numerous immature biliary elements was reduced suggesting a regression of changes induced by the drugs.

Other consistent findings included, tonsillitis, and cystic prostatitis. Small aggregates of mononuclear cells were seen in the lungs. Wherever suture material was encountered, there was a granulomatous response. The renal lymph node was consistently enlarged and dark. This node was not segregated during processing, but nodes were seen in which bile and hemosiderin lade macrophages were prominent, which indicates a cause for the enlarged nodes.

The results of this study indicate the feasibility of protecting the normal liver parenchyma from a supralethal dose of nitrogen mustard by the concomitant administration of nitrogen mustard antagonists. These results offer encouragement for drug therapy of hepatic neoplasia by permitting the administration of a dose level which is presently prohibitive due to toxicity.

VIII. Antiallergic Properties of Radioprotectants

In-vivo

The transfer of allergic antibody (reagin) from man to other primates has been shown to sensitize the skin of recipient primates[1]. Challenge with the appropriate antigen will produce a reaction in the sensitized animal. The allergic reaction can be localized for study purposes by injecting the reaginic sera intradermally to produce a wheal. Intravenous injection of antigen and Evans Blue dye produces a circumscribed blue area due to extra-vasation of dye at the sensitized site. Since reagin has been shown to be thiol sensitive, it should be sensitive to certain thiol-containing compounds. In this procedure 0.1 ml of reaginic sera from clinical patients was injected intradermally into healthy, Macaca mulatta monkeys at three sites per serum dilution. Undiluted serum plus four serial serum dilutions were used on each monkey. Drugh injections (I.V.) are given thirty minutes prior to challenge with antigen, (10-20,000 PNU/ml) plus 2 ml of 1% Evans Blue dye are used for challenging. Serum injection sites were checked thirty minutes after challenge for color changes. Drug effectiveness was based on a comparison of the reaction in drug-tested animals as compared to non-drug-tested controls. Preliminary studies indicated an absence of an allergic response with injections of WR 377 at 500 mg/Kg; WR 638 at 250 mg/Kg; WR 771 at 5 mg/Kg; WR 1616 at 350 mg/Kg; and WR 2347 at 450 mg/Kg. WR 2529 gave a slight diminution of the allergic reaction at 500 mg/Kg. Several studies have been conducted with WR 638 in order to parallel the Phase I clinical workup. Results at various dose levels have been erratic. Control response has also been variable and adjustments in procedure have been made to try to assure a more uniform response. Results using WR 638 are shown in Table IV.

1. Rose, Noel, R., John H. Kent, Robert E. Reissman, Carl E. Arbesman and Pierre Girard. Demonstration of Human Reagin in Monkey. J. Allergy 35, 520-534; 535-546, 1964.

2. Leddy, John P., Geraldine L. Freeman, Ascencion Luz and Richard H. Todd. Inactivation of the Skin-Sensitizing Antibodies of Human Allergy by Thiols. Proc. Soc. Exp. Biol. Med. 111, 7-12, 1962.

In-vitro

In order to more efficiently utilize the animals available for antiallergy drug screening an in-vitro screen has been developed. The procedure is similar to that described by Leddy et al[2]. Two drugs and a control were tested on each monkey. The drug-serum

Table IV

In Vivo Antiallergy - Monkey

Patient	Date	Antigen	Drug WR No.	Dose	Route of Administration	Serum Dilution	Reactivity * Treated	Control
G. L.	14 May 69	Timothy	638	125 mg/kg 2 days	IV	Undiluted	-	+++
						1:2	-	++
						1:5	-	++
						1:25	-	+
						1:125	-	-
B. G.	13 Jun 69	Alterneria (mold)	638	125 mg/kg 2 days	IV	Undiluted	-	++
						1:2	-	++
						1:5	-	+
						1:25	-	+
						1:125	-	-
?	2 Jul 69	Orc. Grass	638	125 mg/kg 2 days	IV	Undiluted	++ +	-
						1:2	+ +	-
						1:5	+ +	-
						1:25	- -	-
						1:125	- -	-

* Diameter of wheal (mm)
Negative -
Slight 1 - 5
+ 5 - 10
++ 10 - 15
+++ 15 - 25
++++ 25 - or >

Patient	Date	Antigen	Drug WR No.	Dose	Route of Administration	Serum Dilution	Reactivity Treated		Control
J. C.	24 Jul 69	Timothy	638	125 mg/kg 2 days	IV	Undiluted	+++		+++
						1:2	+++		+++
						1:5	+++		++
						1:25	++		-
						1:125	-		-
J. C.	1 Aug 69	Orc. Grass	638	125 mg/kg 2 days	IV	Undiluted	+++		-
						1:2	+++		-
						1:5	+++		-
						1:25	+		-
						1:125	-		-
R. V.	2 Sep 69	Ragweed	638	125 mg/kg 2 days	IV	Undiluted	+++	+++	- +++
						1:2	+++	+++	- ++
						1:5	-	+++	- -
						1:25	-	-	- -
						1:125	-	-	- -

Table IV - (Continued)

Patient	Date	Antigen	Drug WR No.	Dose	Route of Administration	Serum Dilution	Reactivity Treated	Reactivity Control
R. U.	2 Sep 69	Ragweed	638	200 mg/kg 2 days	IV	Undiluted	-	-
						1:2	-	-
						1:5	-	-
						1:25	-	-
						1:125	-	-
R. U.	2 Sep 69	Ragweed	638	250 mg/kg 1 day	IV	Undiluted	-	-
						1:2	-	-
						1:5	-	-
						1:25	-	-
						1:125	-	-

Table IV - (Continued)

Patient	Date	Antigen	Drug WR No.	Dose	Route of Administration	Serum Dilution	Reactivity Treated			Control		
S. P.	*10 Dec 69	Ragweed	638	225 mg/kg 1 day	IV	Undiluted	UR	UR	UR	UR	UR	UR
						1:2	UR	UR	UR	UR	UR	UR
						1:5	UR	UR	UR	UR	UR	UR
						1:25	UR	UR	UR	UR	UR	UR
						1:125	UR	UR	UR	UR	UR	UR

* 48 hours for skin binding - used only known reactors.

** UR = Unreadable due to massive reaction.

Table IV - (Continued)

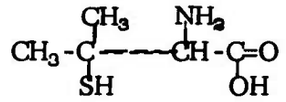
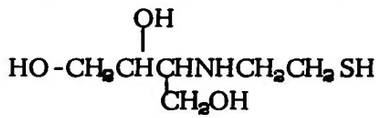
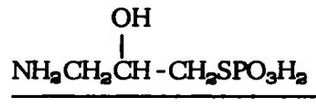
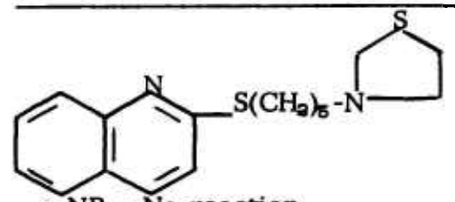
Patient	Date	Antigen	Drug WR No.	Dose	Route of Administration	Serum Dilution	Reactivity Treated			Reactivity Control		
S. P.	15 Jan 70	* Ragweed	638	225 mg/kg 1 day	IV	1:2	+++	-	+++	-	-	+++
						1:10	++	-	+++	-	-	+++
						1:50	-	-	-	-	-	-
						1:250	+	-	+	-	-	++
						1:750	-	-	-	-	-	-

* Antigen diluted from 20,000 PNU/CC to 5000 PNU.

Table IV - (Continued)

Table V

In Vitro Antiallergy Study

Structure	WR No.	Wheal Diameter (mm) Injection Site Treated 1	Treated 2	Control 1	Control 2
	377	NR*	NR	20	17
	2347	16	19	27	24
	77913	Slight **	NR	16	25
$NH_2(CH_2)_5NHCH_2CH_2SH$	1729	16	15	20	17
$NH_2(CH_2)_5NHCH_2CH_2SPO_3H_2$	2823	18	22	20	26
$NH_2-(CH_2)_3-NH-CH_2CH_2SPO_3H_2$	2721	Slight	Slight	27	24
	91488	NR	NR	15	25

* NR = No reaction

** Slight - barely visible

mixture and control serum was injected intradermally in the abdominal area. The antibody was challenged twenty-four hours after injection with an intravenous injection of appropriate antigen plus 1% Evans Blue dye. Activity of the test compound was based on a comparison of wheal size of drug treated serum as compared to that of the control serum. Eight compounds were tested in the one experiment conducted in fiscal year 1970. The results are shown in Table V.

IX. Evaluation of Radiation Compounds as Possible Therapeutics for Wilson's Disease

Wilson's disease, or hepatolenticular degeneration, was orginally described by Wilson in 1912 as a "familial nervous disorder associated with cirrhosis of the liver." Currently the disease is best defined as an inter-relationship between an autosomal recessive inherited defect causing abnormalities in the metabolism of copper. There is a significant increase in the serum copper (bound loosely to albumin) but a deficiency of serum ceruloplasm, a copper protein with weak oxidase activity. The excess copper is deposited in such tissues as the brain, liver, cornea, kidney, and skin. The disturbances of copper metabolism are summarized in Table 1. Clinically, 76% of all disgnosed cases are neurological in complaint, the remainder chiefly involving the liver. A diagnosis can be made with certainty if greenish-brown bands (Kayser-Fleischer rings) can be seen around the limbus of the cornea.

In 1964 Porter compared the distribution of copper in subcellular fractions from Wilson's Disease liver, normal adult liver, and human neonatal liver. Porter found the principal subcellular site of liver copper deposition to be the mitochondrial fraction, with a distinct similarity in the distribution of copper in newborns to that of Wilson's disease. Other researchers such as Evans have found the hepatic-mitochondrial copper distribution and serum ceruloplasm in neonates (rat, bovine) to coincide with that of a Wilson's disease liver. In 1956, Walshe introduced the sulfhydral-containing copper-chelating agent penicillamine as a treatment for Wilson's disease. Penicillamine has an affinity for copper, causing mobilization and excretion of the bound-copper. However, adverse effects have been noted, such as: leukopenia, thrombocytopenia, vitamin B_6 deficiencies and lathyrogenic effects resulting from disordered collagen formation. Possibly other sulfydral compounds may prove to be less toxic and have a greater affinity to chelate copper.

The xperimental animals were rat neonates ranging from 12-48 hours of age (approximately 5-6 litters/experiment). The neonates were anesthetized and then sacrificed. Livers were removed, weighed and

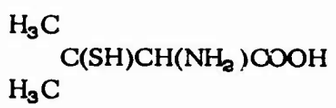
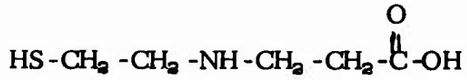
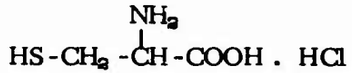
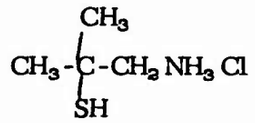
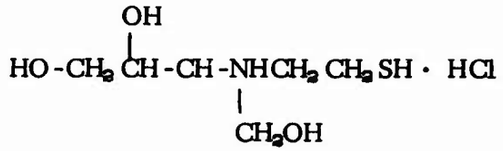
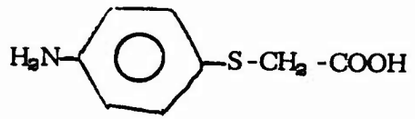
suspended in .25M sucrose. The livers were homogenized and differentially centrifuged to produce a mitochondrial fraction. Equal volumes of D-penicillamine or other test compounds and mitochondria were incubated at 37 degrees C for one hour. A control combined .23M sucrose instead of the drug. After incubation, the samples were centrifuged at 27,000 xg for five minutes. The supernatants were analyzed for copper by atomic absorption spectrophotometry.

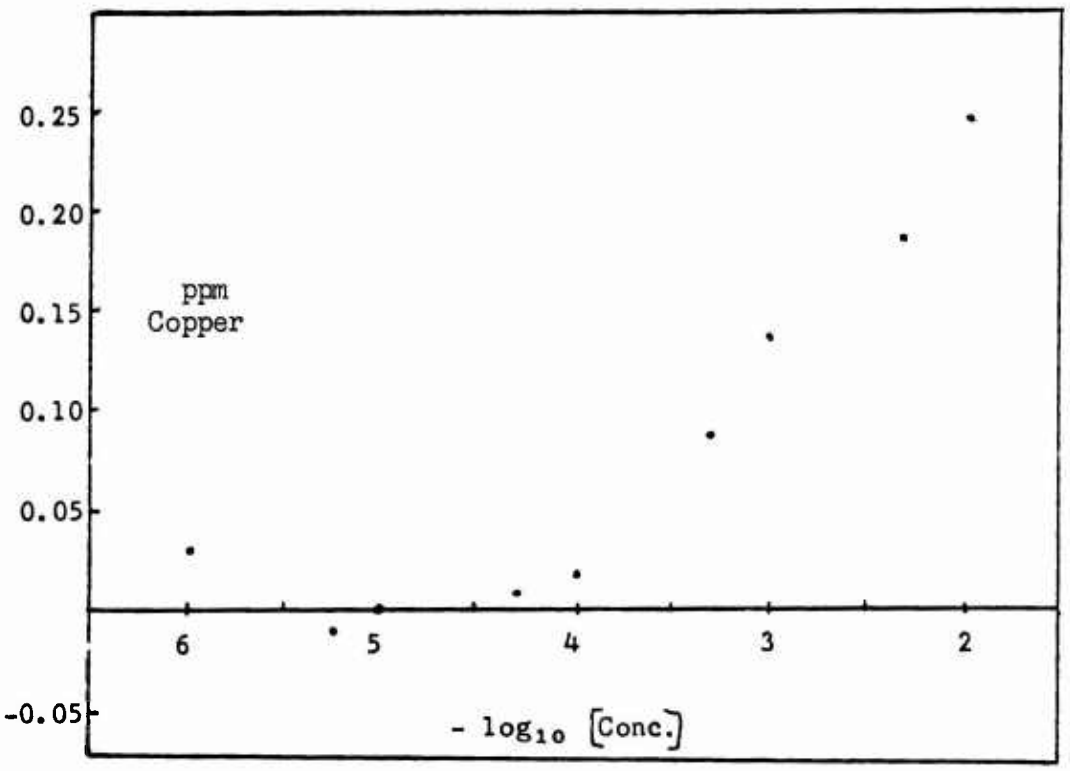
D-penicillamine was effective in removing the bound-copper found within neonatal mitochondria. Several concentrations of D-pennicillamine, 10^{-3}, 10^{-4}, and 10^{-6} M were tested at different time intervals: 0, 10, 20, 30, 45, 60, 90, 120, and 180 minutes. Results showed that at 60 minutes the majority of the chelatable copper was removed. On this basis, a 60 minute incubation was established. At the concentrations of 10^{-2} - 10^{-4} M of D-penicillamine a linear relationship was established between the corresponding amount of copper removed and the logarithm of drug concentration (see Graph 1). In the range of 10^{-4} - 10^{-6} M no marked difference in ability to chelate copper was seen. Thus the concentrations of 10^{-2} - 10^{-4} M were used in studying other sulfhydral compounds. Other compounds tested to date are: N/β mercaptoethyl-β-alanine (WR 2346); L-cysteine hydrochloride (WR 348); dl-thero-3-2-mercaptoethyl-amino 1,2,4 butanetriol HCl (WR 2347); para-aminothiophenoxyacetic acid (WR 15206). Their ability to chelate copper in relation to their corresponding chemical structure, as compared with D-penicillamine is interesting (see Graphs 2-4). Presently, other sulfhydral compounds are being tested in order to obtain more conclusive results on the possible binding mechanism of D-penicillamine to copper and in hopes of discovering a more effective chelator of copper for Wilson's disease patients.

Table 1 - Copper in Wilson's Disease

1. ↓ Total Serum Copper
2. ↑ "Free" or Bound Copper
3. ↓ Ceruloplasm in Serum
4. ↑ Absorption of Copper from G.I. Tract, Decreased G.I. Excretion of Copper, or both.
5. ↑ Urinary Output of Copper
6. ↑ Copper in Tissues - Brain, Liver, Cornea, Kidney and Skin
7. Delay in Clearance of Plasma Copper by Liver

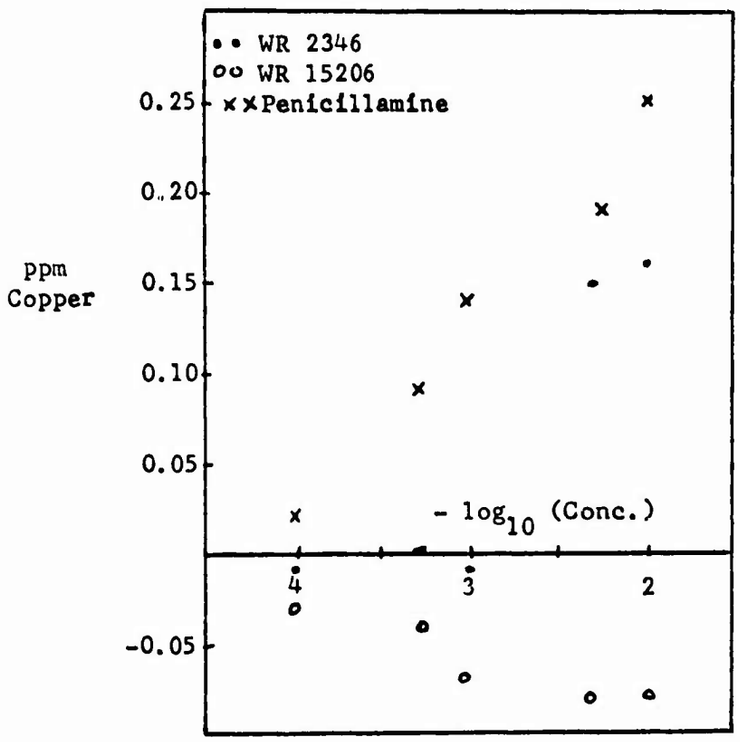
Table 2

WR 377		D-Penicillamine
WR 2346		N-/β-Mercaptoethyl-β-Alanine
WR 348		L-Cysteine Hydrochloride
WR 1553		2-Mercapto-2-Methylamino-Propane HCl
WR 2347		dl-Threo-3-(2-Mercaptoethyl) Amino-1, 2, 4-Butanetrial HCl
WR 15206		Para-Aminothiophenoxyacetic Acid



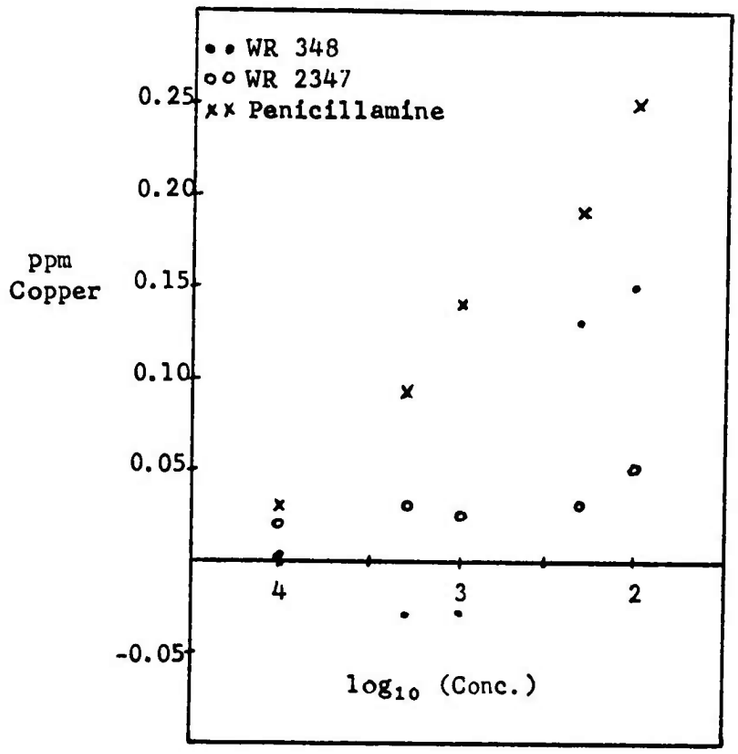
Graph 1

Copper Removed From Neonatal Rat Liver Mitochondria As A Function of Penicillamine Concentration.



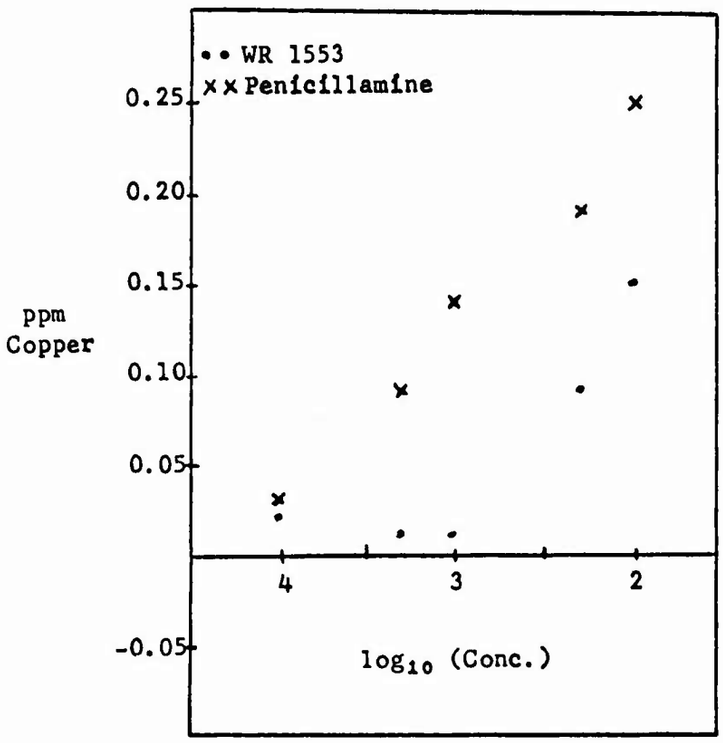
Graph 2

Copper Removal From Neonatal Rat Liver Mitochondria as a Function of Drug Concentration.



Graph 3

Copper Removal From Neonatal Rat Liver Mitochondrial As A Function of Drug Concentration.



Graph 4

Copper Removal From Neonatal Rat Liver Mitochondria As A Function of Drug Concentration.

X. Evaluation of Pit Viper Antivenom Against C. rhodostomo in Albino Mice

The purpose of this investigation was to assay the potency and specificity of a monvalent antisnake venom serum prepared by American Field Service, Thailand against C. rhodostoma. The ability of this antivenom to neutralize lethal effects of pit viper venom was determined.

Materials and Methods

C. rhodostoma venom (lot # CR 1168) was obtained from the American Venom Labs, Jungle Land, Thurmont, Maryland, in a dried lyopnilized state. Prior to use the venom was dissolved in physiologic saline. Freshly prepared solutions of venom were used for each day's testing. The lethal intravenous dose of venom was established in groups of ten mice. All injections were made into tail vein.

The toxicity LD_{50} of the venom was first established by injecting varying doses into tail veins. The relative potency of the antivenom was determined by injecting mice intravenously with lethal doses of venom, followed by a second intravenous injection of .1 ml of the antivenom into same tail vein.

Survivors were those animals alive at twenty-four hours.

Results

The results of these studies are summarized in Tables 1, 2 and 3.

Table 1 #3 shows the effect of graded doses of C. rhodostoma on both percent mortality and survival times in mice. The toxicity LD_{50} as determined by transforming data to log-probit scale and using Miller Tainter method of analysis is shown to be 120 ± 9.2 µg. The ability of pit viper antivenom to protect mice against lethal doses of venom is shown in Table 2. The relative potency of the monovalent antivenom (pit viper) is evaluated as 159%. When the results are plotted (Fig. 1) the LD_{50} of the antivenom-treated mice is shown to be 191 ± 21µ g. In keeping with units established by other investigators, 1 ml of the reconstituted antivenom will effectively neutralize not more than 1.6µg C. rhodostoma venom.

Discussion

Data from these studies indicates that 160µg of C. rhodostoma venom will effectively kill 80-100% of all mice given a single intravenous injection.

Time to death following venom decreases with an increase in the amount injected. These data are consistent with results obtained using other venoms.

Since data on relative potency of this antivenom are lacking, the neutralization capacity was effective since it increased the toxicity LD_{50} of venom by a factor of 1.6.

References

1. Vick, James and Marie Grenan, Evaluation of Antivenoms Against Naja naja and Bungarus Caeruleus Snake Venoms. (To be published)

2. Vick, J. A., C. R. Roberts and M. H. Heiffer. Pharmacological Effects of Lethal Doses of Snake Venoms. Fed. Proc. 27: 707, 1968.

3. Miller, L. C. and M. L. Tainter. Estimation of the ED_{50} and its Error by Logarithmic-Probit Graph Paper. Proc. Soc. Exp. Biol. and Med. 57: 261-264, 1944.

Table 1. Effect on Mortality of Varying Concentrations of Venom.

# Mice	(μg) Venom	(ml) Venom	# Dead	% Mort	S. T. (Min.)
10	100	.50	3	30	18' - 33'
10	100	.20	4	40	38' - 174'
9	120	.30	3	33	20' (2 overnite)
9	120	.20	5	55	25' - 175'
10	140	.35	9	90	10' - 162 ' (1 overnite)
5	140	.20	3	60	13' - 54' (1 overnite)
10	160	.40	10	100	3' - 47'
5	160	.20	4	80	15' - 35' (1 overnite)
10	200	.50	10	100	4' - 70'
5	200	.20	5	100	20' - 30' (2 overnite)

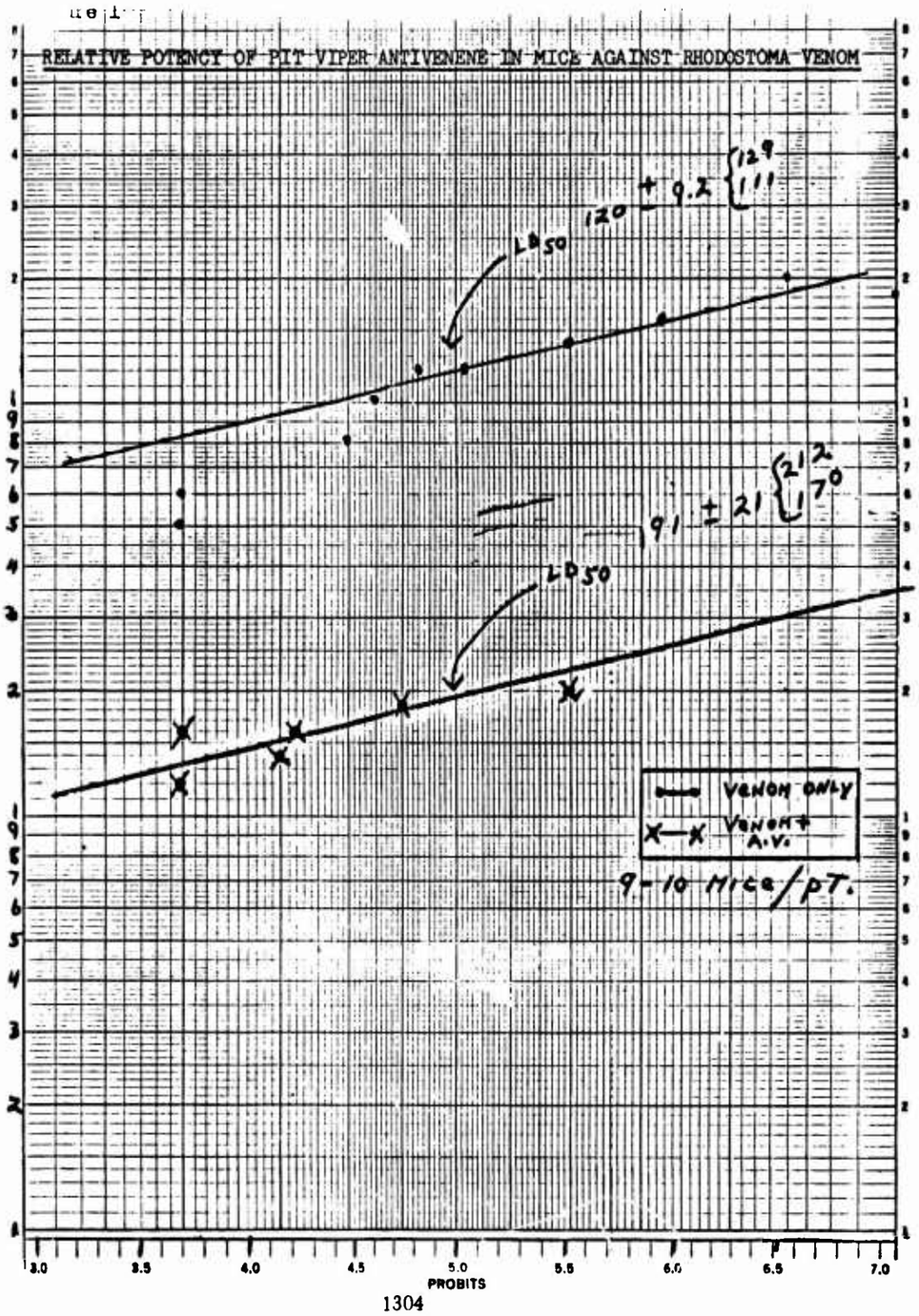
Table 2. Effect of Pit Viper Antivenom on Reversal of Lethality of Rhodostoma Venom in White Mice.
(I. V. Injections)

# Mice	(μg) Venom	(ml) Venom	(ml) Ant. V.	# Dead	% Mort.	S. T. (Min.)
6			.1	0	0	
9	120	.30	-	3	33	20' (2 overnite)
9	120	.20	-	5	55	25' - 175'
10	120	.20	.1	1	10	(Overnite)
10	140	.35	-	8	80	10' - 162'
5	140	.20	-	3	60	13' - 54'
10	140	.20	.1	1	10	(overnite)
6	150	.25	-	4	67	18' (2 overnite)
9	150	.25	.1	2	22	58' - 135'
10	160	.40	-	10	100	3' - 47'
5	160	.20	-	4	80	15' - 35' (1 overnite)
10	160	.20	.1	1	10	(overnite)
10	180	.30	-	10	100	3' - 77' (1 overnite)
10	180	.30	.1	4	40	20' - 30' (1 overnite)
10	200	.5	-	10	100	4' - 70'
5	200	.2	-	5	100	20' - 30' (2 overnite)
10	200	.2	.1	7	70	24' - 67'

[1 ml will neutralize not more than 1.6 mg venom]

Table 3. Summary of Results of Experiments on Lethality of Rhodostoma Venom in Mice. (I. V. Injection)

# Mice	(μg) Venom	(ml) Venom	# Dead	% Mort.	S. T. (Min.)
10	40	.4	0	0	0
10	50	.5	1	10	15'
10	60	.3	1	10	72'
10	80	.4	3	30	18' - 51'
10	100	.5	3	30	18' - 33'
10	100	.2	4	40	38' - 174'
9	120	.3	3	33	20' (2 overnite)
9	120	.2	5	55	25' - 175
10	140	.35	9	90	10' - 162'
5	140	.20	3	60	13' - 54' (1 overnite)
6	150	.25	4	67	18' (2 overnite)
10	160	.40	10	100	3' - 47'
9	160	.40	8	88	8' - 30'
5	160	.20	4	80	15' - 35' (1 overnite)
10	180	.30	10	100	3' - 109'
10	200	.50	10	100	4' - 70'
6	200	.50	5	83	13' - 19' (1 overnite)
5	200	.20	5	100	20' - 30' (2 overnite)
10	240	.40	10	100	5' - 35'


RELATIVE POTENCY OF PIT VIPER ANTIVENENE IN MICE AGAINST RHODOSTOMA VENOM
LD50
120 ± 9.2 {129 / 111
LD50
91 ± 21 {212 / 70
VENOM ONLY
X—X VENOM + A.V.
9-10 Mice/pt.
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
PROBITS

XI. Pharmacology of Radioprotectants

Experiments were conducted to define descriptively the general pharmacology of antiradiation drugs developed under this program. Data from these studies continue to illustrate that there is no overlying single pharmacological action of these agents and that each antiradiation drug is unique with respect to its own pharmacological properties. Considerable effort has been expended in the exploitation of WR 2823, WR 1729, and WR 149024, with particular emphasis on the application of these drugs to the potential therapeutic efficacy in the treatment of incidental injuries incurred on the nuclear battlefield. These agents represent a series of compounds having antiradiation actions which also possess remarkable properties to prevent or to reverse mortality and other pathophysiology related to nuclear battlefield injuries. Specifically, WR 2823 is being developed for Phase I and Phase II testing in man as a potential drug for the treatment of hemorrhagic shock. WR 2823 and WR 149024 have been shown to be effective in preventing pathophysiology of lethal endotoxin injections in the dog. WR 149024, WR 2823, WR 2721 and WR 33278 are effective in preventing death due to traumatic injury (Noble Collip Drum Trauma) or endotoxin injection in mice. WR 149024 and WR 2823 are effective in preventing death and acute cardiovascular changes due to anaphylaxis in mice. WR 149024 and WR 2823 are effective in preventing acute hypotension, elevated blood histamine level, and death from intravenous injections of endotoxin in dogs. WR 2823 AB has been formulated for eventual use in man. Stability tests are under way, sterility and pyrogen testing have been completed. The formulated material has been proven for efficacy and safety in animals. Subacute toxicity studies are under way, preliminary to beginning Phase I testing in man. We have not been able to demonstrate that WR 2823, WR 1729, or WR 149024 can prevent mortality due to tourniquet injury in mice. WR 2823 acts to prevent the decrease in cholonic temperature in mice subjected to Noble Collip Drum Trauma. WR 2823 could not be shown to have a significant effect on blood sugar levels in mice not subjected to Noble Collip Drum Trauma; however, WR 2823 does prevent blood glucose rise following Noble Collip Drum Trauma. WR 2823 has no effect on hematocrit in mice. WR 2823 does not have an addative effect to the therapeutic properties of blood reinfusion in monkeys subjected to irreversible hemorrhagic shock. A thin layer chromatographic procedure has been developed for the study of the diaminothios and their derivatives. The metabolism and excretion patterns of WR 109342 are being studied to evaluate the possible pharmackinetic mechanism involved. WR 2823 has been shown to be a selective alpha adrenergic blocking drug in the femoral vascular bed.

XII. Summary and Conclusions

Emphasis in the contract synthesis program has been on the latentiation of the thiols. Excellent oral activity has been obtained in certain of the 3-pyridinyloxyalkyl thiazolidines in mice. Five compounds were carried through to dog studies and two to monkey studies during the year. On compound, WR 97913, gave good protection to the dog; it was not tested in monkeys. Several agents, in addition to their antiradiation properties, have assumed increasing importance from the standpoint of preventing death due to hemorrhagic, traumatic, or endotoxin shock. These are scheduled to be studied in the clinic shorty. Studies of ancillary benefits which may accrue from the use of these agents, such as the possibility of use in Wilson's Disease, arthritis, and as an antiallergy drug, have received only modest attention.

Project 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 055, Chemical protection against irradiation

Publications

Vick, J. A., W. Shipman and R. Mundy, Pharmacological Studies of the Major Fractions of Bee Venom. Fed. Proc. 29: 778, 1970.

Vick, J. A., Pathophysiology and Treatment of Endotoxin and Exotoxin Shock in the Primate. "Use of Non-Human Primates in Drug Evaluation." University of Texas Press. Chapter IV, 1969.

Vick, J. A., M. H. Heiffer and D. Jacobus, Treatment of Hemorrhagic Shock with WR 2823, a New Alpha Adrenergic Blocking Agent. Physiologist. 12: 383, 1969.

Heiffer, M. H., E. H. Herman, J. A. Vick, G. E. Demaree, R. L. Mundy and D. G. Reynolds, On the Alpha-Adrenergic Blocking Properties of WR 2823. Fed. Proc. 28: 611, 1969.

Vick, J. A., and J. Lipp, Effect of Cobra and Rattlesnake Venoms on the Central Nervous System of the Primate. Toxicon. 8: 33, 1970.

Slotta, K. H., and J. A. Vick, Identification of the Direct Lytic Factor from Cobra Venom as Cardiotoxin. Toxicon. 6: 167, 1969.

Harris, L. W. and J. A. Vick, Effects of 2-Pyridinium Aldoxine Methochloride and Atropine in Relation to Elevation of Blood pH in Soman-Poisoned Dogs. Biochem. Pharm. 18: 427, 1969.

Harris, L. W. and J. A. Vick, Effects of Intravenous Perfusion of Tris Buffer Amino Methone on Dogs Poisoned with Soman and Treated With Atropine and PAM. Biochem. Pharm. 18: 409, 1969.

Vick, J. A., Pathophysiological Studies of Ten Poisonous Snake Venoms."Animal Toxins." Pergamon Press, 1969.

Demaree, G. E., J. S. Frost, and M. H. Heiffer. The Effect of 1,18-Diamino-6,13-Diaza-9,10-Dithiaoctadecane Tetrakis Hydrochloride (WR 149024) on the Response to Ovalbumin in Sensitized Mice. Fed. Proc. 29: 420, 1970.

Klayman, D. L., M. M. Grenan, and D. P. Jacobus. Potential Anti-radiation Agents. II. Guanidinoalkanethiosulfuric Acids. J. Med Chem. 12: 723, 1969.

Klayman, D. L., M. M. Grenan, and D. P. Jacobus. Potential Anti-radiation Agents. III. N-Substituted Aminoethanethiosulfuric Acids. J. Med Chem. 13: 251, 1970.

Klayman, D. L., and R. J. Shine. A New Synthesis of Selenoureas and Selenothiocarbamic Esters from Thioureas. J. Org. Chem. 34: 3549, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6472	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	H. Term	U	U	NA	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A824	00	056
b. CONTRIBUTING				
c. [redacted]	CDOG 1212B(21)			

11. TITLE (Procode with Security Classification Code)

(U) Protective Effect of Aminothiols against Ionizing and Neutron Radiation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

014100 Radiobiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 03	Cont	DA	C. In-House

17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	8	240
FISCAL YEAR CURRENT 70	5	150

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Biochemistry
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Angel, LTC C. R.
TELEPHONE: 202-576-2211
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Copeland, E. S. Ph.D.
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procode EACH with Security Classification Code)

(U) Radiation Protective Agents; (U) Drug Tolerance; (U) Chemoprophylaxis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procode text of each with Security Classification Code.)

23. (U) The definition and quantitation of chemical, biochemical and physiological changes occurring after administration of chemical compounds that show reduction in mammalian mortality after exposure to ionizing and neutron radiation.

24. (U) Basic biochemical mechanisms of radioprotective drug action at the cellular, organ and whole body levels are being explored in bacteria, mammalian cell cultures, and small mammals. An integrated multidisciplined effort involves chemistry, biochemistry, physiology and pharmacology leading to definition of the mechanism of action of aminothiols in cellular and mammalian systems. Radioisotopic techniques for distribution, retention and excretion of radiation modifiers are employed where applicable.

25. (U) 69 07 - 70 06 Structure-function studies of high purity aminothiol radioprotectors continue. Following the inactivation of the Division of Nuclear Medicine, on 31 Dec 69, it is expected that the structure-function studies will be consolidated within the program of the Division of Biochemistry and into work unit 070 and will be reported under DA 0A 6430. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1498 (1 MAR 68) PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BLANK PAGE

Project 3A062110A824, IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00, Ionizing Radiation Injury, Prevention and Treatment

Work Unit 056, Protective effect of aminothiols against ionizing and neutron radiation

Investigators.
Principal: LTC C. R. Angel, MSC
Associate: E. S. Copeland, Ph.D,; A. Einheber, Ph.D.; CPT R. J. Jandacek, MSC; R. T. Lofberg, Ph.D.; E. C. Richardson, M.S.; LTC H. M. Swartz, MC; R. E. Wren, B.S.

Description.

The overall objectives of this work unit are (1) to determine the mechanism of radioprotective action of aminothiols through performance of studies utilizing a broad variety of methods; (2) to study absorption, fate, excretion, and duration of effect of selected compounds with a view toward their possible future use in humans; (3) to measure the effects of treatment with these drugs, either alone or in combination with radiation, on animal response to other types of trauma; and (4) to utilize the available drugs of this class to explore the basic mechanisms by which ionizing radiation damages biological systems.

1. Animal studies.

As previously reported (WRAIR Annual Report 1968-69), WR 2823B at a level of 50 mg/kg body weight protects mice against Noble-Collip Drum Trauma (NCDT) when given intraperitoneally, 15 minutes prior to the trauma. Since WR 2823B is a chemical compound that forms equilibrium mixtures of disulfide and thiol functional groupings, a study involving 700 mice was instituted to determine whether different lots of this compound differed in protective efficacy and to determine what relative proportion of disulfide thiol complex was related to the protective effect against shock. Three different lots, B (laboratory use), AB (formulated for human testing), and AC (preformulation material) were tested in the mouse model system. All three were high in reducing mortality and no statistical significance could be demonstrated between the lots examined.

2. Structure-function studies in bacteria.

In previous WRAIR Annual Reports studies were reported which established a close relationship between radioprotection and the free radical production of ionizing radiation. These studies have been designed to examine in detail the relationship between chemical structure and radioprotective activity. A number of compounds have been synthesized and are currently used in these studies.

These studies were extended to the 3, 4, 5 and 6 carbon analogs of MEA and preliminary data on the effect of these compounds on both biological and free radical parameters determined. These data, while most interesting and of considerable potential importance in the understanding of radiation protection and the development of a clinically effective radioprotector, is of a preliminary nature. Because of the termination of this project confirmatory experiments cannot be accomplished at the WRAIR and considerable caution should be exercised in the interpretation of these data.

Each compound was prepared in lots of 40-50 grams and rigorously purified. The physical constants of the C4 did not agree with literature values but its identity was confirmed by elemental analysis, functional analysis and NMR studies. It is probable that the single literature value is in error and this compound may not have been adequately prepared previously.

Excellent protection, irrespective of carbon chain length from 2-6 was found in bacteria irradiated at 1°C in either oxygen or nitrogen. Protection decreased monotonically with chain length when the irradiations were carried out at -196°C in oxygen or nitrogen but the C6 analogs still offered considerable protection. In nitrogen equilibrated samples the ESR results indicated that the longer the chain, the less effective the compounds were in reducing the number of radicals on bacteria. In oxygen, an additional effect was found, in which the longer chain analogs showed an increased amount of energy stabilization on the compounds.

These results indicate that the mode of protection by these compounds at -196°C is probably via elimination of radiation-induced direct effects without necessarily transferring the energy to the compounds. Energy transfer did not enhance protection. At 1°C a second mechanism of protection appeared to predominate and this mechanism was independent of the distance between the nitrogen and sulfur atoms.

X-ray crystallography studies of these compounds indicated that the relationship between the N and S atoms in the 2 and 3 carbon analogs was quite different, with considerable interaction between them in the 2 carbon but little or none in the 3 carbon compound. It is not clear if these conformational differences extend to physiological conditions.

Summary and Conclusions.

Different lots of the shock drug WR 2823 were examined in the mouse model system for their relative protective efficacy in reducing mortality due to drum trauma. In the three lots of compound compared, no significant difference between lots were found.

The studies with the 2-6 carbon analogs of MEA suggest that the efficacy of the aminothiols does not halt abruptly at 3 carbons and an

important group of clinically protective agents may have been overlooked. The mechanism of radioprotection of these compounds does not appear to be energy transfer but rather a catalytic type of effect resulting in a decrease in the number of radiation-induced radicals left on target molecules.

Project 3A062110A824, IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00, Ionizing Radiation Injury, Prevention and Treatment

Work Unit 056, Protective effect of aminothiols against ionizing and neutron radiation

Literature Cited.

1. Copeland, E. S. and Swartz, H. M.: Radical formation in cysteamine-HCl gamma-irradiated in the dry state and in frozen aqueous solution. Intern. J. Radiat. Biol. 16: 296-300, 1969.

2. Einheber, A., Wren, R. E. and Klobukowski, C. J.: The influence of certain radioprotective chemicals on the mouse's response to tourniquet injury. J. Trauma 10: 322-330, 1970.

3. Einheber, A., Wren, R. E. and Dobek, A. S.: Mortality, morphologic changes, and saline therapy after scald injury of germfree mice and Pseudomonas-free conventionalized mice, with or without Proteus mirabilis: An inquiry into a possible noninfective role of the microbial flora. J. Trauma 10: 135-152, 1970.

4. Einheber, A., Wren, R. E. and Klobukowski, C. J.: Interference of hepatic drug metabolism in Plasmodium berghei-infected mice and its therapeutic modification: A study of hexobarbital sleeping time and phenobarbital-induced liver stimulation. Exp. Parasit. 27: June 1970.

5. Richardson, E. C. and Swartz, H. M.: Effects of aminothiols on survival and radiation induced free radicals in E. coli B/r. Bact. Proc. 65, 1969.

6. Swartz, H. M., Copeland, E. S. and Dingman, W. D.: Characterization of the free radicals induced by gamma irradiation of E. coli. Radiat. Res. May 1970.

PROJECT 3A663713D829
MALARIA PROPHYLAXIS

Task 00
Malaria Investigations

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6517	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	106
b. CONTRIBUTING				
c. ~~XXXXXXXXXX~~ DOG	1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Antigenic Fractionation, Serology of Malaria (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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 NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 70	3	100
CURRENT FISCAL YEAR 71	3	100

19a. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: MERONEY, COL W. H.

TELEPHONE: 202-576-3551

19b. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of CD&I

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: SADUN, E. H., Sc.D.

TELEPHONE: 202-576-3308

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P. DA

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Plasmodium; (U) Immunity; (U) Erythrophagocytosis; (U) Autoimmunity; (U) Diagnosis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To isolate and purify various protein antigens from plasmodia. To characterize these substances immunochemically, to relate immunochemical characteristics to biologic activities, such as protective immunity, diagnostic specificity, cross reactions with normal host tissue components, etc.

24(U) Separate parasite proteins by physical and chemical means. Determine the presence and activity of metabolic antigens in the plasma of acutely infected animals and human patients. Analyze the fractionated proteins by both classical and new methods.

25(U) 69 07 - 70 06 Some aspects of the relative role of antibodies and cell mediated response can be uncovered by using antilymphocyte globulin and malarial antiserum. In experiments with P. berghei in rats animals treated with antilymphocyte globulin and normal serum had notably higher parasitemias and mortality rates than those treated with antilymphocyte globulin and P. berghei antiserum. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

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

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 106, Antigenic fractionation, serology of malaria

Investigators.
Principal: Elvio H. Sadun, Sc.D., Lib. Doc.
Associate: W. H. Hildreth, MAJ S. H. Lourie, MC; SP5 R. B. Tomlinson

Some aspects of the relative role of antibodies and cell mediated response can be uncovered by using antilymphocyte globulin and malarial antiserum. In experiments with P. berghei in rats, animals treated with antilymphocyte globulin and normal serum had notably higher parasitemias and mortality rates than those treated with antilymphocyte globulin and P. berghei antiserum.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 106, Antigenic fractionation, serology of malaria

Literature Cited

1. References

None

2. Publications

Sadun, E. H., Gore, R. W., Wellde, B. T. and Clyde, D. F.: Malarial antibodies in human volunteers. A comparison of the soluble antigen fluorescent antibody (SAFA) and indirect hemagglutination (IHA) tests using as antigen Plasmodium falciparum parasitized erythrocyte lysates. Mil. Med. 134(No. 10):1294-1299, 1969.

Wellde, B. T., Stechschulte, D. J., Schoenbechler, M. J. and Colgate, W. A.: An indirect hemagglutination test for malaria using an antigen from the lysate of parasitized erythrocytes. Mil. Med. 134(No. 10):1284-1293, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6503	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	K. Completion	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	107
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Procede with Security Classification Code)*

(U) Serodiagnostic Tests for Human Malaria (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

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

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	2	50
FISCAL YEAR CURRENT 70	2	50

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Inst of Res

ADDRESS:* Washington, D C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* Walter Reed Army Inst of Res
Div of CD and I

ADDRESS:* Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Fife, E. H., Jr.

TELEPHONE: 202-576-3544

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: vonDoenhoff, A. E., Jr.

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Malaria; (U) Complement; (U) Complement Fixation Tests; (U) Antigens; (U) Serodiagnosis; (U) Serology; (U) Biochemistry; (U) Hemolysis

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) Unrecognized malaria infections in returnees whose clinical symptoms and parasitemias have been suppressed by prophylactic drugs an ever increasing military medical problem. Present efforts directed to development of serologic methods for detection of these occult infections and for differential diagnosis. Improvement of specificity and sensitivity of tests by physicochemical fractionation of antigens. Isolation, characterization and in vivo response to lytic factor obtained from malaria parasites, and its possible role in clinical malaria.

24. (U) CF technics are used to determine the efficacy of antigen fractionation procedures and evaluate sensitivity and specificity of purified products. Osmotic fragility of erythrocytes, hematocrits, febrile reactions and C-prime levels used as criteria for appraising in vivo response to plasmodial lytic factor. Technical problems include limited availability of parasite material and separation of parasites from erythrocytes.

25. (U) 69 07 - 70 06. Methods developed for effective separation of malaria parasites from host red cell elements. Purified fractions from erythrocyte-free parasites showed excellent sensitivity and specificity in CF tests. Now have capability for differential serodiagnosis of falciparum, vivax and quartan malaria. Comprehensive in vivo studies established possible role of plasmodial lytic factor in abnormal erythrocyte fragility, rbc destruction and febrile reactions in malaria. Basic objectives of work unit accomplished. Field studies to ascertain efficacy of procedures for detecting occult infection in returnees and determining persistence of antibodies following cure to be incorporated in another work unit. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 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

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 107, Serodiagnostic tests for human malaria

Investigators.
Principal: Earl H. Fife, Jr., M.S.
Associate: Albert E. vonDoenhoff, B.S.

Description.

This work unit is concerned with the isolation and characterization of plasmodial antigens, studies on the antigenic structure of various plasmodia, investigations of serologic patterns developed during the course of infection, and elucidation of immune mechanisms associated with this disease. In vitro as well as in vivo methods are employed. In vitro methods are used in (1) development of procedures for separating malaria parasites from host blood components; (2) isolation, purification and identification of plasmodial antigens by physicochemical and serologic methods; and (3) development, improvement and evaluation of serologic procedures for detection of antibodies and for following antibody patterns in infected hosts. In vivo studies include (1) the role of antigen and antibody in certain immunopathologic conditions associated with malaria infection; (2) production of specific antibodies to characterize experimental antigen fractions and to investigate the antigenic relationships of various species of Plasmodium; and (3) investigations on the immunogenicity of the purified antigen fractions.

Progress.

The basic objectives of this work unit have been accomplished. Therefore, it is recommended that the work unit be terminated and any future studies along these lines incorporated in Work Unit 172 titled "Sero-recognition of microbial infections". The present final report briefly summarizes the principal findings and contributions made during the course of these studies. These findings were reported in detail in previous reports on this work unit.

1. Isolation and fractionation of serologically active malaria antigens. For years investigators were unable to effectively separate malaria parasites from host erythrocyte components, and antigens prepared from parasite harvests containing red cell stromata showed a high incidence of nonspecific reactivity, low specific reactivity, and often were anticomplementary. These problems ultimately were resolved by investigators in the Department of Serology (1), who observed that the red cell membranes could be preferentially fragmented without damage to

the parasites, by passing the parasitized blood through a French pressure cell under carefully controlled pressure. The red cell fragments were exceedingly small and could be removed by washing the parasite harvest with saline.

Complement fixing antigens were prepared by solubilizing the parasites by passage through the French pressure cell at high pressure, and isolating the major antigen fraction by filtration through a Sephadex G-200 gel column. Antigens prepared in this manner showed excellent sensitivity and specificity and had no detectable traces of red cell contaminants. Moreover, the antigens proved to be species specific, showed little or no heterologous cross reactivity, and provided a reliable basis for differential serodiagnosis of human malaria. During the course of these studies the capability was developed for the serodiagnosis of vivax, falciparum and quartan malaria.

2. Serodiagnostic tests for malaria. The purified plasmodial antigen fractions were evaluated in CF tests adapted to the Microtiter system. The latter innovation significantly reduced the quantities of reagents required to perform the tests and facilitated testing large numbers of sera. Evaluation of a large group of sera from Montagnard tribesmen residing in an area hyperendemic for malaria revealed that the CF test provided a more accurate index of the malaria experience in semi-immunes than did examination of a single thick or thin blood film. The latter often were negative in individuals giving high CF titers with one or more of the plasmodial antigens. Moreover, the CF test results strongly suggested that many had intercurrent vivax and falciparum infections that were not demonstrable in the blood film examinations. Extensive experience with sera from individuals known to be free from malaria indicates that it is highly unlikely that reactions of the magnitude exhibited by the Montagnards could be simply nonspecific in nature.

3. Preservation and storage of malaria parasites and antigens. In the early phase of these investigations, storage of parasite harvests and antigens presented a difficult problem. Neither could be lyophilized, and a gradual loss of antigenic activity was observed in products stored at -60°C. These problems ultimately were overcome by incorporating 2% polyvinyl pyrollidone (PVP) in the preparations. Parasite harvests preserved with 2% PVP and held at -60°C, showed no evidence of deterioration after storage for more than 2 years; CF antigens prepared from these stored parasites were comparable to those obtained from freshly harvested organisms. Similarly, antigen fractions that were stabilized with 2% PVP and lyophilized, showed no change in serologic properties after storage at 3°C for more than 2 years. The excellent stability and long storage life of these products assure ready availability of free parasites for antigen production, and make it feasible to prepare and store large volumes of antigen that would be required for mass-testing. At the present time, reasonable supplies of P. falciparum antigen and

P. knowlesi antigen (the latter for detection of vivax malaria) are on hand. Initial supplies of P. malariae antigen have been depleted. However, it is proposed to prepare additional amounts of this antigen if a critical evaluation is undertaken.

4. Isolation and characterization of a lytic component of P. knowlesi. During investigations on the fractionation of malaria antigens on Sephadex G-200 gel columns, it was observed that certain low molecular weight components from the column possessed lytic properties. In view of the possible role of this lytic factor (LF) in the host-parasite relationships and pathology in malaria infections, efforts were made to characterize the factor and investigate its functional activity. In vitro studies on the kinetics of LF-induced hemolysis revealed that the temperature and time of incubation as well as the concentration of LF all influenced the hemolytic activity of the factor. However, regardless of the temperature or LF concentration, little or no hemolysis occurred during the first 4 hours of incubation. On the other hand, hemolysis progressed in a linear fashion after the initial lag phase, and spontaneously terminated after incubation for 21 hours, even though some intact cells remained in the reaction mixture. The pre-lytic lag phase suggested that a "preconditioning" action of some nature must occur before lysis could take place.

The spontaneous termination of lysis after incubation for 21 hours was shown not to be due to depletion of the lytic factor; further treatment of the remaining cells with fresh LF failed to induce further lysis. However, kinetic studies on a mixture of old cells labeled with Fe^{55} and young cells tagged with Fe^{59} revealed that the majority of unlysed cells remaining after incubation for 21 hours with LF bore the Fe^{59} label. Thus, it appeared that the young cells were more resistant to LF induced lysis and that hemolysis spontaneously terminated when the population of older cells was depleted.

Physicochemical analyses of the LF revealed the following characteristics. The factor is a relatively small molecule and appears to have a molecular weight of less than 5000. Quantitative chemical analyses for protein (Lowry test) indicated the presence of a small amount of protein. However, in view of the low molecular weight of the LF, it was believed that these reactions were due to amino acids and/or small peptides rather than protein per se. Trace amounts of carbohydrate also were detected. The principal component of the LF appeared to be lipid in character, with a high cholesterol content. Thin layer chromatography revealed a variety of lipids belonging to the following classes: phospholipids, free fatty acids, cholesterol, cholesterol esters, and triglycerides.

In vivo studies also were conducted to determine whether certain clinical features of malaria could be induced by injecting the LF into experimental animals. In ancillary studies in this Department (2), it

was observed that the nonparasitized as well as parasitized erythrocytes showed increased osmotic fragility in P. knowlesi infections. Moreover, the osmotic fragility progressively increased with each cycling of the parasite in the acute stages of the infection. It was of interest, therefore, to determine whether this phenomenon could be artificially produced by injecting LF into experimental animals. Hamsters receiving single or multiple i.v. injections of LF were used in these studies. Contrary to expectations, the osmotic fragility of the erythrocytes decreased rather than increased for a period following injection of the LF. The cells collected one-half hour after injection were considerably more resistant to hypotonic lysis than the controls. Resistance progressively increased through hour 2 and remained at a relatively high level through the fourth hour. However, there was a significant decrease in resistance after the fourth hour, and cells collected 5 hours after injection were more fragile than the controls. Erythrocytes from hamsters receiving 3 injections of LF over a 48 hour period and exsanguinated 4 hours after the last injection, showed osmotic fragility values comparable to those of animals receiving a single dose of LF and exsanguinated 4 hours later. However, the hematocrit values of the animals receiving the multiple injections of LF were considerably lower than those of the other groups. These observations suggested that considerable in vivo hemolysis had occurred and indicated that the anemia associated with acute malaria could be simulated by multiple injections of LF.

It was also observed that i.v. injection of LF induced a febrile response in the recipient hamsters. Since recurrent fever is a characteristic of malaria in a nonimmune host, the pyrogenic properties of the LF were investigated. A group of hamsters was given 3 injections of LF at 24 hour intervals, and the rectal temperatures were recorded at specified intervals after each injection. Following each injection, the temperature progressively rose during the succeeding 4 hours, but always returned to normal before the subsequent injection was administered 24 hours later. It was further noted that the degree of febrile response increased with each injection; the maximum temperature observed after a given injection was always significantly greater than that attained with the previous injection.

Results of the foregoing in vivo experiments suggest that the lytic factor isolated from malaria parasites may in part contribute to certain clinical features of malaria. The ability of the LF to increase the osmotic fragility and ultimately lyse red cells indicates that the factor may play a role in the anemia associated with acute malaria infections. The findings further suggest that the LF could be responsible for the febrile reaction associated with the cycling of the parasites.

Summary and Recommendations.

Methods have been developed for effective separation of malaria

parasites from host red cell elements and purified antigen fractions from these parasite harvests have exhibited excellent sensitivity and specificity in complement fixation tests. Antigen prepared by these methods are species specific and appear to provide a capability for the differential serodiagnosis of falciparum, vivax and malariae malaria. Moreover, tests on semi-immunes residing in malaria endemic areas revealed that the serologic tests were superior to the examination of a single thick or thin blood film for appraising the malaria experience of such individuals. The ability of the CF tests to identify infected service men in whom clinical malaria has been suppressed by prophylaxis or those likely to show recrudescent malaria has not been established. In addition, the persistence of antibodies following radical cure is not known.

Coincidental with the fractionation of specific complement fixing antigens from the erythrocyte-free malaria parasites, it was observed that a low molecular weight component of the parasites posessed hemolytic properties. In vivo studies revealed that i.v. injection of the lytic factor induced certain clinical manifestations characteristic of malaria in non-immunes; the factor increased the osmotic fragility of the erythrocytes and produced a febrile response.

With the development of specific complement fixation tests for human malaria, the basic objectives of this work unit have been accomplished. It is believed that further research and development along these lines is unwarranted. Rather, it is recommended that continued studies be directed to critical evaluation of the procedures already developed, appraising their efficacy for detecting individuals with occult infection, and determining the persistence of antibodies following radical cure. It is further recommended that the present work unit be terminated and that future evaluations of the procedure be included with another work unit (Sero-recognition of microbial infections).

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 107, Serodiagnostic tests for human malaria

Literature Cited.

1. References

(1). D'Antonio, L.E., vonDoenhoff, A.E., and Fife, E.H. A new method for isolation and fractionation of complement fixing antigens from Plasmodium knowlesi. Proc. Soc. Exper. Biol. & Med., 123 : 30, 1966.

(2). Fogel, B.J., Shields, C.E., and vonDoenhoff, A.E. The osmotic fragility of erythrocytes in experimental malaria. Am. J. Trop. Med. & Hyg., 15 : 269, 1966.

2. Publications

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6506	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	108
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Effects and Mechanism of Action of Chemotherapeutic Agents

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	c. PROFESSIONAL MAN YRS	d. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	3	70
FISCAL YEAR CURRENT 71	11	250

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Biochemistry

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Angel, LTC C. R.

TELEPHONE: 202-576-2211

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Siu, P. M. L. Ph.D.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Plasmodium; (U) Enzymes; (U) Carbonic Anhydrase; (U) Plasmodium falciparum; (U) Drug Effects; (U)Primaquine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Basic biochemical activities of malarial parasites will be studied to provide further data for evaluation of drug-resistance of plasmodium falciparum. Analogues of antimalarial drugs will be synthesized where not available for biochemical studies.

24. (U) Plasmodium berghei will be used as a test organism to evaluate intermediary metabolism of the malarial parasite and responses to antimalarial agents. Special synthesis of additional antimalarial compounds will be done.

25. (U) 69 06 - 70 06 CO_2 fixation enzymes have been implicated as an allosteric enzyme. Phosphenol pyruvate (PEP) carboxylase and PEP carboxykinase have been separated and purified. The most stable enzyme appears to be the phosphenol pyruvate enzyme. Mechanism of inhibition of the CO_2 fixation by selected antimalarials in the presence of purified enzymes. Studies on the subcellular location of chloroquine-binding sites in chloroquine-sensitive P. berghei showed the site of highest affinity to be located within the parasite membrane or in some other insoluble cellular component. A lower affinity site was found to be in the soluble contents of the parasite. Activities within this work unit are consolidated and entitled, Biochemical Effects and Mechanisms of Action of Chemotherapeutic Agents. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 108, Study of malaria and antimalaria therapy

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: CPT P. A. Kramer, MSC; LTC T. K. Li, MC;
J. E. Matusik, B.S.; P. M. L. Siu, Ph.D.

Description.

The objectives of this work unit are to examine biochemical variations in enzymic constituents of the malarial parasite and the interaction of specific antimalarial compounds on the parasite and the host. Activities within the work unit are subdivided into parasite studies and the effect of antimalarial agents on the metabolism of the host.

Progress.

1. Parasite studies.

Definition of the metabolism of the malarial parasite and the location of binding sites that lead to the explanation of antimalarial drug action are important efforts. Plasmodium berghei is used as the model test system for the study of carbon dioxide fixation and the location of chloroquine binding sites.

a. Carbon dioxide fixation in Plasmodia, mechanism of action and control. Phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase activities have been reported earlier in Plasmodia. The carboxylase activity has been partially purified devoid of carboxykinase activity. The metal requirements of the reaction was studied. We have shown that oxaloacetate is formed in the presence of either Mg^{++}, Mn^{++} or Co^{++}. Other metal ions such as iron, copper, mercury, nickel, selenium, chromium, zinc and vanadium are ineffective. It was found, however, that ferrous, ferric, cupric and mercuric ions inhibited the formation of oxaloacetate by CO_2 fixation. The ferrous and ferric ions inhibited noncompetitively with Mg^{++} for the carboxylase. The K_i values were calculated for ferrous and ferric ions and shown to be 1.45×10^{-3} M and 1.41×10^{-3} M, respectively.

The substrate affinity constants were determined for the carboxylase reaction. Normal enzyme kineteics were observed for phosphoenolpyruvate, and Mg^{++} in the presence of the carboxylase. We have found earlier, however, with an enzyme preparation possessing carboxykinase activity, that abnormal enzyme kinetics were observed with Mg^{++}. It

is not yet known whether these two activities are carried out by the same or different proteins. This aspect is under investigation in our laboratory. It would be interesting, for instance, if both the carboxylase and the carboxykinase activities were carried out by the same protein. If this were true, then it is conceivable that the enzyme in one form catalyzes the carboxylase reaction and in another form would catalyze the carboxykinase reaction. The apparent K_m value for PEP was calculated to be 1.05×10^{-3} M; for Mg^{++}, 3.0×10^{-3} M; for Mn^{++}, 1.0×10^{-4} M; and for Co^{++}, 2.6×10^{-4} M. The relative affinities of the metal ions for the carboxylase are in the order of $Mn^{++} > Co^{++} > Mg^{++}$.

The effects of small molecules on the carboxylase reaction were studied. Unlike the bacterial carboxylase, the plasmodial enzyme was not affected by the addition of acetylCoA, fructose diphosphate and cyclic 3',5'-AMP. Avidin also had no effect on the formation of oxaloacetate in concentrations of 30 ug and 60 ug per ml of reaction mixture. A rapid loss of enzymic activity occurred, however, in the presence of added urea.

Evidence was obtained for the structural modifications of phosphoenolpyruvate carboxylase from Plasmodia. The partially purified carboxylase applied to a cellulose phosphate column in the absence of added Mg^{++}, was washed with a gradient of 0.05 M - 0.8 M phosphate buffer containing 0.5 M glucose, and 0.001 M EDTA. There was only one peak of enzymic activity and the total recovery was 62%. On the other hand, when the same partially purified carboxylase, stored at -12° for 7 days was chromatographed in a similar manner, but in the presence of 0.001 M $MgCl_2$, two enzymic activity peaks appeared. The total recovery when assayed immediately was 48%. After these fractions were stored at -12° overnight, only one enzymic peak emerged when assayed and the total recovery increased to 92%. The eluates without added Mg^{++} were also assayed the next day and found to possess the same activity. These results with those of urea strongly suggest that the phosphoenolpyruvate carboxylase from Plasmodium berghei is composed of subunits and is an allosteric enzyme.

b. Subcellular location of chloroquine in chloroquine sensitive Plasmodium berghei. This investigation centered on the determination of the subcellular location of chloroquine binding sites within the parasitized cells. A protocol was established for the differential fractionation of the host cell and parasite, and a system devised for producing relatively "clean" membrane-wrapped parasites--preparations free of white blood cells, platelets and the majority of erythrocytic ghost.

The effects of this differential lysis on the equilibrium distribution of chloroquine between cells and an external aqueous medium in vitro were then determined in an attempt to determine which fractions displayed binding activity at various concentrations of the drug. Numerous

unsuccessful attempts were made to solubilize a high affinity binder from the parasite membranes.

Mouse erythrocytes parasitized with CS P. berghei were examined while in equilibrium with concentrations of chloroquine sufficient to titrate either the binding site at $K_A = 10^8\ M^{-1}$ (high affinity) or $K_A = 10^5\ M^{-1}$ (middle affinity). When the high affinity site was saturated with the drug and the red blood cell contents lysed away with saponin, the chloroquine remained in the parasites. In fact, the membrane-wrapped parasites themselves were capable of concentrating the drug to the same extent as intact parasitized cells, illustrating the lack of need for red blood cell components in high affinity binding. When the parasites obtained above by saponin lysis of the cell were themselves lysed in distilled water, the majority of the chloroquine remained with the parasite membranes. Furthermore, studies of this effect at various concentrations of drug revealed that the affinity constant of the parasite membranes for chloroquine was identical to that of the intact cells, only the maximal binding capacity of the membranes was altered by lysis.

The membranous binding site was also shown not to be DNA and to be deficient, as expected, in chloroquine resistant strains.

The middle affinity site behaved quite differently. It was released to quite a large extent (> 50%) by saponin lysis of the RBC and was almost completely removed by saponin and water lyses combined. Hence this site appears to be located in the cytoplasmic or nuclear portion of the parasites.

2. Effect of malaria chemotheropeutic agents upon alcohol metabolism and alcohol dehydrogenase.

Clinical impressions that alcohol ingestion by individuals taking CP tablets results in untoward side effects suggests that this is an area worthy of both basic and clinical investigation. In particular, it would be important to examine specifically whether or not compounds such as chloroquine, primaquine, dapsone and others are inhibitory to liver alcohol dehydrogenase and to characterize the nature of such interactions.

a. Interaction of chloroquine, primaquine and other aminoquinoline congeners with horse liver alcohol dehydrogenase. Crystalline horse liver alcohol dehydrogenase was chosen as the model enzyme system for study because it is available commercially in a highly purified form and it is very similar to the human liver enzyme in its physical and catalytic properties. At pH 8.5, alcohol dehydrogenase activity was inhibited more than 95% by 7×10^{-5} M primaquine, whereas the same

concentration of chloroquine did not inhibit at all. Fifty percent inhibition by primaquine occurs at 2×10^{-6} M, giving an apparent inhibition constant of 2×10^{-6} M. The inhibition is immediate and reversible by dilution, and is not increased by prior incubation of the enzyme with primaquine. Detailed kinetic studies confirmed that primaquine is a good inhibitor ($K_I = 1.7 \times 10^{-6}$ M) and showed, in addition, that it is non-competitive with respect to both the coenzyme, NAD, and the substrate, ethanol.

It was also found that upon binding to alcohol dehydrogenase, the absorption maximum of primaquine at 260 mu undergoes a shift of 13 mu towards the long wavelength region. This spectral shift is analogous to the change observed when primaquine is placed into a nonpolar solvent, e.g., dioxane, indicating that primaquine binds to hydrophobic regions of the enzyme molecule. Moreover, the spectral shift allowed the measurement of the stoichiometry of the interaction of primaquine with alcohol dehydrogenase, as well as the dissociation constant, K_D. By means of 4-cell difference spectrophotometry, it was found by titration that 1 molecule of primaquine binds to each of the two active enzymatic centers of the enzyme and that $K_D = 2 \times 10^{-6}$ M, in good agreement with the kinetic data.

A series of 8-amino-6-methoxyquinoline analogs were then examined, to determine which portions of the primaquine molecule were important to the interaction of primaquine with alcohol dehydrogenase, and hence inhibition. The data indicated that substitution on the 8-amino group by an aliphatic side chain was critical, as was also the terminal primary amino function. Significantly, chloroquine and desethychloroquine did not inhibit at all, even up to a concentration of 1×10^{-3} M, nor did dapsone.

Similar studies on purified enzyme from human livers are underway.

b. Inhibition of ethanol metabolism in man following the ingestion of a single CP tablet. Since the alcohol dehydrogenase reaction is rate-limiting in the metabolism of alcohol and primaquine is a potent, non-competitive inhibitor of the enzyme, the physiologic significance of the interaction of primaquine with alcohol dehydrogenase might be manifest in vivo. This can be accomplished by measuring the disappearance rate of alcohol from serum, following a loading dose of ethanol. A pilot study with 7 volunteers has thus far been conducted. Subjects in a fasting condition were given 40-70 ml of alcohol orally, and their serum alcohol concentrations were measured q.1/2 hour by means of gas chromatography. Four hours before testing, the subjects were given either a placebo or a CP tablet. Each subject served as his own control. In 4 of the 7 subjects, a significant decrease in the rate of alcohol

removal was observed following the ingestion of the CP tablet, indicating a retardation of alcohol metabolism after ingestion of the CP tablet. These studies provide a rational basis for the clinical impression that the CP tablet adversely affects the alcohol tolerance of its users. Presently, a larger-scale study is in progress to determine the duration of action of a single CP tablet and the effect of repeated weekly doses of the CP tablet upon alcohol metabolism.

Summary

Studies on carbon dioxide fixation in Plasmodia show the following:

a. Phosphoenolpyruvate carboxylase from Plasmodium berghei undergoes structural changes when stored in the presence of magnesium ion.

b. The carboxylase from Plasmodia in contrast to bacterial carboxylase was unaffected by acetylCoA, fructose diphosphate or cyclic 3',5'-AMP. Rapid loss of enzymic activity occurs in the presence of urea.

c. Iron, copper and mercury inhibit carboxylase, Km values were determined for the enzyme in presence of magnesium, cobalt and manganese.

Binding sites for chloroquine are divided into high affinity and middle affinity sites. The high affinity site appears associated with parasite membrane and is not DNA. The middle affinity site is associated with cytoplasm and nucleus of the parasite. Primaquine is a potent inhibitor of alcohol dehydrogenase in horse liver preparations. A pilot study in man showed that ingestion of the CP tablet may result in the retardation of ethanol metabolism.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 108, Study of malaria and antimalaria therapy

Literature Cited.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6471	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	112
b. CONTRIBUTING				
XXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Field Studies on Drug Resistant Malaria (TH)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 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

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	3	177
FISCAL YEAR CURRENT 71	3	177

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 2012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Altstatt, LTC L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Diggs, LTC C. L.

NAME: Colwell, MAJ E. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Drug-resistant Malaria; (U) Falciparum Malaria; (U) Chloroquine; (U) Resistance; (U) In-vitro models; (U) Clinical Research

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 variables which influence the prevalence of chloroquine-resistant falciparum malaria in a human population in Southeast Asia thus adding to our basic knowledge of drug-resistant malaria and providing a setting whereby new antimalarials may be tested efficiently.

24. (U) Study sites are identified in Thailand where malaria transmission occurs throughout the year. The prevalence of drug-resistant falciparum malaria is estimated using in vitro technique. Variables such as rainfall, temperature, vector density and human immunity are measured.

25. (U) 69 07 - 70 06 We have substantiated that falciparum malaria remains hyperendemic in certain comparatively inaccessible areas in Thailand. Logistical and administrative arrangements have been made through the Royal Thai Government whereby the diseases may be studied in these areas. The in vitro technique as the measure of chloroquine resistance is under study in a field unit presently. For technical reports see SEATO Annual Progress Report, 1 Apr - 31 Mar 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 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

BLANK PAGE

Project 3A663713D829 MILITARY MEDICAL RESEARCH PROGRAM S.E.ASIA

Task 00, Malaria Investigations

Work Unit 112, Field studies on drug resistant malaria

Investigators.
Principal: Carter L. Diggs, LTC, MC
Associate: Prajim Boonyakanist; Tan Chongsuphajaisiddhi, M. D., Ph.D.; Nipon Chuanak; Kanjika Devakul, M.D., Ph.D.; Carter L. Diggs, LTC, MC; Robert Gentzel, SSG; Douglas J. Gould, Ph.D.; Bruce A. Harrison, CPT, MSC; Y. M. Huang, Ph.D.*; Ratanaporn Kasemsuthi, B.Sc.; Kol Mongkolpanya; Vanchai Numsuwankijkul, B.Sc.; Larp Panthusiri; Katchrinnee Pavanand, M.D.; Barnyen Permpanich, R.N.; E.L. Peyton; Vajri Promtatvethi, M.B., B.S. (Lond), F.R.C.S., DA; Rampa Rattanarithikul; John E. Scanlon, Ph.D.*; Sunthorn Sirivanakorn, Ph.D.*; Prasit Sookto; Michael F. Sullivan, CPT, MSC; Stanley W. Theune, MSG; Atthasit Vejjajiva, M.B., B.S. (Lond), M.R.C.P.; Prapit Venatanasesth, M.D.; Chirapan Vinagupta; Robert Weidhorn, SFC

---

* Southeast Asia Mosquito Project, Smithsonian Institution, Washington, D.C.

Serologic Studies in Human Malaria

Principal Investigator: Carter L. Diggs, LTC, MC

Associate Investigators: Robert Gentzel, SSG
Robert Weidhorn, SFC
Prasit Sookto
Chirapan Vinagupta

OBJECTIVE The original objective of this study was to compare the serologic response after (a) adequate treatment of the initial attack (U.S. troops) and (b) multiple episodes of malaria (sera from endemic areas).

DESCRIPTION Since antigen usable without further purification was not obtainable, lysates of washed P. falciparum chimpanzee erythrocytes were obtained from WRAIR and attempts were made to isolate antigenic fractions to be employed with the soluble antigen fluorescent antibody (SAFA) test (Sadun et al). Limited trials at isolation by DEAE Sephadex chromatography (Sadun et al) and more extensive efforts by G-200 Sephadex Chromatography (Gore, personal communication) were made. Fractions were monitored by absorption at 280 mμ. Serologic testing was by the SAFA test using sera from patients with proven malaria.

PROGRESS In our hands, fractionation of lysate on DEAE Sephadex resulted in poor yields of protein; therefore, we turned to G-200 Sephadex, A total of four runs were made. The lysate was eluted in two major peaks as determined by absorbence at 280 mμ; the first, eluted with the excluded volume, was colorless and opalescent; the second contained the bulk of the hemoglobin. Four eluate pools were prepared from material eluted prior to the second peak (hemoglobin): fraction 1 was the entire first absorbence peak; fractions II and III (FII and FIII) were prepared from the eluate between the two absorbence peaks; fraction IV (FIV) was taken from the ascending portion of the hemoglobin peak. SAFA tests employing these fractions showed no difference between normal and malaria sera in three of four chromatographic runs (#1, 3 and 4); in the second run FI and FIII gave some evidence of antigenic activity; FI yielded higher fluorescence in 5 of 8 tests malaria sera than any of the 8 normal control sera; FIII gave a stronger reaction than the controls in 4 of the 8 cases. However, results obtained on retesting five and seven days later suggested some less of activity of the FI antigen. Further tests will be required to clarify this point.

Possible reasons for our failure to isolate active antigen consistently

are (a) the lack of facilities for preparative chromatography in the cold and (b) the relatively lengthy period of storage of the crude lysate before use (more than 6 months).

Despite arrangements made with several units, only a few serum samples from U.S. troops have been obtained.

This project will be terminated on 1 July 70 due to departure of the principle investigator.

SUMMARY Attempts to isolate antigen for the malaria SAFA test have been only partially successful.

Interaction of Plasmodium falciparum and Canvalescent Serum in vitro

Principal Investigators: Katchrinnee Pavanand, M.D.
Carter L. Diggs, LTC, MC

Assistant Investigators: Barnyen Permpanich, R.N.
Vanchai Numsuwankijkul, B.Sc.
Stanley W. Theune, MSG
Robert Gentzel, SSG
Nipon Chuanak

OBJECTIVE: The studies of Cohen et al[1], and subsequently, other workers, demonstrate clearly an important role for antibody in immunity to Plasmodium falciparum infections. Exploitation of these findings requires extensive study of numerous aspects of the protective effect including its mechanism and specificity. Study of the problem in the intact experimental animal or patient poses many problems which could be circumvented by the availability of an assay of the protective antibody in vitro. Recent development in Cohen's laboratory in London indicate that such an assay might be feasible for P. knowlesi.[2] His data suggest that inhibition of plasmodial development occurs through reaction of antibody with the extracellular merozoites. If this is the case, the development of an in vitro assay could be approached by achieving repenetration in vitro and then studying the inhibition of the phenomenon by patient serum. Implicit in these requirements is the ability to detect the repenetration event with certainty. The present study was designed to (a) explore the possibility that in vitro erythrocyte penetration by P. falciparum can be detected by a marked recipient cell technique and (b) study, if feasible, the effect of serum from patients with a history of recurrent malaria on penetration in vitro.

DESCRIPTION The system for detection of penetration in vitro involves the inclusion of fetal erythrocytes in the culture; these cells can later be distinguished from adult cells by differential elution of hemoglobin. The presence of a parasite within a fetal cell is taken as evidence of penetration in vitro.

Washed erythrocytes from heparinized cord blood and from patients with acute falciparum malaria were suspended in tissue culture medium 199 modified by the addition of $NaHCO_3$ (1.7 mg/ml). Penicillin G (400 units/ml), and streptomycin (400 µg/ml) and equilibrated with 5% CO_2 in air. 3 Additional glucose was added in various amounts as described below. The pH of the medium immediately after gassing was 6.7-6.9. Mixtures of the cell suspensions were prepared so that the final total erythrocyte concentration was 1×10^8/ml with 1% of the cells parasitized. In most experiments, equal numbers of adult and fetal cells were employed; if necessary, erythrocytes from a normal adult donor were used to reduce the final parasitized cell concentration. Compatible normal serum was included in each culture. The culture mixtures were incubated without agitation at 37^0C in disposable plastic serological microtitration trays sealed with cellophane tape. The trays were sterilized prior to use by ultraviolet irradiation. After an appropriate interval, thin blood films were prepared and fixed with ethanol. The hemoglobin of the adult erythrocytes was eluted with citrate buffer (pH 3.4 ± 0.05); on treatment of the slides with eosin, only the fetal cells were stained[4]. The preparations were counterstained with Giemsa's stain for visualization of the parasites. Sera were collected from patients with a history of recurrent malaria. All sera and all parasites were obtained in Cholburi Province. Globulin fractions of sera were prepared by precipitation in 1/3 saturated $(NH_4)_2SO_4$ in the cold; the redissolved preparations were dialyzed exhaustively against saline.

PROGRESS Experiments have been performed with 28 isolates of P. falciparum in 24 cases, progressive development with infection of fetal cells was observed. Penetration was detected as early as 24 hours and was usually maximal at 48 hours. However, since there was considerable variation in the rate of development, preliminary observations on control preparations were used to decide on the optimal time for harvest; on occasion this was as late as 60 hours after initiation of culture. All intermediate stages of asexual development were observed in adult (donor) cells. On one occasion parasites identified as immature gametocytes were seen after 24 hours of culture. Parasites in fetal cells were usually small rings, although on a few occasions somewhat larger trophozoites were observed.

The percentage of fetal erythrocytes parasitized was variable but values as high as eight per cent were recorded. Thin blood films prepared from adult erythrocytes alone and from fetal erythrocytes alone served as controls on the specificity of differential hemoglobin elution. Adult cells were essentially devoid of hemoglobin after elution, whereas fetal cells showed uniform uptake of eosin. Control slides of cell mixtures prepared prior to incubation at 37°C showed no parasitized hemoglobin containing cells.

Thirteen sera from patients with histories of repeated attacks of malaria were studied for an effect on penetration of fetal cell by P. falciparum in vitro. In these experiments, the medium consisted of equal parts of modified 199 and the test serum. Supplemental glucose (100 mg% final concentration) was added to that already present in the culture medium. Eight replicate cultures were employed for each serum sample. The mean percentage of fetal erythrocytes parasitized was determined for each serum and compared with the value for normal serum used in a control set of cultures. Experiments were performed on two P. falciparum isolates. It is apparent that all of the test sera allowed less fetal cell penetration than the controls.

Since it was recognized that inhibition of penetration could be caused by a variety of serum components (or deficiencies of serum components) efforts were made to adapt the system for testing of serum fractions. Experiments were performed to test the effect of varying proportions of serum, culture medium and normal globulin on fetal cell penetration. Again eight-fold replicate cultures were employed. The supplemental glucose in the culture medium prior to admixture with serum and globulin was 200 mg%. The results are summarized in Table II. The data indicates that the globulin preparations cannot substitute for serum; however, when serum is present at a concentration of 0.25 ml per ml of mixture (25% serum) penetration is comparable in extent to that obtained with 50% serum. On this basis further experiments were conducted with 25% serum, 50% globulin, and 25% 199.

The results of two experiments on the effect of varying amounts of added glucose are summarized in Table III. With the exception of one mean value, both experiments indicate that approximately 200 mg% of added glucose is optimal. Greater or lesser amounts seem to be inhibitory. The similarity in the extent of penetration obtained in the two experiments is probably fortuitous.

Studies of the effect of globulin prepared from the serum of patients with a history of recurrent malaria are now in progress.

SUMMARY (1) An experimental system which allows detection of invasion of erythrocytes by P. falciparum in vitro has been devised. Human fetal erythrocytes are employed as the recipient cells. The lower solubility of fetal hemoglobin in acid is exploited as a cell marker by which the fetal cells can be distinguished in artificial admixture with adult erythrocytes. Parasites in the previously uninfected fetal cells serve as evidence of invasion in vitro.

(2) Fewer fetal cells were penetrated in the presence of thirteen sera from patients with a history of recurrent malaria than in a normal control serum. With one of these sera no fetal cell penetration was observed.

(3) It has been shown feasible to perform these culture experiments in the presence of a globulin fraction of serum; experiments testing the effect of globulin from patients with a history of recurrent malaria are in progress.

Table 1. Inhibition of penetration of fetal erythrocytes by *P. falciparum in vitro* by serum from patients with a history of recurrent malaria.

Serum No.	Mean percentage of fetal cells parasitzed	
	Exp. 1	Exp. 2
1	.20	3.5
2	.86	5.6
3	.53	4.0
4	.60	3.6
5	.40	-
6	.27	-
7	. 0	-
8	.43	-
9	.54	-
10	-	4.9
11	-	4.5
12	-	3.35
13	-	6.8
Control	1.6	8.8

Table II. Effect of varying proportions (by volume) of culture medium 199, normal serum and normal serum globulin on the percentage of fetal erythrocytes parasitized *in vitro*.

% Globulin	% 199	% serum							
		0		10		25		50	
0	25	NS	NS*	0.3	0.2	0.4	0.1	1.0	0.3
	50	0	0	0	0.02	0.5	0.03	1.8	0.5
25	25	0.1	0	0.1	0.2	0.7	0.3	1.6	1.2
	50	0	0	0	0.05	0.4	0.6		
50	25	0	0	0.1	0.2	1.6	1.1		
	50	0	0						

* NS-slides not satisfactory.
Units are mean per cent fetal erythrocytes parasitized. The two values in each block are from two experiments with different *P. falciparum* isolates. Values greater than 0.1% are rounded to the nearest 0.1%.

Table III.

Added glucose final concentration mg%	Average % fetal erythrocytes parasitized	
	Experiment No. 1	Experiment No. 2
50	0.10	-
100	0.17	-
150	0.32	-
200	0.05	0.40
250	0.38	-
400	-	0.30
600	-	0.15
800	-	0.15
1000	-	0.20

The Fate of Fibrinogen in Malaria

Principal Investigators: Tan Chongsuphajaisiddhi, M.D.,Ph.D.
Ratanaporn Kasemsuthi, B.Sc.
Kanjika Devakul, M.D., Ph.D.

Associate Investigators: Prapit Vevatanasesth, M.D.

OBJECTIVE: There is growing evidence that intravascular coagulation may be important in the pathogenesis of malaria. The objective of the present study is an evaluation of fibrinogen disappearance in Plasmodium coatneyi infected monkeys. If evidence for intravascular coagulation is found, efforts to study the phenomenon histologically will be made.

DESCRIPTION: Rhesus monkeys (Macaca mulatta) were used. Fibrinogen, prepared from the plasma of normal monkeys by the method of repeated salt fractionation by 2.05 M ammonium sulfate, was labelled with radioactive iodine (I-131) and injected intravenously into the monkeys to be studied. Blood samples were taken at various intervals for the determination of plasma radioactivity. Further details of the procedure were presented in the SMRL Annual Report 1969. Plasma volume and fibrinogen concentration were determined by standard methods. To obtain estimates of the amount of fibrinogen being degraded, fibrinogen turnover rates were calculated according to the expression $V_p \ C_f \ K_1$ where V_p is the plasma volume, C_f the plasma fibrinogen concentration, and K_1 the first order rate constant for I-131 fibrinogen disappearance. Urine samples were also collected for radioactivity measurements.

PROGRESS: A control study of fibrinogen metabolism was done in six normal monkeys. Plasma radioactivity, expressed as the percentage of the initial value at zero time (by extrapolation) was shown to have a biological half life between 30-39 hours. The cumulative radioactivity excreted in the urine (expressed as the percentage of the injected dose) in 6 days varied from 42% to 77% of the injected dose.

In three monkeys (MS67, PK20 and PK28), infected with Plasmodium coatneyi, the study was performed at 13, 15 and 16 days after the infection respectively. The parasitemias were 0.7%, 0.8% and 0.8% and the hematocrits 23%, 29% and 20%. Plasma radioactivity was shown to have a biological half life of 24, 32 and 34 hours; i.e., two of the three monkeys gave values within the normal range. The plasma volumes, fibrinogen concentrations and fibrinogen turnover rates for the normal and infected animals are given in Table 1. Both plasma volume and plasma fibrinogen concentrations were higher in two of the

three infected animals. Earlier studies (SMRL Annual Report 1968) also indicated expanded plasma volumes during chronic coatneyi malaria. Thus it appears that on a weight basis, more fibrinogen was degraded in the infected animals than in the controls; this is expressed in the table in terms of fibrinogen turnover rates. More data is required to determine whether or not these changes are representative of P. coatneyi infected monkeys in general.

Urinary excretion of I-131 in the infected monkeys was similar to that in the controls. Thus, in six days, 72%, 54% and 58% of the injected dose was excreted by the three monkeys.

SUMMARY: Fibrinogen turnover rates were greater in three monkeys with P. coatneyi infections than in control animals. These findings can be interpreted in terms of increased fibrin formation in the infected monkeys. Further study is needed to evaluate the generality of the observation.

Table 1. Fibrinogen turnover in normal and Plasmodium coatneyi infected monkeys.

Monkey No.	Weight Kg.	Plasma volume ml/kg	Plasma fibrinogen concentration mg/ml	Fibrinogen turnover rate mg/kg/hr
Normal				
MS 91	2.25	28.5	4.3	2.8
MS 92	2.40	46.5	3.0	2.7
MS 93	2.65	36.5	3.7	2.7
MS 95	2.70	35.3	2.3	1.9
PK 20	4.20	30.4	3.8	2.5
PK 28	4.90	40.7	2.1	1.5
Infected				
MS 67	6.83	44.5	3.5	4.2
PK 20	3.95	80.1	4.5	7.9
PK 28	4.90	57.7	11.4	13.4

Malaria and the Nervous System: Cerebral Haemodynamics and Metabolism in Patients with Malaria and Central Nervous System Symptoms
The Response of the Diseased Cerebrovascular System to 5% Carbon Dioxide Inhalation and Hyperventilation

Principal Investigator: Athasit Vejjajiva
M.B., B.S. (Lond.), M.R.C.P.

Associate Investigator: Vajri Promtatvethi
M.B., B.S. (Lond.), F.R.C.S., D.A.

OBJECTIVE: The objectives of this study are to investigate the physiologic factors which influence the cerebral blood flow and metabolism with emphasis on techniques for experimentally increasing blood flow; to evaluate the possibility of modifying those factors which are said to cause brain dysfunction in "C.N.S. malaria."

DESCRIPTION: See previous Annual Report.

PROGRESS: In our previous report, it was concluded that the administration of 5% carbon dioxide and acetazolamide in combination had an additive effect in increasing the cerebral blood flow in patients with occlusive cerebro-vascular disease. In practically all those patients, there was predominant clinical and angiographic involvement of the carotid arterial system.

During the period under report, sixteen patients with lesions in various parts of the central nervous system due to vascular and non-vascular disorders were studied. These included one patient each with Parkinson's Disease, hepato-cerebral degeneration, uremia, motor neuron disease, multiple sclerosis, malignant hypertension, two patients with Takayasu's arteritis, three patients with upper brain stem lesion. Cerebral haemodynamics were determined before and after 5% carbon dioxide inhalation.

It was found that in all except the two patients with upper brain stem lesions, carbon dioxide produced a significant increase in cerebral blood flow. Those two patients and six others with decerebrate rigidity previously studied (see previous Annual Report), who failed to respond to carbon dioxide, seemed to substantiate the recent hypothesis based on animal study that the upper brain stem played an important role in the regulation of cerebral blood flow.

Mosquito Fauna of Thailand

Principal Investigators : Bruce A. Harrison, CPT, MSC
Y.M. Huang, Ph.D.*
E.L. Peyton*
Rampa Rattanarithikul
John E. Scanlon, Ph.D.
Sunthorn Sirivanakarn, Ph.D.*

Assistant Investigators : Prajim Boonyakanist
Kol Mongkolpanya
Larp Panthusiri

OBJECTIVE : To collect, identify, catalogue and redescribe all of the mosquito species of Thailand. Information is also assembled on the distribution, larval habitats, and other aspects of the bionomics of the various species. The eventual goal is the production of monographs on the mosquitoes of the area, together with keys, handbooks and other identification aids, for use of workers in public health and associated fields.

DESCRIPTION : Mosquitoes are collected from many areas of Thailand in connection with various studies on arboviruses and malaria. Additional collections of a specialized nature are made to obtain correlated series of larvae, pupae and adults for illustration and taxonomic study. These have consisted mainly of collections of mosquito larvae from all types of habitats; these larvae have been reared individually so as to recover a correlated series of cast skins and adults. All of the reared material is later identified and processed at SMRL in Bangkok. The majority of this material is shipped to the Smithsonian Institution for study by specialists in the Southeast Asia Mosquito Project (SEAMP).

PROGRESS : During this period 938 mosquito collections were made in 8 provinces of Thailand, the British Crown Colony of Hong Kong and the Philippines. These collections resulted in 13,624 pinned adults, 13,365 slide mounts of larvae, larval and pupal skins and 27 slide mounts of terminalia and other structures. The mosquito collections made on Mindoro and Luzon in the

* Address: Southeast Asia Mosquito Project, Smithsonian Institution, Washington, D.C.

Philippines and in the British Crown Colony of Hong Kong provided material for study and comparison with other species of the Anopheles in the minimus complex present in Thailand. Results of mosquito collections made during this period are given in detail in the following sections.

Aedes : The majority of the work on this genus was concentrated on species belonging to the subgenus Stegomyia. Immature stages were collected primarily from bamboo oviposition cups set out in a variety of habitats and from artificial containers in and around houses. Adults were obtained in diurnal and crepuscular collections of mosquitoes attracted to human bait. During the previous reporting period 14 species of Stegomyia were recorded from Thailand, including a possible new species near A. unilineatus. This latter species has subsequently been recognized as a species new to science and has been named Aedes seatoi in honor of this laboratory.* Aedes seatoi closely resembles A. albopictus in the adult stage and its larval stages are very similar to those of A. aegypti. Because of its possible confusion with those two dengue vectors, more knowledge of the ecology and behavior of A. seatoi is urgently needed. Aedes seatoi is currently known from Angthong, Chieng Mai, Chon Buri, Kanchanaburi, Nakhon Sawan, Prachinburi and Saraburi provinces (Fig. 1), however, its distribution is probably much wider throughout Thailand and Southeast Asia for it has recently been collected in Malaya.** Adults of A. seatoi have been collected biting man in Saraburi and Prachinburi provinces between 1800-1900 hours. Thus far, all larvae of this species have been collected from water accumulated in nodes of bamboo, except for two collections from the axils of banana plants and one from a clay water jar. Aedes seatoi is most frequently collected in the vicinity of villages surrounded by orchards (jack fruit, mango, papaya and banana) and bamboo groves.

Aedes scutellaris, first recognized in Thailand 4 years ago, has since been collected close to the sea in four provinces (Phuket, Prachuab Khirikhan, Surat Thani and Trat) and inland along the Chao Phaya river in Nonthaburi province (Fig. 1). Two morpho-

---

* Huang, Y.M., Proc. Ent. Soc. Wash. 71: 234 (1969)
** Personal Communication : S. Ramalingham

logic forms have been noted in a colony of A. scutellaris, established with material from Prachuab Khirikhan, which are distinguished by differences in the mesepimeral scales. Currently, efforts are being made to solve this taxonomic problem by a study of the progeny reared from females of each of the morphologic variants. Little is known of the vector potential of this mosquito in Thailand, although Aedes scutellaris is reputed to be an important vector of dengue viruses in New Guinea.

The utility of bamboo oviposition cups in the collection of Stegomyia mosquitoes was demonstrated during a survey in Angthong province during the dry season this past year. This and 3 adjacent provinces were suffering from a severe drought at the time of the survey, and all natural larval habitats of Stegomyia mosquitoes were dry, except for a few water jars at houses. A total of 227 bamboo cups were set out in 8 areas for a period of 21 days. Eighty one (35.7 per cent) of the cups yielded eggs of five species of Stegomyia (A. aegypti, A. albopictus, A. gardneri imitator, A. pseudoalbopictus and A. seatoi), indicating that adult females were present in the area even under the adverse conditions existing there at the time (Table 1).

Anopheles : Studies on the distribution, ecology and systematics of Anopheles species in Thailand were continued during this period. Two well known malaria vectors from the Indian subregion, Anopheles culicifacies and An. stephensi, were collected in Amphur Mae Sariang, Mae Hong Son province. The collections of An. stephensi were made in a small village near the Salween river, while An. culicifacies was found in the town of Mae Sariang as well as in rural areas. Previous collections of An. stephensi have been made in Chieng Mai and Chieng Rai provinces, while An. culicifacies has been recorded by SMRL from Ayutthaya, Chieng Mai, Chon Buri, Kanchanaburi, Lampang, Lamphun and Tak provinces.

Studies on the minimus group of species in Thailand were continued during this period. Eight members of this complex of closely related species, An. aconitus, An. minimus, An. pampanae, An. culicifacies, An. jeyporiensis, An. fluviatilis, An. filipinae and An. varuna, have been recorded from Thailand. The first two species have been incriminated as vectors of malaria in

Thailand, while the others have not been shown to be of any importance in malaria transmission here. The members of this complex are separable on the basis of a few morphologic characters which show considerable variation in Thai populations. Some workers doubt that the last three species above really occur in the kingdom, but specimens of these species continue to turn up in SMRL collections. In an effort to determine the degree of variation in morphology existing amongst these species, a study of the progeny from females typical of each member species was undertaken. Approximately 4,000 progeny from An. aconitus and An. minimus females have been reared to the adult stage. Specimens resembling An. filipinae and An. varuna have been found in sibling broods from both An. aconitus and An. minimus parents, while specimens that would be identified as An. fluviatilis with present keys, together with intermediate forms, have been reared from typical An. minimus females. These fluviatilis forms closely resemble the An. fluviatilis collected by SMRL in northern Thailand and in Hong Kong. Adults of An. jeyporiensis resembling both of the subspecies, An. j. jeyporiensis and An. jeyporiensis candidiensis, were reared from larvae collected in Hong Kong's New Territories, but no morphologic differences were noted in the larval and pupal stages. The above results indicate that five members of the minimus complex may validly be considered to occur in Thailand, An. aconitus, An. culicifacies, An. jeyporiensis, An. minimus and An. pampanai. The last species still remain the rarest member of the complex in Thailand. During the past year a few adults of this species were collected biting humans and buffalo in Buriram and Prachinburi provinces. Previous collections by SMRL have been made in Chantaburi, Nan and Prachinburi provinces.

Since colonies of both An. aconitus and An. minimus can be maintained by the forced insemination technique, an attempt to determine the amount of genetic compatibility existing between these two species was made by crossing the two by this method. A total of 122 crosses were made, of which 76 were between An. aconitus males and minimus females and 46 between minimus males and aconitus females. Eggs were obtained from 8 minimus females, or which approximately 170 hatched. All except three of the larvae from these eggs died before the third instar. Two of the three developed into males while the third (a female)

developed only as far as the pupal stage. Eggs were obtained from only one of the aconitus females in these experiments, and these failed to hatch. These results are similar to those obtained by Kitzmiller*and his coworkers in crossing attempts involving European and North American species of the Anopheles maculipennis complex.

Culex : During this period special emphasis was placed on a study of the species within the subgenus Culex. This subgenus includes C. tritaeniorhynchus and C. gelidus, vectors of Japanese encephalitis virus in Thailand. Another member of the subgenus C. annulus, which occurs widely throughout Thailand, is a suspect in the transmission of JE virus on Taiwan.** Culex tritaeniorhynchus and C. annulus belong to a complex of species, the vishnui subgroup, which are separable most often in the larval stage only; identification of adults of these species is often impossible, creating serious problems is determining the source of JE virus in pools of these mosquitoes. During October 1969 larval surveys were begun in 5 districts of Chiengmai province that were affected by an epidemic of Japanese encephalitis earlier that year. Three species in the vishnui subgroup, C. annulus, C. pseudovishnui and C. tritaeniorhynchus, were collected in these surveys. The first and third species were commonly encountered in these collections, but larvae of C. pseudovishnui were more rarely encountered. Certain habitat preferences by larvae of Culex species were noted during these collections. Larvae of members of the pipiens group were found more frequently in small to moderate sized oviposition sites, while members of the sitiens group (including the vishnui subgroup) were collected more often in moderate to large bodies of water (Table 2). Furthermore, both C. gelidus and C. quinquefasciatus were collected from polluted water in most instances.

* In : Genetics of Insect Vectors of Disease. 1967 p. 151
** J. Med. Ent. 6: 327

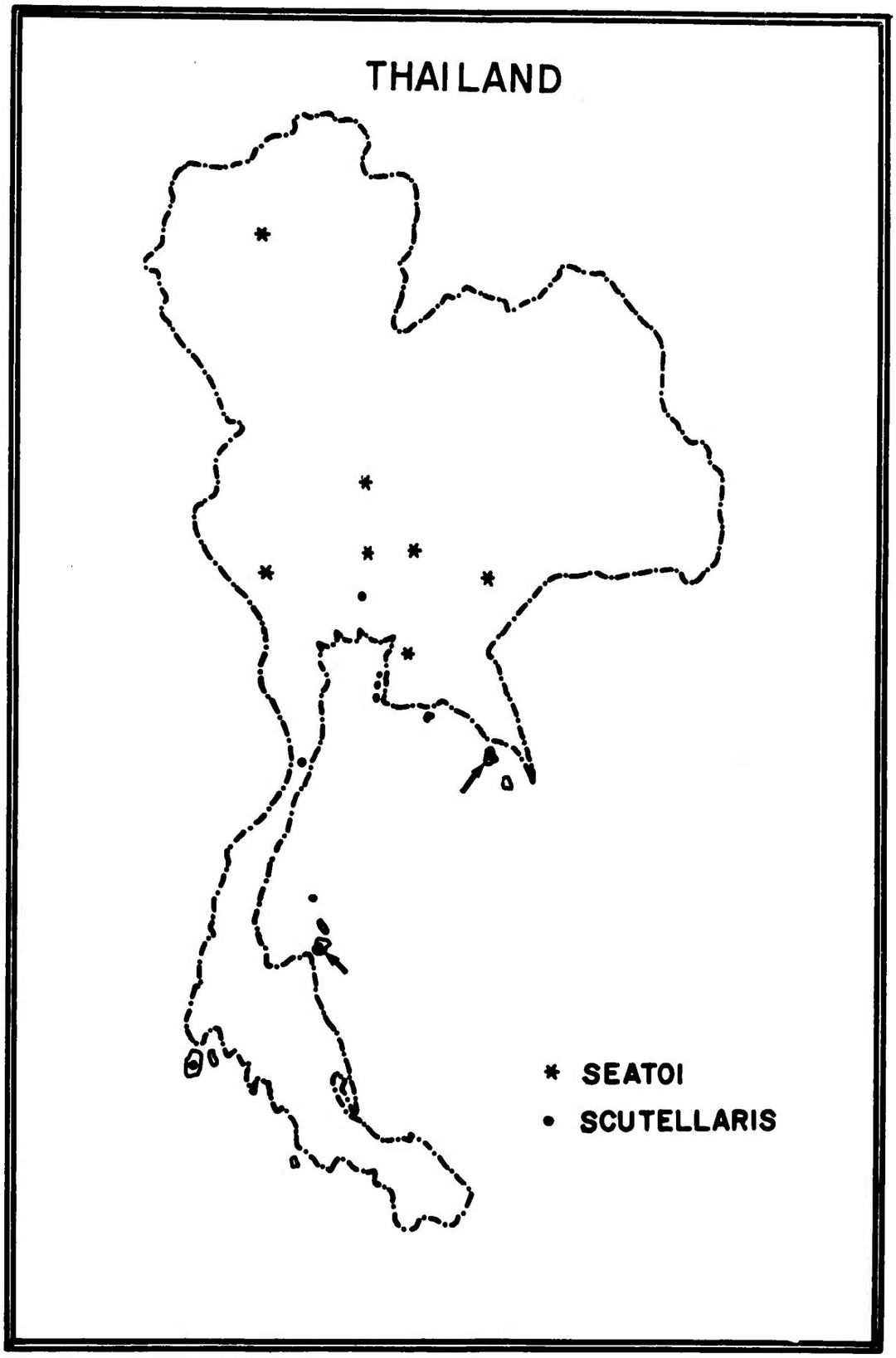


FIG. 1

Table 1. Distribution and association of Stegomyia species in 90 bamboo oviposition cups, Angthong - 1969

Species	Number with cups	Per cent
A. aegypti	2	2
" seatoi	1	1
" albopictus	48	53
" albopictus + aegypti	2	2
" albopictus + gardnerii imitator	2	2
" albopictus + pseudalbopictus	2	2
" albopictus + seatoi	13	14
" albopictus + Tripteroides	7	8
" albopictus + aegypti + seatoi	2	2
" albopictus + g. imitator + seatoi	1	1
" albopictus + Tripteroides +seatoi	1	1
Species other than Stegomyia	9	10

Table 2. Frequency of Culex (Culex) collections in Chieng Mai province by habitat and size of habitat, 1969-1970.

		Small				Moderate			Large					Percent		
		Animal Foot Print	Wheel Track	Stump Hole	Artificial Containers	Well	Ditch	Ground Pool	Pit	Small Pond	Rice field	Swamp	TOTAL	Small	Moderate	Large
pipiens group	fuscocephala	26	1	-	-	1	8	11	2	1	6	1	57	47.4	35.1	17.5
	hutchinsoni	7	-	-	-	1	1	1	-	-	-	1	11	63.6	27.4	9.1
	quinquefasciatus	-	-	1	6	1	5	-	-	2	-	-	15	46.7	40.0	13.3
sitiens group	bitaeniorhynchus	1	-	-	-	-	1	2	-	1	-	-	5	20.0	60.0	20.0
	gelidus	-	-	-	-	-	3	3	1	4	-	-	11	0	60.0	40.0
	tritaeniorhynchus	7	-	-	2	1	12	5	3	4	11	-	45	20.0	40.0	40.0
	annulus	3	-	1	1	1	8	10	1	4	7	1	37	13.5	51.3	35.1
	pseudovishnui	1	-	-	-	1	-	1	-	-	2	-	5	20.0	40.0	40.0

Mosquito Vectors of Malaria

Principal Investigators : Douglas J. Gould, Ph.D.
Bruce A. Harrison, CPT, MSC
Michael F. Sullivan, CPT, MSC

Assistant Investigators : Prajim Boonyakanist
Larp Panthusiri

OBJECTIVE : To investigate the bionomics and population dynamics of those species of Anopheles responsible for transmission of human malaria in Southeast Asia.

DESCRIPTION : Specific factors being studied in the attempt to define the actual and potential vector species present in Thailand include determination of host preferences, susceptibility to infection with malaria, flight range, longevity and patterns of biting activity.

PROGRESS : During this period, malaria surveys were conducted in Buriram and Prachinburi provinces. A persistent foci of vivax malaria in Tambon Lam Duan, Amphur Krasang in northeastern Buriram province prompted the first survey from 19-25 May. This trip was a follow up to surveys reported in the previous year. Neither An. minimus nor balabacensis has been collected in this area in recent years, thus the identity of the malaria vector is not known. A total of 759 anophelines were collected, with ten man-biting collections yielding 241 mosquitoes and two buffalo-biting collections yielding 518 mosquitoes. An. annularis accounted for 624 of the adults, of which 70% were collected from buffalos. A regional Malaria Eradication Project team made 401 blood smears from residents in two villages in the Tambon during this period. All smears were negative for malaria. Dissections from 643 mosquitoes were also negative for malaria, thus the identity of the vector there is still not known.

The second survey was prompted by reports of a malaria epidemic in Amphurs Aranyaprathet and Kabinburi, Prachinburi province. This survey was divided into two trips. The first trip (26 June-6 July) involved 21 collections of adult anophelines, 4 from buffalo,

9 from human bait and 8 collections of mosquitoes resting on vegetation around houses in both districts. A total of 632 mosquitoes were collected, of which 187 were An. balabacensis, one of the primary malaria vectors in Thailand. Seven balabacensis out of 182 dissected contained malaria parasites.

The second trip (19-29 July) into Prachinburi concentrated on a malaria epidemic in Tambon Thung Pho, Amphur Kabinburi. Thick Blood films, taken at random from 327 residents of 7 villages, were examined by the Department of Epidemiology and 157 (48%) were found to have malaria parasites. Of these, 131 (83%) had Plasmodium falciparum, 21 (13%) had P. vivax, and 4 (2.5%) had mixed P. falciparum-P. vivax infections. One P. malariae infection was found. During this period 19 adult mosquito collections were made, 10 from human bait, and 9 collections of mosquitoes resting in houses or in vegetation near houses. These collections yielded 1,602 mosquitoes, of which 1,099 were An. balabacensis. Four different collecting methods were used to obtain adult balabacensis. A comparison of the efficiency of these 4 methods is as follows :

A) biting man-inside house -- 0.5 mosq./man/hour
B) biting man-outside house -- 1.3 mosq./man/hour
C) resting-inside house -- 3.5 mosq./man/hour
D) resting-outside vegetation near house -- 1.4 mosq/man/hour

No explanation can be given at present for the disparity between "biting man-inside" and "resting-inside" collections. A total of 1,035 balabacensis were dissected during the trip and 9 were positive for malaria parasites.

Project 3A663713D829 MILITARY MEDICAL RESEARCH PROGRAM S.E.ASIA

Task 00, Malaria Investigations

Work Unit 112, Field studies on drug resistant malaria

Literature Cited.

References:

1. Sadun, E. H. and Gore, R. W., Exptl. Parasitol. 23:277, 1968.

2. Cohen, S., McGregor, I.A., and Carrington, S.P., Nature 192: 733, 1961.

3. Cohen, S., Butcher, G.A., and Crandall, R.B., Nature 223:368, 1969.

4. Trigg, P.I., Parasitology, 59:925, 1969.

5. Betke, von K., and Kleihauer, E., Blut, 4:241, 1958.

6. Huang, Y.M., Proc. Ent. Soc. Wash. 71:234 (1969).

7. Genetics of Insect Vectors of Disease. 1967 p. 151.

8. J. Med. Ent. 6:327

Publication:

1. Vejjajiva, A., et al.: The Combined Effect of Carbon Dioxide and Acetozolamide on Cerebral Blood Flow in Occlusive Cerebrovascular Diseases. Excerpta Medica. International Congress Series, No. 193, 821, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 0A 6537	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	114
b. CONTRIBUTING				
XXXXXXX CDOG	1412A(2)			

11. TITLE (Precede with security Classification Code)

(U) Malaria Program Supervision (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012100 Organic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 07	CONT	DA	C. IN-HOUSE

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	9	630
	CURRENT 71	9	630

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202/576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Division of Medicinal Chemistry
Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Rothe, COL W. E.

TELEPHONE: 202/576-2280

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Sweeney, Thomas R., Ph.D. DA

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Drugs; (U) Biology; (U) Chemistry

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To manage, to integrate, and provide quality control for the Drug Research Program on Malaria, both in-house and by contract.

24 (U) To define areas requiring investigation, to develop suitable contract proposals, to follow progress by correspondence or site visits, to guide direction of investigation, to provide for exchange of information, and to continually check findings for verification through independent agencies (both in-house and contract). Two outside advisory groups are utilized.

25 (U) 69 07 - 70 06 Close supervision, through guidance and integrated evaluation of productivity, was continued for forty-nine contracts in the areas of chemical synthesis and drug preparation and for twenty-three biological contracts concerned with the preclinical aspects of anti-malarial efficacy and drug safety.

Preclinical workups continued on two new Investigational New Drug (IND) basic documents with seven IND supplements in preparation. Five meetings of advisory groups were organized and conducted to examine specific facets of antimalarial drug development and utilization. In-depth metabolism and safety studies were instigated following clinical feed-back on five drugs under active study in man. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 114, Malaria program supervision

Investigators
Principal: COL William E. Rothe, VC
Associate: Thomas R. Sweeney, Ph.D.

The Contract Chemical Synthesis Program

During FY-70 there was a maximum of 54 active contracts; only 24 of these will be carried beyond 30 June 1970. During the year 5 new contracts were let. There are, in addition, 4 strictly preparations facilities for obtaining large quantities of desired compounds; one of these was added during the year; one preparations laboratory contract expired with the fiscal year. Finally, there is one contract to study the feasibility of the large scale photochemical synthesis of the phenanthrenemethanols and one to synthesize radioactivity tagged compounds for metabolite studies. At present there are 12 contracts with academic institutions, 9 with industry and 7 with research houses.

During FY-70 there was a total of approximately 1500 compounds submitted from the synthesis contracts, of which 900 were target compounds. As of the end of FY-70 the average cost per target compound is $2300. During the year the number of compounds requested from the preparations laboratories was 12, 22 and 6 in small, medium and large quantities, respectively. Compounds received numbered 5, 14 and 9 in the same quantitites. Chemical publications and submitted manuscripts numbered 61.

Actively pursued synthesis areas can be broken down as a percentage of obligated funds as follows:

Area	Percent
Quinoline methanols	6
Phenanthrene methanols	29
Other amino alcohols	15
Naphthothiophene	
Pyridine	
Aminohydroxy acephenanthrene	
Quinolizone	
Amino hydroxy, dihydrophenanthrene	
Diazaphenanthrene	
Benzacridine	
Aminoquinoline type	6
Antifols and antimetabolites	21
Polyhalogen compounds	6

Miscellaneous
Hydrozamic acids 17
Oxazines
Pyridol derivatives
Bix thiocyanato phenois
Quinoline quinones
Regression analyses
Benzothiopyrans

The main thrust of the synthesis program is with the antifols (antimetabolites) and the aminoalcohols. The phenanthrene aminoalcohols continue to hold the most promise as a source of useful drugs. Two very promising areas that have emerged are the 4-amino-2-stilbazole type and the 2,4-diamino-5-arylthioquinazolines; these are being explored. Areas that have not lived up to expectations are the hydroxamic acids, the polyhalogeno compounds, the pyridol derivatives and the benzothiopyrans.

There have been three feasibility studies on new approaches to large quantities of the substituted phenanthrenes during the year. One, the conversion of a phenanthrene decarboxylic acid to a bis trifluoromethyl phenanthrene through a reaction involving sulfur tetrafluoride, after a reasonable try, was abandoned because of very difficult chemistry, difficult product separation problems and low yields. A study of the formation of the desired phenanthrene through a thermal ring closure is still in progress. The most promising approach at this time is the photochemical ring closure which appears to be entirely feasible for intermediate scale reactions and possibly adaptable to very large scale operations.

The synthesis program has increased tremendously the effort expended within the Division on the preparation of material for patent application. It is deemed important to expedite the patent applications in order to speed up publications and the exchange of information between contractors and between WRAIR and potential contractors. Preparation of the material in fairly complete form can most quickly be done within the Division because therein resides the intimate knowledge and familiarity with the chemistry and biology involved.

Acquisition of Chemicals

New chemicals for screening, other than those from the synthesis program, continue to be received. Although gift compounds trickel in at about the same rate as last year, the compounds received under the No Dollar Agreement increased appreciably because of the efforts of the Division in securing the release of several thousand compounds from a commercial source.

Biology

Primary screening of all drugs received during the report period was accomplished. Drugs showing promise in the primary screens were further

examined in more definitive secondary systems. Emphasis in the secondary screens were placed on effectiveness against resistant strains, against preerythrocytic forms, and by oral administration.

Progress

a. Primary Screens

1. All drugs received were submitted to the University of Miami for testing in the blood induced Plasmodium berghei - mouse screen. A total of 23,000 drugs were evaluated, and of these, 1295 exhibited significant activity. Drugs normally were tested at three levels with active drugs being retested at six levels. Twenty-four thousand, five hundred (24,500) drugs were also tested in the blood induced P. gallinaceum - chick system. Of these, 297 were active.

2. The in vitro systems at the University of West Virginia were discontinued during the contract period. A total of 6037 assays on 461 compounds were run in the carbohydrate metabolism system. Nine hundred and eight were active. In the system measuring inhibition of nucleic acid synthesis, 5486 assays were conducted on 4221 compounds. Greater than 40% inhibition occurred with 408 compounds.

3. The system based on inhibition of sporozoite formation in the salivary glands of mosquitoes which was operated by Insect Control and Research in Baltimore was discontinued during the report period. In the system using Aedes aegypti mosquitoes and Plasmodium gallinaceum parasites, a total of 29,275 examinations were conducted on 17,644 drugs in this system. Of these, 1662 were active at .10% drug concentration and 307 at .01% drug concentration or lower. A total of 3773 tests were conducted on 337 drugs using Anopholes stephensi mosquitoes and P. cynomolgi parasites. Three hundred and sixty drugs showed activity with 337 of them showing activity at .01% or lower drug concentration. An attempt was made to ship frozen sporozoites to Dr. Rane at the University of Miami for use in a sporozoite-chick test system. Although there was some successful transmission, the results proved too variable to use in a routine test system. A limited capability for this type of testing is being maintained at the National Naval Medical Center under the supervision of Dr. L. A. Terzian. During the report period, Dr. Terzian tested 80 compounds. Dr. Terzian can employ various strains of P. vivax and P. falciparum as well as P. gallinaceum and P. cynomolgi.

b. Secondary Screens

1. The major effort at Illinois Institute of Technology Research Institute was concentrated in the test system employing sporozoite induced P. berghei infections in mice. By varying the time of administration of drug, some insight can be obtained as to whether the test drugs act as a causal prophylactic or curative agent. Testing against resistant organisms was discontinued at IITRI, however, these organisms have been banked by cyropreservation so that testing could be renewed upon short

notice if it became desirable to do so. IITRI continues to act as a repository for various strains of malaria, providing these parasites to other investigators when requested. Tests conducted during the reporting period and number found active are as follows:

Test System	Total Cmpds. Tested	Active
Secondary	11	4 PO 5 SQ
CHL Res.	39	13
DDS Res.	10	7
TRZ Res.	57	29
Sporo-Mouse	22	6 Prophylactic only 6 Blood Schizont only 8 Both

2. The test system at the University of Georgia under the supervision of Dr. Paul Thompson which employs drug resistant P. berghei in mice was expanded to a throughput of six compounds per week. This is adequate to conduct all preliminary screening against drug resistant organisms. Strains resistant to chloroquine, triazine, DDS, and pyrimethamine are available. Since drugs are administered in the diet or by gavage, this system also provides an estimate of the efficacy of the drug by the oral route of administration. The following is a summary of work conducted during this report period:

	# Drugs
P Strain	101
TRZ Strain	54
DDS Strain	13
CHL Strain	66

Onset and Duration of Action Studies - 4

3. Drugs which were possible folic acid antagonists were submitted to Dr. C. C. Smith at the University of Cincinnati for determination of folic acid inhibition and the reversibility of the action. A total of 1449 assays were conducted on 483 compounds. Of these, 322 inhibited folic acid synthesis. This inhibition was reversed with 166 compounds when folic or folinic acid was added to the system. A total of 87 assays were conducted on 29 compounds against drug resistant organisms. Twelve compounds showed cross resistance.

Conclusions

During the report period, there was a concerted effort to make the drug screening program more efficient and economical, eliminating those systems which were not absolutely essential. Primary screening now is concentrated in the system at the University of Miami. Secondary screening against drug resistant P. berghei is being carried out at the University of Georgia, however, testing can be resumed at Illinois Institute of Technology Research Institute on short notice.

Pharmacology and Clinical Program

During the past year the Pharmacology Department supervised the contracts responsible for preclinical studies of antimalarial drugs scheduled for clinical trial. These contracts included studies on the metabolism of these agents, as well as the toxicity. The Department of Pharmacology also provided direct communication with the clinical centers involved in Phase I and Phase II drug trial. There are 12 primary contracts concerned with the preparation of preclinical information; ten concerned with metabolism and toxicology of candidate compounds, one concerned with the formulation and one concerned with the purity and potency of the formulated materials. Three clinical contracts were added during this period. During the past year the Department of Pharmacology prepared one "Notice of Claimed Investigation Exemption for a New Drug" as well as 14 supplements. Details on each of these drugs are reported in their respective claims.

Phase I studies were completed for WR 5677, 33063, 30090, and 40070 as well as combination studies with WR 6798 + WR 1544 + WR 2975. Pharmacokinetic studies were done on WR 6798 and WR 5677. Prophylactic studies have been completed on a combination of WR 6798, WR 1544 and WR 2975. A Phase I study involving three day medication with WR 5949 and WR 4629 in combination has been completed. A study is being conducted in an attempt to identify the metabolites of WR 448 which are responsible for the methemoglobinemia. Phase II studies are in progress for WR 33063 and WR 30090.

Summary and Conclusions

The so-called third phase of the Malaria Program - the use of human plasmodia for the selection of agents to be developed either by chemical derivation or through preclinical study - is operating satisfactorily. The efficacy of new agents is routinely determined at the preclinical level against both drug sensitive and drug resistant P. falciparum. The Chicago in vitro test is available for rapid screening of small samples, while the Aotus/falciparum system is employed for critical evaluation of candidate drugs. The use of these systems permits the selection of appropriate chemical development of new structure leads which do not show cross resistance against human strains of interest.

The Malaria Program continues in a balanced fashion, but at a reduced level of effort commensurate with budget restrictions. Several interesting drugs are well along in their clinical evaluation, and many promising candidates are progressing through the drug development regime.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 114, Malaria program supervision

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6513	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	123
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Biological studies on anopheline vectors of malaria (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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: Not Applicable

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	1	35
	CURRENT 71	1	30

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, D.C. 20012

ADDRESS:

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL, W. H.

TELEPHONE: 202 - 576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of CD and I

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic participation)

NAME: Ward, Dr. R. A.

TELEPHONE: 202 - 576-2553

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Bergman, CPT E. A.

NAME: Neal, T.J., BS DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Anopheles; (U) Colonize; (U) Mosquitoes; (U) Vectors.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) Development of new anopheline control procedures through a knowledge of vector biology and ecology.

24. (U) Establishment of laboratory colonies of anophelines from areas of strategic importance. Evaluation of rearing procedures through alteration of environmental factors. Study of mosquito pathogens on mosquito behavior, especially as related to disease transmission.

25. (U) 69 07 - 70 06. Establishment of new anopheline colonies: A. freeborni and a Pakistan strain of A. stephensi. A new mass rearing procedure has been developed for A. stephensi. Although mean dry weight of adults is less in the mass rearing method than in the conventional small pan procedure, adult longevity is probably greater with the new technique. Nosema-infected A. quadrimaculatus showed no significant difference in their ability to transmit simian malaria as compared with uninfected controls. The mortality of infected mosquitoes was extremely high, with none living beyond 12 days; thus precluding malaria transmission. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 20 Jun 70.

* Available to contractors upon originator's approval.

DD FORM 1 MAR 68 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 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 123, Biological studies on anopheline vectors of malaria

Investigators
Principal: Ronald A. Ward, Ph.D.
Associate: LTC Bruce F. Eldridge, MSC; CPT Louis C. Rutledge, MSC; CPT Edward A. Bergman, MSC; David E. Hayes; Talmadge J. Neal, B.S.; Ronald Lowe, Ph.D.*, and Kenneth Savage, M.S.*

Description

Biological studies of anopheline mosquitoes are performed, using material maintained in the insectary at this Institute. Studies include evaluation of rearing techniques and their effect on research results, development of new rearing techniques, the effect of biological agents on anopheline mosquitoes, and nutritional studies of artifically reared anophelines.

Progress

1. Laboratory rearing of anophelines

By carefully washing all anopheline eggs with distilled water, and keeping all water containers in which larvae and pupae are maintained or handled scrupulously clean, the level of infection of Nosema stegomyiae, a microsporidian parasite, has been reduced to the point where it is of no significance in rearing procedures.

Several strains and species of Anopheles were added to the insectary. With new species of culicines colonized from nature for the first time (reported elsewhere), the list of colonized material maintained has been substantially increased. Species presently maintained are shown in Table 1.

2. Evaluation of rearing procedures

Last year, a procedure was developed which permitted the mass rearing of anopheline mosquitoes in large (30 X 54") stainless steel trays. The chief advantage of the system was that many of the handling procedures, such as separation of larvae from pupae, and transfer of pupae into smaller containers, could be accomplished using equipment and techniques which saved literally hours of time. Before the procedure could be adopted as the standard rearing procedure, however, it was necessary to evaluate the quality of the mosquitoes produced, as evidenced by body weight, longevity, and activity level.

---

* Insects Affecting Man and Animals Laboratory, U.S. Dept. of Agriculture, Gainesville, Florida

Preliminary studies were done on the assumption that optimum larval density was related to surface area of the breeding water. The area of large pans are approximately 10 times that of the small pans. Consequently 10 times as many larvae were initially placed in the large pan as in the small pans (2500 vs. 250). Larvae were then fed twice daily, with as much food being added each time as the larvae would consume before the next feeding.

Results indicate that anophelines reared using the new procedure (large pan) are definitely lighter in weight than those reared in the conventional manner (small pan). There is not, however, any observable decrease in adult longevity. In fact, a longevity experiment performed with Anopheles stephensi showed that large pan males and females lived longer, on the average, than did conventionally reared controls.

Table 2 shows the mean dry weight of males and females of 2 anopheline species reared by the 2 procedures. The mean weights of all comparable groups differ significantly between large pan rearing and small pan rearing. The difference between small and large pan male An. stephensi is not significant.

Table 3 indicates the time to 50% mortality for the same experimental groups.

3. Effect of Nosema infection on malaria transmission

Preliminary investigations at the U.S. Department of Agriculture Insects Affecting Man and Animals Research Branch Laboratory in Gainesville, Florida indicated a possible interference effect of Nosema stegomyiae (a microsporidian protozoan parasite of mosquitoes) upon malarial transmission. Test results with Anopheles quadrimaculatus showed that Nosema-infected mosquitoes were far less efficient experimental vectors of an avian malarial parasite (Plasmodium gallinaceum) than non-infected controls.

A collaborative project was undertaken by the USDA laboratory and the Department of Entomology, DCD&I to determine if this also occurred in mosquitoes infected with the simian malarial parasite, P. cynomolgi. First stage A. quadrimaculatus were exposed to Nosema spore suspensions in Gainesville, Florida and the resultant adult mosquitoes were shipped by air to WRAIR for experimental malaria studies with the simian malarial parasite. Control lots of mosquitoes were reared in Florida and simultaneously sent to WRAIR.

The level of Nosema-infection ranged between 50 - 95% in material received at WRAIR. After receipt, the adults were kept in the insectary for 1 - 4 days and then fed on infected monkeys. Malarial oocyst dissections were made 8 days after infection. In some instances, the microsporidian infection was so severe that only a small sample or no mosquitoes were available on the 8th day for dissection. In a series

of 6 experiments, using 2 strains of P. cynomolgi, there were no significant differences in mean oocyst counts among groups of Nosema-infected and control lots of A. quadrimaculatus simultaneously fed on infected monkeys (Table 4). Despite this lack of response on oocyst development by day 8, the fact that virtually no microsporidian-infected anophelines survived until day 12, a time at which the sporozoites would normally be infective, indicates that this parasite might be an effective means of interrupting malaria transmission.

Conclusions and recommendations

1. Procedures are being used in the WRAIR insectary which minimize the adverse effects of infections of anopheline mosquitoes by Nosema stegomyiae. A new mass rearing procedure for anophelines has received a preliminary evaluation which indicates that the mean dry weight of resulting adults is less when this procedure is used in contrast with the established procedure. It was determined that Nosema-infected Anopheles quadrimaculatus mosquitoes showed no significant reduction in their ability to transmit Plasmodium cynomolgi parasites as compared with uninfected controls. The mortality of infected mosquitoes was very high, however, with virtually none living as long as 12 days. This in itself would serve to present malaria transmission.

2. Further evaluation of the mass rearing technique should be done. Other biological characteristics of anophelines should be measured in Nosema-infected and non-infected adult mosquitoes.

TABLE 1

Mosquito Colonies Maintained at WRAIR

Species	Source	Status
Armigeres subalbatus	Singapore	Subcolony
" "	Japan	"
Aedes aegypti	Koh Samui	"
Aedes canadensis	Maryland	Not established
Aedes polynesiensis	Samoa	Subcolony
Ae. scutellaris malayensis	Singapore	"
Anopheles quadrimaculatus	Florida	"
Anopheles freeborni	California	"
Anopheles balabacensis	Thailand	"
Anopheles stephensi	India	"
" "	Iran	"
" "	Pakistan	"
Culex salinarius	Maryland	Established
Culex restuans	"	Not established
Culex tritaeniorhynchus	Japan	Subcolony
Culiseta melanura	Maryland	Not established

TABLE 2

Dry Body Weights of 2 Species of Anopheles Resulting from 2 Different Rearing Procedures*

	Large Pan		Small Pan	
Species	Males	Females	Males	Females
An. stephensi	0.387 ± 0.007	0.502 ± 0.011	0.402 ± 0.014	0.590 ± 0.014
An. quadri-maculatus	0.402 ± 0.014	0.620 ± 0.030	0.526 ± 0.019	0.840 ± 0.036

* Numbers shown are mean ± standard error of approximately 25 individuals each.

TABLE 3

Time (Days) to 50% Mortality for 2 Species of Anopheles Resulting from 2 Different Rearing Procedures

	Large Pan		Small Pan	
Species	Males	Females	Males	Females
An. stephensi	20.2	21.2	13.4	14.0
An. quadri-maculatus	9.0	12.0	11.4	11.4

TABLE 4

Effect of Microsporidian Infection of Anopheles quadrimaculatus on Susceptibility to Plasmodium cynomolgi (RO/PM and "B" strains). (Malarial susceptibility is assayed in mean number of malarial oocysts per dissected gut)

Date	Parasite strain	Mean number oocysts	
		Nosema-infected	Non-infected
12 Mar 70	RO/PM	7.3	1.1
13 Mar 70	RO/PM	4.5	0.9
13 Mar 70	"B" strain	0	10.8
26 Mar 70	RO/PM	2.5	6.6
4 May 70	"B" strain	2.0	4.6
7 May 70	"B" strain	0.3	0.1

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 123, Biological studies on anopheline vectors of malaria

Literature Cited

1. References - none

2. Publications

1. Rutledge, L.C. Some Effects of Fumagillin on Anopheles stephensi. Mosquito News 30 (In press), 1970.

2. Ward, R.A. and Scanlon, J.E. (Editors). Conference on Anopheline Biology and Malaria Eradication. Misc. Publ. Entomol. Soc. Amer. 7: 1, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6514	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	124
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Biological Studies of mosquito malaria infection and transmission (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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: Not Applicable

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	1	35
FISCAL YEAR CURRENT 71	1	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, D.C. 20012

ADDRESS:

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202 - 576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Div of CD and I
Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Ward, Dr. R. A.

TELEPHONE: 202 - 576-2553

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Rutledge, CPT L.C.

NAME: Hayes, D.E. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Anopheles; (U) Genetics; (U) Mosquitoes; (U) Plasmodium; (U) Susceptibility; (U) Falciparum malaria; (U) Aotus

23. TECHNICAL OBJECTIVE. 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Development of genetic and environmental means of interrupting malaria transmission through an understanding of factors influencing susceptibility of anopheline vectors to malaria and of factors determining transmission efficiency of vectors. Also, the development of test systems for the evaluation of antimalarial drugs.

24. (U) Studies are conducted on the infectivity of human and closely related simian malarial parasites to various mosquito vectors of SE Asian areas. Attempts to transmit falciparum malaria to lower primates are made. Matched feedings of anophelines on gametocytemic hosts are conducted, followed by dissections of samples of the mosquitoes at intervals thereafter to determine level and progress of mixed strain infections. Mass selection procedures are used to select more efficient and less efficient vectors of malaria.

25. (U) 69 07 - 70 06. Two new strains of chloroquine resistant falciparum malaria from Vietnam and the Philippines have been established in splenectomized Aotus monkeys through serial passage of infected blood. Attempts to infect anopheline mosquitoes have been negative to date and it is believed that gametocyte immaturity is the responsible factor. Studies with the P. cynomolgi - rhesus monkey system have served to characterize, identify and quantitate a number of factors affecting the transmission of malaria to mosquitoes. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 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 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 124, Biological studies of mosquito malaria infection and transmission

Investigators
Principal: Ronald A. Ward, Ph.D.
Associate: CPT Louis C. Rutledge, MSC; CPT Stephen C. Hembree, MSC; CPT Anthony J. Johnson, VC; David E. Hayes

Description

Studies are conducted on the infectivity of human and simian malarial parasites to various mosquito vectors of malaria. Special emphasis is placed on the role of genetic and environmental factors in mosquito susceptibility. Mosquito transmission of falciparum and vivax malaria to lower primates is attempted. The results of these studies are applied to the development of test systems for the evaluation of antimalarial drugs.

Progress

1. Transmission of human malaria to monkeys

In an attempt to establish cyclical passage of falciparum malaria in Aotus trivirgatus 2 additional isolates of this parasite (PER strain - Philippines and Brai. strain - Vietnam) were obtained from Dr. David Clyde, University of Maryland School of Medicine. Since both these isolates had recently been transmitted to human volunteers by mosquito bite, it was postulated that the probability of establishment of mosquito transmission with such strains would be greater than with a strain such as the Aotus - Camp which had an extensive history of consecutive blood passages in subhuman primates. The details of the infection of Aotus are indicated in Table 1. 4/5 animals became patent while 1 monkey (395) remained negative 49 days after inoculation. Immature gametocytes were extremely abundant in the 3 positive splenectomized Aotus and 26 separate feeding trials of Anopheles species were performed upon these monkeys. In all instances, the mosquitoes exhibited no malarial oocyst development on the 8th day after an infective blood meal the time when midgut dissections were performed. The Brai. and PER strains have been serially passed into new Aotus and certain of these animals are being treated with an immunosuppressent (Imuran) in an attempt to achieve a higher degree of gametocyte maturity through the suppression of the lymphocyte-macrophage system.

Experiments on the cyclical transmission of the Chesson strain of vivax malaria which were described in the 1969 WRAIR Annual Report were continued. 3 consecutive monkey-mosquito-monkey cycles were completed by the end of 1969. A comparison of the infection of P. vivax in Aotus

by blood transfer or sporozoite transmission is shown in Table 2. Gametocytes appeared 3 weeks later in animals infected by mosquito bite as contrasted to blood passage of parasites. The overall gametocyte levels were similar in both groups while the peak gametocytemia occurred about 10 days after the appearance of the first gametocytes. It was possible to infect mosquitoes on Aotus from the first day gametocytes were visible, and in one instance, mosquitoes were infected one day prior to the first sign of gametocytes on blood films.

Considerable variation was present in the infectiousness of individual monkeys to pools of anophelines (Table 3). This was similar to the situation experienced in feeding anophelines on other malarial parasites in different host systems. Gametocytes from sporozoite-induced infections were equally infectious to anophelines in comparison with blood passaged infections. In the former, 25/46 pools of mosquitoes applied (54.3%) were infected, while in the latter category 41/96 (42.7%) were infected. Although the pool infection rates were similar, there was a marked difference in the quantitative oocyst counts (Table 2). Mosquitoes fed on blood-infected Aotus had peak mean oocyst counts of 2.8/mosquito while the counts from monkeys infected with sporozoites averaged 10.7/mosquito.

Transmissions have been made with 3 vector species: Anopheles b. balabacensis, A. quadrimaculatus and A. stephensi. A. b. balabacensis is the most efficient vector with 3 times the number of oocysts developing on the mosquito midgut as in the other 2 anophelines. A typical oocyst count for the former species is 60/mosquito, for the latter 2 species, 20. In general, P. vivax, gametocytes in Aotus are less infective to anophelines than vivax gametocytes in man or chimpanzees by a factor of 3 or 4. This does not however, appreciably affect the establishment of cyclically-passed Chesson vivax in a monkey system.

2. Transmission of simian malaria

The Anopheles stephensi - Plasmodium cynomolgy - rhesus monkey host-parasite system is used as the standard laboratory model for malarial studies due to its close resemblance to human vivax malaria, ease of mosquito infection and transmission, and a consistant source of a hardy simian host.

A paper is being prepared giving time series analyses of the infectiousness of 5 rhesus monkeys which were infected with cynomolgi malaria by mosquito bite. Periods of gametocytemia ranged from 13-14 days (mean = 23.6) in individual monkeys. The mean gametocyte count over the period of gametocytemia ranged from 420-680 gametocytes/mm^3 of blood (mean = 550) and the mean oocyst count in mosquitoes fed during the same period ranged from 5.5-64.8 oocysts/mosquito (mean = 36.4). Mean gametocyte survival rates (ratio of mean number of 8 day oocysts to mean gametocyte counts) ranged from 0.6 to 5.0% (mean = 2.8%). These figures

provide a measure of the comparative efficiency of the transmission of P. cynomolgi.

Within individual monkeys gametocytemia follows the tertian periodicity of parasitemia in general, and this periodicity is rather closely reflected by the daily transmission record (oocyst counts). With respect to the primary attack and short-term relapses (recrudescenses), however, the situation differs. The several attacks (primary attack and recrudescences) differ in infectiousness to mosquitoes. Typically, those that occur near the middle of the course of gametocytemia produce greater numbers of oocysts on a per-gametocyte basis. At this time, moreover, greater numbers of gametocytes are usually present.

When paired gametocyte and oocyst counts are considered the linear correlation is quite low (range 0.0 to 0.6; mean = 0.18). The lag correlation does not significantly improve this situation. However, when the data are fitted to a second-order polynomial, much better correlations are observed (range 0.3 to 0.8; mean = 0.65). The regression equations deriving from this model imply infection threshold values on the order of 10 - 100 gametocytes per mosquito blood meal and optimal values of 3000 - 9000 gametocytes per mosquito blood meal, depending on the individual monkey. Gametocyte levels greater than 7000 per mosquito blood meal have not been observed.

3. Analysis of malarial oocyst distributions

Among similar mosquitoes exposed to equal risk of infection, the coefficient of variation in oocyst counts is on the order of 100%. Frequency distributions of individual counts within such series show a marked clumping of oocysts within individual mosquitoes. Since this clumping is a measure of the relative susceptibility of individual mosquitoes to infection, an attempt is being made to identify the applicable frequency distribution so that dispersion constants can be computed. Some 170 series of oocyst counts derived from experiments with 6 species of Plasmodium, 6 species of mosquitoes and 3 species of vertebrates are being coded and punched for machine processing. The Division of Biometrics and Information Processing is preparing a computer program for fitting the Poisson and negative binomial distribution to oocyst data.

4. Genetic aspects of mosquito susceptibility

Direct experimental evidence of genetic control of the susceptibility of Anopheles stephensi to P. cynomolgi is being sought through inbreeding experiments. 25 inbred lines of this species (Iran strain) were established in November 1969. Two of these lines have been carried through 7 generations of inbreeding and these now appear to be firmly established. These 2 inbred lines are now being compared with the parent (stock) strain with respect to their susceptibility to infection with P. cynomolgi. It is expected the coefficient of variation

in oocyst counts in these 2 lines will be reduced. This reduction would be a measure of the importance of the genetic component of susceptibility to infection. The possibility exists also that an inbred line might prove relatively resistant to malarial infection.

Conclusions and recommendations

1. Two new strains of chloroquine resistant falciparum malaria from Vietnam (Brai. strain) and the Philippines (PER strain) have been established in splenectomized Aotus trivirgatus through serial passage of infected blood. Attempts to infect anopheline mosquitoes have been negative to date and it is believed that gametocyte immaturity is the responsible factor. Treatment of animals with immunosuppressant drugs or antilymphocyte serum may prolong gametocyte survival until they attain a level of maturity which is suitable for infecting mosquitoes.

2. Cyclical transmission of Chesson vivax malaria has been achieved in Aotus and the problem of gametocyte infectivity and mosquito susceptibility in this host-parasite system has been discussed. Future progress is largely dependent upon the procurement of adequate quantities of Aotus monkeys throughout the year.

3. Studies with the P. cynomolgi - rhesus monkey system have served to characterize, identify and quantitate a number of factors affecting the transmission of malaria to mosquitoes. The phenomenon of individual variation in the susceptibility of anophelines to malaria infection is being critically examined and the genetic basis of such variation is being investigated. Most previous work in this area has been done with avian malaria and culicine mosquitoes. It is recommended that the present studies be continued due to the close similarity of cynomolgi malaria to human vivax malaria and the applicability of the information acquired to attempt to establish a mosquito-transmissible strain of P. falciparum in monkeys.

TABLE 1

Infection of *Aotus trivirgatus* with *P. falciparum*-infected blood from human volunteers. All monkeys were splenectomized prior to infection except 967 which had an intact spleen.

Monkey no.	Parasite strain	Prepatent period (days)	Day of peak parasitemia	Maximum parasitemia (%RBC)	No. of mosquito infection trials	Day of death
67	PER	17	27	5.5	17	-
388	PER	17	25	44.0	4	27
967	PER	12	-	< 0.01	0	-
369	Brai.	10	24	62.0	5	24
395	Brai.	-	-	-	0	-

TABLE 2

Infection of splenectomized Aotus trivirgatus with Plasmodium vivax (Chesson strain)

	Route of infection			
	Blood transfer (n = 7)		Sporozoite transmission (n = 4)	
	Mean	Range	Mean	Range
Day of 1st gametocytes	17.4	9-26	40.3	28-64
Day of 1st mosquito infection	21.7	16-27	40.3	28-64
Day of peak gametocytemia	26.3	10-40	51.8	45-68
Day of peak mosquito infection	33.9	20-91	46.5	35-69
Peak gametocyte count/mm^3 blood	2170	800-3800	2680	1500-4100
Highest mean oocyst count/ mosquito	2.8	0.1-5.9	10.7	2.4-20.2

TABLE 3

Infection of mosquito pools (Anopheles stephensi from P. vivax (Chesson strain) infected Aotus trivirgatus

	Route of infection			
	Blood transfer		Sporozoite transmission	
Aotus no.	No. pools infected / No. pools fed	% infected	No. pools infected / No. pools fed	% infected
1	5/15	33.3	1/17	5.9
2	1/14	7.1	7/11	63.6
3	7/31	22.6	13/14	92.9
4	8/10	80.0	4/4	100.0
5	5/9	55.6	55.6	
6	5/5	100.0		
7	10/12	83.3		
TOTAL	41/96	42.7	25/46	54.3

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 124, Biological studies of mosquito malaria infection and transmission

Literature Cited

1. References - none

2. Publications

1. Rutledge, L.C., Gould, D.J., and Somchit, L. A Technique for Estimating Total Malarial Sporozoites in Mosquitoes. Mosquito News 30:64, 1970.

2. Rutledge, L.C., Gould, D.J., and Tantichareon, B. Factors Affecting the Infection of Anophelines with Human Malaria in Thailand. Trans. Roy. Soc. Trop. Med. Hyg. 63:613, 1969.

3. Rutledge, L.C., Hayes, D.E., and Ward, R.A. Plasmodium cynomolgi: Sources of Variation in Susceptibility of Anopheles quadrimaculatus, A. balabacensis and A. stephensi. Exper. Parasitol. 27:53, 1970.

4. Ward, R.A. Comparative Studies on Falciparum and Vivax Malaria in Subhuman Primates. Parassitologia, 1970.

5. Ward, R.A., Rutledge, L.C., and Hickman, R.L. Cyclical Transmission of Chesson Vivax Malaria in Subhuman Primates. Nature 224:1126, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6515	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	125
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Taxonomy and Ecology of Disease Bearing Mosquitoes of Southeast Asia (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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: Not Applicable
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	1	35
FISCAL YEAR CURRENT 71	1	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, D. C. 20012
ADDRESS:

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202 - 576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of CD and I
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Eldridge, LTC B. F.
TELEPHONE: 202 - 576-3719
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Tyson, CPT W. H.
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Anopheles; (U) Aedes; (U) Mosquitoes; (U) Ecology; (U) Taxonomy; (U) Vectors

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To produce keys, guides, and other guides for the identification of mosquitoes which are vectors of diseases of military importance. Emphasis is on vectors of malaria and arbovirus diseases such as dengue. Also, to obtain data on the ecology, biology, and disease transmission potential of these mosquitoes.

24. (U) Mosquitoes are collected by military and civilian cooperating agencies and are forwarded to a joint WRAIR-Smithsonian Institution team for identification. This team intensively studies these collections, as well as collections in established museums, and publishes keys, guides, and other identification aids which are returned to SE Asia for use by entomologists engaged in control and survey operations. Team also accumulates and makes available disease transmission and biological data.

25. (U) 69 07 - 70 06. Revision of genus Aedeomyia completed and submitted for publication. Revision of Aedes, subgenus Muscidus completed and submitted for publication. Final draft of monograph of Anopheles of Thailand completed. Experimental hybridization experiments with geographic strains of Anopheles stephensi demonstrated genetic control of several biological characteristics, including egg structure, fecundity, feeding behavior, susceptibility to malaria, and longevity. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

BLANK PAGE

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 125, Taxonomy and ecology of disease bearing mosquitoes of Southeast Asia

Investigators
Principal: LTC Bruce F. Eldridge, MSC
Associate: CPT William H. Tyson, MSC; Ronald A. Ward, Ph.D.; CPT Louis C. Rutledge, MSC, John E. Scanlon, Ph.D.*; MAJ John F. Reinert, MSC**

Description

Mosquitoes are collected in Southeast Asia by cooperating military organizations and other groups. Other supplementary materials are obtained from existing collections in museums and other institutions. After study taxonomic revisions and descriptions are prepared for all of the mosquitoes of Southeast Asia, with emphasis on the species of medical importance. Sections of the work are published as completed, and keys of value to military entomologists are prepared as required. The eventual aim of the project is the publication of a series of monographs completely describing the mosquitoes of the area. In addition collection and ecological data are recorded later for collation with published data on the ecology of the various species. Laboratory crosses are made among members of wide-ranging species or related species to detect the presence of mating barriers which might affect the specific status of various taxa. Studies under this work unit are performed in conjunction with the Smithsonian Institution under U.S. Army Medical Research and Development Command Contract MD-2672. This report covers the in-house portion of the work only.

Progress

1. Anopheles of Thailand

The book-length work: Anopheles mosquitoes of Thailand is in the final stages of preparation and should be published during calendar year 1970 as scheduled. This work involves a thorough revision of the anopheline fauna of Thailand, including a complete description and illustration of all life cycle stages of species from that country.

2. Aedes of Southeast Asia

Study has been completed on 2 subgenera of this genus (Diceromyia and Muscidus) and taxonomic revisions are published or in press.

---

* University of Texas, School of Public Health, Houston
** Department of Entomology, University of Florida, Gainesville.

In the case of Diceromyia, keys are published for all oriental species. One species is described as new to science. The subgenus is reviewed generally on a world-wide basis, and the zoogeographical distribution is established.

The complete revision of the subgenus Muscidus is finished and awaits only final checking of illustrations.

3. Aedeomyia of Southeast Asia

The revision of this unusual and interesting genus has been essentially completed. Publication will occur in early FY 71.

4. Experimental taxonomy of Anopheles stephensi strains

Genetic disparity among 3 strains of Anopheles stephensi from India, Iran and Iraq was demonstrated by showing that they differ in egg structure, fecundity, feeding behavior, susceptibility to malaria and longevity. The 6 reciprocal genetic crosses of the 3 strains were made, and the fecundities of the 6 parental crosses and of the 6 types of F_1 hybrids were determined and compared with those of the 3 parental strains (Table 1). While increased fecundity was observed in certain parental crosses, reduced fecundity was observed in others. Similar effects were found to be due to decreased or increased egg development or hatch rate, or both. Oviposition rates did not vary greatly, and no abnormality in gonadal development was found. Since sterility of the order of 50% occurs in certain crosses (Table 1), it seems possible that genetic control methods might be feasible for this species. An analysis of egg measurements indicate that Anopheles stephensi mysorensis Sweet and Rao cannot be considered a valid subspecies.

5. Hybridization of Anopheles species

Interspecies crossing experiments with Anopheles stephensi and A. balabacensis have been conducted. Male A. stephensi mate freely with female A. balabacensis in cages, and the reciprocal cross can be accomplished by use of forced-mating techniques. In both of these crosses egg development and oviposition occurs more or less normally. Fully mature embryos can be dissected from eggs after 2 or more days. In one instance, hatching has occurred.

6. Crossing of Armigeres subalbatus strains

This species has an extremely wide distribution in Asia, from Japan and China, through to India and South to the Malayan peninsula. In portions of its range it is of significance as a filariasis vector. Preliminary crosses made between strains from Japan and Singapore are completely interfertile without any evidence of a genetic barrier.

Conclusions and recommendations

Taxonomic revisions of several groups of mosquitoes have been completed. In addition, biological criteria have been employed to elucidate natural relationships among geographic strains of anophelines. Such techniques should be employed to a much greater extent in the future, especially in the case of geographic strains differing in vectorial capacity. Taxonomic reviews of anophelines in areas other than Thailand are badly needed. Revision of the genera Culex, Aedes (especially Stegomyia), and Mansonia should receive top priority.

TABLE 1

Fecundities of Parental Crosses, Within-strain Matings and F_1 Hybrids of 3 Geographic Strains of Anopheles stephensi. Figures Following Each Mean are SE of the Mean

	Parental Crosses		
♀ Partner	India ♂	Iran ♂	Iraq ♂
India	101.7 ± 12.4 progeny	46.7 ± 9.6 progeny	50.5 ± 10.0 progeny
Iran	64.4 ± 8.8	46.4 ± 10.3	41.4 ± 9.9
Iraq	56.0 ± 11.8	31.5 ± 9.5	51.1 ± 11.1

	F_1 Hybrids		
♀ Parent	India ♂	Iran ♂	Iraq ♂
India	101.7 ± 12.4 progeny	64.9 ± 9.5 progeny	73.0 ± 11.3 progeny
Iran	80.2 ± 12.3	46.4 ± 10.3	42.4 ± 7.6
Iraq	59.0 ± 11.4	36.3 ± 8.2	51.1 ± 11.1

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 125, Taxonomy and Ecology of disease bearing mosquitoes of Southeast Asia

Literature Cited

1. References - none

2. Publications

1. Reinert, J.F. Contributions to the Fauna of Southeast Asia - VI. Genus Aedes, Subgenus Diceromyia Theobald in Southeast Asia. Contrib. Amer. Entomol. Inst. 5(5) 43 pp., 1970.

2. _____. The zoogeography of Aedes (Diceromyia) (Diptera: Culicidae). J. Entomol. Soc. South Africa. 33:129, 1970.

3. Rutledge, L.C., Ward, R.A., and Bickley, W.E. Experimental hybridization of geographic strains of Anopheles stephensi (Diptera, Culicidae). Ann. Entomol. Soc. Amer. 33:1024, 1970.

4. Tyson, W.H. Notes on African Aedes, Subgenus Muscidus (Diptera: Culicidae). J. Entomol. Soc. South Africa. 33:82, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6516	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	126
b. CONTRIBUTING				
[redacted]	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) In vitro Cultivation of Mosquito Tissues and Malarial Parasites (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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: Not Applicable

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	1	35
FISCAL YEAR CURRENT 71	1	30

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, D.C. 20012

ADDRESS:

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202 - 576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of CD and I

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Schneider, I. Ph.D.

TELEPHONE: 202 - 576-3049

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Aedes; (U) Anopheles; (U) Culex (U) Mosquitoes; (U) Plasmodium; (U) Tissue Culture; (U) Immunology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To develop reliable in vitro procedures by which large quantities of relatively pure malarial sporozoites can be produced for malaria vaccine development studies. Also, to develop mosquito tissue culture systems for studies on pathogen invasion and growth dynamics in invertebrate tissue.

24. (U) Development of culture media which will support growth of invertebrate stages of malaria parasites. Development of various techniques for the isolation and purification of individual stages of parasite. Evaluation of mosquito life cycle stages for suitability for establishing primary cultures.

25. (U) 69 07 - 70 06. Separation of the various erythrocytic stages of Plasmodium cynomolgi has been substantially increased by substituting a discontinuous density gradient for the linear gradient previously used. The separation of gametocytes from mature schizonts is not complete and efforts are continuing to refine the technique still further. Tissues and cells from one additional mosquito species. Culex restuans, have been placed in primary culture. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 126, In vitro cultivation of mosquito tissues and malarial parasites

Investigators

Principal: Imogene Schneider, Ph.D.
Associate: CPT Darrell E. Bay, MSC

Description

This investigation involves 3 major areas: (1) the use of an in vitro system for the collection of large numbers of sporozoites free or virtually free of mosquito tissue, (2) the use of density gradients to isolate the sporozoite stage from mosquitoes and the gametocyte stage from the other blood forms and (3) the development of primary and established cell lines from a selected number of mosquito species for subsequent utilization in arbovirus research.

Progress

1. Cultivation of malaria parasites and mosquito tissues

Prior work on culturing the mosquito phases of malaria parasites has made it abundantly clear that little information is gained using the oocyst stages. Assuming a reasonably adequate medium, the older oocysts, having already differentiated, will mature in vitro and release their sporozoites within 12 to 48 hours. The use of younger oocysts, which would place more stringent demands on the culture system, is theoretically desirable but unfortunately impractical due to the great difficulty of dissecting such stages from the mosquito host. It therefore seemed advisable to attempt the culture of the initial, e.g., gametocyte, stages assuming these could be obtained in sufficient numbers The technique being employed to isolate the gametocytes from the other blood stages is that of density gradient centrifugation.

Plasmodium cynomolgi blood forms, predominently gametocytes and schizonts, after isolation on density gradients were cultured in medium alone or together with cells from an established line of Anopheles stephensi. The culture chambers, microtest tissue culture plates, were prepared one day in advance. Thirty of the wells were filled with 15 µl of medium, the remaining wells with an equal amount of medium containing A. stephensi cells (10^5 cells/ml). Approximately 1 µl of blood forms was added to each well the following day. Four series of 60 cultures each were completed but all were unsuccessful. Further attempts will be postponed until the gradient technique can be perfected (see 2a below).

Attempts to obtain adequate numbers of P. cynomolgi ookinetes for culturing, using the capillary tube method were also unsuccessful.

2a. Isolation of the gametocyte stages from the other blood forms

Rowley et al. reported the partial separation of the blood forms of Plasmodium berghei in different fractions of a bovine serum albumin gradient. In linear gradients having densities varying from 1.04 to 1.09 gm/cm^3 the ring forms predominated in the heaviest fractions and the mature schizonts in the lightest fractions with the trophozoites banded in between. The gametocytes were apparently distributed more or less randomly in the upper half of the gradient. In attempting to use identical gradients for isolating the various blood stages of P. cynomolgi a number of difficulties were encountered. Since a large number of blood cells were found at the bottom of the tube following centrifugation the density of the heaviest fraction was increased from 1.09 to 1.17 gm/cm^3 (at 4°C). Although this adjustment resulted in moving the densest fractions well above the bottom of the tube, considerable difficulty was met in preparing such gradients with a conventional gradient former due to the viscosity of the bovine serum albumin. The use of Renografin-albumin gradients mitigated this problem but introduced a far more serious one; namely, the blood cells had somewhat crenated outlines after a run. Eleven subsequent gradients were prepared in which the percentages of Renografin and albumin were altered while retaining the appropriate densities. The percentages having the least adverse effect on the blood cells were 15% BSA/ 20% R (1:1) and 30% BSA/60% R (1:1).

The use of a linear gradient has been temporarily by-passed in favor of a discontinuous gradient. Seven runs have been made with the latter. Since these gradients are prepared by hand the viscosity of the albumin presents little problem. Gradients ranging from 40 to 80 percent in 10 percent increments are presently used. The separation of gametocytes from mature schizonts is still not complete and efforts are contunuing to refine the gradient still further.

Two other difficulties have not been completely resolved. It is impossible to fractionate the gradient by bottom puncture since the blood, whether heparinized or defibrinated, has a tendency to cling to the sides of the tube as it drains. Withdrawing the fractions from the top or by side puncture both have disadvantages but presently the latter method is used. The other problem involves the removal of the albumin once the appropriate fractions are isolated and prior to being placed in culture. Repeated washing followed by centrifugation effectively removes the albumin but results in the loss of numerous cells. Volumes of diluent, speed and length of centrifugation and numbers of cells retained are now being quantitated in order to obtain maximum clearance of albumin at acceptable levels of cell loss.

2b. Isolation of malaria sporozoites on density gradients

It is anticipated that the sporozoites isolated from either the culture system or from the gradients will serve as a source of malarial antigens for immunological studies.

Further refinements have been made in this technique primarily to assure as tight a banding of the sporozoites as is possible with the gradient forming materials used, namely, Renografin (methylglucamine diatrizoate with additives) and bovine serum albumin-fraction V. The linear gradients now routinely used are prepared from 15% BSA/ 20% R (1:1, density = 1.06 g/cm^3) and 30% BSA/ 56% R (1:1, density = 1.15 g/cm^3). Sporozoites of both Plasmodium gallinaceum and P. cynomolgi band most heavily in fractions 11 and 12 (density = 1.12 g/cm^3).

Four gradients can be run concurrently using the HB-4 rotor in an RC-2 refrigerated centrifuge with each gradient capable of handling material from 1000 mosquitoes. On the average, 4 x 10^7 P. gallinaceum sporozoites can be recovered from the peak fractions of any one gradient (data obtained when donor mosquitoes had a 70-90% infection rate with a mean oocyst count of 75 per midgut). Since the isolation procedure is considered reasonably efficient the yield can be most readily increased by using larger quantities of mosquitoes.

To avoid overloading any one gradient, a continuous flow system is now being used with an SS-34 rotor. Six runs have been made thus far with the maximum mosquito sample tested being 5000. Recycling of the diluent, e.g., the lower density mixture, through the flow system an average of 4 times has been required to ensure transfer of the majority of the sporozoites to the tubes. The necessity for recycling will probably be eliminated once the appropriate flow rate is determined. Assuming that problem can be solved the system should be capable of handling a 10-fold increase in same size without overloading.

3. Development of primary and established cell lines from mosquitoes of the genus Culex

The utilization of insect cell cultures for studying various aspects of the pathogenic cycles of viral, protozoan and bacterial agents having an insect vector or reservoir is well recognized. Unfortunately, the number of lines currently available for such studies is decidedly inadequate. In a number of instances, the lack of appropriate cell cultures has left investigators with little alternative but the use of an atypical pathogen-vector culture system. The tenuous nature of many of the conclusions drawn from such studies is readily apparent, especially so in those instances where the pathogen-vector relationship is a highly specific one. In anticipation of conducting comparative studies involving in vivo versus in vitro invasion and replication of a number of arboviruses, cells from several species of the genus Culex differing markedly in their vector capacity were selected for culturing.

Forty-four primary cultures of Culex tritaeniorhynchus were initiated from August to November 1969. Methods of collecting and sterilizing the eggs, mincing the 1st stage larvae and subsequent trypsin treatments were identical to those used for initiating cell lines from Anopheles stephensi. The medium was identical to that used for the Anopheles lines with minor exceptions: the concentration of sucrose was lowered, those of glucose and NaCl raised and Bactopeptone added.

The pattern of development in the primary cultures was identical to that first described for Aedes aegypti cells. Cell growth, in the form of spherical monolayers issuing from the ends of the larval fragments, was evident within a week after the cultures had been set up. After 4 to 6 weeks in primary cultures, the spheres were excised, subjected to mild trypsin treatment and reseeded into the same flasks. Subcultures were made approximately 2 weeks later, the inoculum containing a minimum of 10^4 cells/ml. The cells do not attach firmly to the surface of the glass flask and can be removed by pipetting.

The cells range from 5-17μ in diameter and 28-60μ in length. Epithelial-like, round and fibroblast cells are present with the latter predominating. Confluent monolayers are not formed as of the 8th subculture but rather the cells grow in large scattered patches. Preliminary chromosome counts indicate the majority of cells are diploid (2n=6).

Eighteen primary cultures of C. salinarius were initiated between December 1969 and February 1970. Development of these cultures differed from those of C. tritaeniorhynchus only in that the cell spheres usually reached greater diameters and could be dislodged from the larval fragments by gentle pipetting of the medium. In primary cultures, the cells are predominently epithelial in appearance and quite uniform in size, ranging from 10-20μ in diameter and 25-45μ in length. Subculturing has not yet been attempted.

Conclusions and recommendations

1. A culture system has yet to be designed which will support the growth and differentiation of the entire mosquito cycle of the plasmodia. The more advanced stages, e.g., the older oocysts, are readily obtainable and can be placed in culture without difficulty. Since they have completed most of their development in vivo culturing such stages provides relatively little information on the adequacy of the medium or of the culture system as a whole. The reverse holds true for the younger stages but, unfortunately these stages are not readily obtainable. Utilizing the technique of density gradient centrifugation for isolating the gametocytes from the other blood forms shows much promise and emphasis should be placed on making it truly effective for this purpose.

2. The isolation of massive numbers of sporozoites on density gradients is perfectly feasible and limited only by the numbers of infected mosquitoes available. Small adjustments in the technique may have to be made but future work should concentrate on using the sporozoites so isolated for EM, immunological and other studies.

3. In all likelihood, cells from any given mosquito species could survive and proliferate in culture with the media and methods now available. Primary cell cultures are most easily initiated from explants of the 1st stage larvae. Subculturing the cells and establishing cell lines is possible but, of course, much more difficult and time consuming.

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 126, In vitro cultivation of mosquito tissues and malarial parasites

Literature Cited

1. References

(1) Rowley, P.T., Siddiqui, W.A. and Geiman, Q.M. Separation of Malarial Parasites According to Age by Density Gradient Centrifugation. J. Lab. Clin. Med. 70:933-937, 1967.

2. Publications

1. Schneider, I. Establishment of Three Diploid Cell Lines of Anopheles stephensi (Diptera: Culicidae). J. Cell Biol. 42:603, 1969.

2. Schneider, I., and D.H. Chen. Isolation of Malaria Sporozoites on Renografin-Serum Albumin Gradients. Amer. Zool. 9:451, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6518	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	127
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Test Systems for Plasmodium falciparum (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	D. In-House

17. CONTRACT/GRANT NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 70	2	70
CURRENT FISCAL YEAR 71	2	70

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: MERONEY, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division CD&I

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: SADUN, E. H., Sc.D.

TELEPHONE: 202-576-3308

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P.

NAME: DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Chimpanzee; (U) Immunity; (U) Chloroquine; (U) Gamma Globulin
(U) Isotope; (U) Susceptibility; (U) Owl Monkey

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) Study susceptibility of chimpanzees and other primates to infections of human malaria. Study the characteristics of drug resistant strains, provide high density of parasites for morphological and biochemical studies. Conduct physiological and pathological studies of malaria and provide test animals for chemotherapeutic and immunological investigations.

24(U) Infect splenectomized, drug treated chimpanzees and other primates with plasmodia of human origin. Observe the extent and duration of parasitemias, study the response of different strains to chemotherapy, study susceptibility to reinfection with homologous and heterologous strains.

25(U) 69 07 70 06 Plasmodium falciparum infections in owl monkeys, Aotus trivirgatus are always fatal within 5 and 18 days depending on size of inoculum. While parasitemia is below 5-10 percent only ring forms seen. Above this all erythrocytic forms are present. Sometimes spontaneous bleeding occurs from nose and intestinal tract and clotting times at needle punctures are increased greatly. Usually at death 40-60 per cent of RBC's are parasitized. Most striking hematologic change is in reduction of number of platelets. A few days before death platelet counts fall to from 20 to 40 thousand. Following serum biochemical values changed significantly during infection: total protein decreased, glutamic oxalacetic transaminase increased, glucose decreased and complement decreased. Variety of lesions found in liver, lung and kidney similar to human cases of P. falciparum, but normal monkeys had lesions which made evaluation difficult. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 127, Test systems for Plasmodium falciparum

Investigators.
Principal: Elvio H. Sadun, Sc.D., Lib. Doc.
Associate: CPT J. S. Anderson, VC; CPT A. J. Johnson, VC; SP5 A. Reyes, Jr.; B. T. Wellde

1. Parasitologic, hematologic, biochemical and pathologic investigations of Aotus monkeys infected with Plasmodium falciparum.

After a period of adaptation, the Malayan Camp. strain of Plasmodium falciparum produces consistent predictable infections in intact Aotus monkeys. At present the infection is almost always fatal with animals dying with a parasitemia of 40-80% five to eighteen days after inoculation, depending on the size of the challenge. While fewer than 10 percent of the red-blood cells are parasitized, ring forms predominate in the peripheral blood and mature forms in the blood of deep organs. As higher parasitemias are reached all erythrocyte forms are present in the peripheral blood and aggregates of erythrocytes containing mature trophozoites and schizonts are commonly seen in the blood of the deep organs. Although immature gametocytes were seen in monkeys in early passages they appear only rarely now. Spontaneous hemorrhages occur in the infected monkeys and are most common from the nasal and gastro-intestinal tracts. Baseline hematologic values from approximately 75 non-malaria infected monkeys have been determined and compared to those of infected monkeys. Progressive decreases occur in red cell parameters (RBC, Hgb, Hct) as the infection progresses. White blood cell numbers appear to be depressed early in infection but may rise to high levels in animals surviving longer than twelve days. This rise is accompanied by an increase in the number of mononuclear cells. Platelets were reduced to 10-20 percent of their original numbers. Serum transaminase levels in 50 non-infected monkeys were compared with those of infected animals. Relatively high SGOT (mean 154) and SGPT (mean 47) were found in normal monkeys. These values agree with those of others who reported high transaminase levels in the sera of new world monkeys. Infected monkeys showed terminal increases in both SGOT (mean 283) and SGPT (mean 124) levels. Serum urea nitrogen levels were increased four-fold in terminal serum samples when compared to controls. Fasting glucose levels were depressed in infected monkeys and levels of serum complement appeared to fall. Total serum protein and electrophoretic determinations were made also. High levels of alpha-2 globulins were found in both groups of animals. About one-third of all monkeys showed an albumin polymorphism which consisted of a split or double albumin peak which consistently appeared on repeated tests. Malaria infected monkeys had lower total protein values which were reflected in reductions in both albumin and gamma globulins.

A variety of lesions were observed in the experimental monkeys; many of these seen in the liver, lung, and kidneys were like those described from human cases infected with P. falciparum. Lesions found in "normal" monkeys made evaluation of tissue responses to malaria difficult.

Table 1

Hematology of "Normal" Aotus Monkeys

	No. Animals	Mean	S.D.*	Median	Range
RBC (/mm^3)	157	5.17	.84	5.04	3.50-7.74
Hct (%)	157	42.0	5.4	42.0	31.0-56.0
Hemoglobin (gm %)	60	14.3	1.1	14.2	11.9-16.0
Reticulocytes (%)	78	2.4	1.7	2.3	0.1-10.6
MCV (μ^3)	157	82.4	11.9	81.6	61.7-118.4
MCH (Picograms)	60	26.9	3.1	26.5	21.7-37.7
MCHC (%)	60	34.1	3.1	34.3	29.6-39.4
Platelets (/mm^3)	63	397.1	109.4	390.0	204-734
WBC's (/mm^{3}x10^3)	157	12.7	4.7	11.7	3.2-28.5
Differential (%)					
Neutrophils	157	55.4	7.6	58.0	13-91
Eosinophils	157	9.5	9.2	7.0	0-37
Mononuclear lymphoid	157	35.3	18.3	35.0	5-80
Basophils	157	<0.1		0.0	0-1

*Standard Deviation

Table 2

Typical Hematologic Values of Owl Monkey Infected with Plasmodium falciparum (Animal No. 617)

Day	% Parasitemia	RBC	Hct.	Hgb.	% Retic.	Platelets	WBC	Differential		
								Neut.	Eos.	Mononuc.
0	----	5.63	47	15.0	2.2	604	9.7	44	10	46
3	----	4.95	41	14.0	2.9	448	7.2	71	6	23
6	----	4.97	37	12.8	0.5	272	9.4	37	0	63
9	>1	4.65	39	13.4	0.1	88	8.0	20	0	80
12	1.0	5.12	37	12.0	2.0	64	13.7	26	0	74
15	25.0	3.40	32	9.9	4.4	30	11.1	44	16	40
16	33.0	3.25	28	8.1	4.1	62	42.0	34	4	62

Table 3

Typical Serum Biochemical Values of Owl Monkey Infected with Plasmodium falciparum (Animal No. 617)

Day	% Parasitemia	Total Protein	Grams/100 ml					Transaminase		Glucose mg %
			Albumin	Alpha-1	Alpha-2	Beta	Gamma	SGO	SGP	
0	---	9.0	3.5	0.5	2.1	1.2	1.7	197	72	110
3	---	8.4	3.7	0.4	1.6	1.0	1.6	170	44	225
6	---	7.5	3.1	0.6	1.6	0.9	1.4	179	62	142
9	<1	7.6	2.9	0.5	1.7	1.3	1.2	197	66	215
12	1	8.8	3.6	0.5	1.8	1.6	1.3	162	66	270
15	25	6.1	2.4	0.5	1.2	1.0	1.0	268	182	70
16	33	6.3	2.5	0.4	1.3	1.3	0.7	326	158	60

Table 4

Serum Proteins of Aotus Monkeys gms/100 ml

	Normal			Infected		
	No. Animals	Mean	Range	No. Animals	Mean	Range
Total protein	75	7.0	4.9-10.2	21	6.1	4.7-7.6
Albumin	75	2.7	1.2-4.2	21	2.2	1.5-3.2
Alpha-1 globulins	75	0.3	0.2-0.5	21	0.3	0.2-0.6
Alpha-2 globulins	75	1.3	0.7-2.4	21	1.2	0.7-1.7
Beta globulins	75	1.0	0.6-1.5	21	1.1	0.5-2.0
Gamma globulins	75	1.8	1.0-2.9	21	1.1	0.5-1.8

Table 5

Serum Chemical Values of Aotus Monkeys

	Normal			Infected		
	No. Animals	Mean	Range	No. Animals	Mean	Range
Transaminase						
SGOT	75	153	49-326	16	282	118-575
SGPT	75	47	21-121	16	124	66-275
Serum urea nitrogen Mg%	56	14	7-26	28	59	19-170
Uric acid Mg%	56	0.5	0-2	28	1.4	0-5
Glucose Mg%	43	113	27-220	5	30	16-51

Table 6

Naturally Occurring Blood Parasites

No. Animals Examined	Incidence of:		
	Malaria	Microfilaria	Trypanosomes
209	0	5	2

Intestinal Parasites of Aotus Monkeys
(51 Specimens)

Protozoa	
Trichomonas sp.	7
Giardia sp.	24
Entamoeba sp.	4
Iodamoeba sp.	1
Chilomastix sp.	1
Helminth ova	
Nematodes	5
Trematodes	1
Cestodes	1

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 127, Test systems for Plasmodium falciparum

Literature Cited

1. References

None

2. Publications

Hickman, R. L.: The use of subhuman primates for experimental studies of human malaria. Mil. Med. 134(No. 10):741-756, 1969.

Ward, R. A., Rutledge, L. C. and Hickman, R. L.: Cyclical transmission of Chesson vivax malaria in subhuman primates. Nature 224:1126-1127, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6519	70 07 01	DD-DR&E(AR)36

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	128
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Natural and Acquired Immunity in Rodent Malaria (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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 NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	2	70
FISCAL YEAR CURRENT 71	2	70

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Resea.

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: MERONEY, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of CD&I

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: SADUN, E. H., Sc.D.

TELEPHONE: 202-576-3308

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P.

NAME: DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Antibody; (U) Rodents; (U) Susceptibility; (U) Immunity; (U) Plasmodium; (U) Splenectomy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To evaluate the role of humoral and cellular factors in determining susceptibility of hosts to malaria, for the maintenance of the complete life cycle of malaria in the laboratory, to find a laboratory animal suitable for the production of large amounts of infected blood for immunological and biochemical studies.

24(U) To test a variety of rodent species for natural susceptibility to P. berghei. Attempt to increase susceptibility by splenectomy and chemical treatment. Standardize the course of infections quantitatively. Evaluate the mechanism of antibody action on host and parasite, and characterize antibodies responsible for these activities. Study the effects of antibody on the parasite and on the host.

25(U) 69 07 - 70 06 Twenty to 60 percent of mice immunized with irradiated parasites survived challenge. Some mice resisted challenge for 8 months. Immunized mice developed mild reticulocytosis, slight fall in hematocrit, markedly enlarged spleens and elevated gamma globulin. Mice splenectomized before immunization were not protected when challenged, but they were slightly resistant when splenectomized after immunization. Three of 7 Aotus monkeys resisted challenge after immunization with irradiated Plasmodium falciparum. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 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.

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 128, Natural and acquired immunity in rodent malaria

Investigators.
Principal: Elvio H. Sadun, Sc.D., Lib. Doc.
Associate: N. T. Briggs; CPT L. T. Quigg, MSC; SP5 A. Reyes, Jr; B. T. Wellde

1. Resistance to malaria infections produced by irradiated parasites.

The exposure of P. berghei infected erythrocytes to radiation levels of 19 Kr or greater prevented the replication of the parasites in both rats and mice. Injections of these irradiated cells stimulated a resistance to challenging infections. Rats given one injection of irradiated parasitized cells developed a significant resistance which was strengthened by repeated immunizations. Although mice showed no protection after one or two immunizations they developed increasing levels of resistance when from three to ten immunizing injections were given. From 20-60 percent of the immunized mice survived the challenging infections while control mice always succumbed to the infection. Immunized mice appeared to control the parasitemia by limiting parasites mainly to reticulocytes while parasites in control mice invaded large numbers of mature erythrocytes. After immunization some mice showed resistance to challenge for as long as 8 months. Mice immunized with irradiated parasitized cells developed a mile reticulocytosis, a slight fall in hematocrit values, markedly enlarged spleens and elevated gamma globulin levels. Passively transferred serum from immunized mice conferred a slight but significant suppression of parasitemia in test mice.

Several experiments were done to evaluate the role of the spleen in the increased resistance in immunized mice. Mice which were splenectomized before immunization showed no protection after challenge when compared to controls. When splenectomy was performed after immunization only a slight resistance to challenge was observed.

Immunization with erythrocytic forms of P. berghei yoeli did not prevent the establishment of infections after sporozoite challenge, but the infections were rapidly controlled and parasites were cleared from circulation much sooner than in control mice.

Immunization of Aotus monkeys with P. falciparum infected erythrocytes irradiated at 25 Kr was also shown with 3 and 4 immunizations. Three of 7 immunized monkeys survived a challenging infection while 13 controls succumbed with high parasitemias. Some immunized monkeys developed hemagglutinating antibody titers before challenge and surviving monkeys showed rapidly rising titers which persisted for at least 12 weeks.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 128, Natural and acquired immunity in rodent malaria

Literature Cited

1. References

None

2. Publications

Briggs, N. T. and Wellde, B. T.: Some characteristics of Plasmodium berghei "relapsing" in immunized mice. Mil. Med. 134(No. 10):1243-1248, 1969.

Stechschulte, D. J.: Cell-mediated immunity in rats infected with Plasmodium berghei. Mil. Med. 134(No. 10):1147-1152, 1969.

Stechschulte, D. J.: Plasmodium berghei infection in thymectomized rats. Proc. Soc. Exp. Biol. & Med. 131:748-752, 1969.

Stechschulte, D. J., Briggs, N. T. and Wellde, B. T.: Characterization of protective antibodies produced in Plasmodium berghei infected rats. Mil. Med. 134(No. 10):1140-1146, 1969.

Wellde, B. T., Ward, R. A. and Ueoka, R.: Aspects of immunity in mice inoculated with irradiated Plasmodium berghei. Mil. Med. 134 (No. 10): 1153-1164, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6520	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	129
b. CONTRIBUTING				
c. CONTRIBUTING	DOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Host Responses to Malaria (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	D. In-House

17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 70	2	70
FISCAL YEAR CURRENT 71	2	70

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: MERONEY, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Division of CD&I
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: SADUN, E. H., Sc.D.
TELEPHONE: 202-576-3308
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: MOON, A. P.
NAME: DA

21. GENERAL USE
Foreign intelligence not considered

22. KEYWORDS (Precede each with Security Classification Code)
(U) Malaria; (U) Gamma globulin; (U) Biochemistry; (U) Antibody; (U) Fluorescent; (U) Isotope; (U) Metabolism

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To study the physiological pathology of malaria including the enhancement of non-specific resistance to infection and to determine how energy requirements are met within the parasite.

24(U) Study the effect of infection on the uptake and distribution of radioisotope-labelled amino acids, study the levels of enzyme activity in tissue extracts and alterations in protein and free amino acid constituents of blood and urine, study the development of relapses, and the pattern of parasitemias and fluorescent antibodies prior to, during, and following therapy. Investigate the use of immune gamma globulins as an adjuvant to chemotherapy in humans infected with drug resistant malaria.

25(U) 69 07 - 70 06 When erythrocyte free preparations of Plasmodium knowlesi were incubated with glucose C14 and pyruvate C14, quantities of glucose carbon incorporated into C02, keto acids and lipids were low and of doubtful significance. Volatile acids, neutral volatile compounds and lactate comprised the major end-products. Acetate and formate were identified as important components of the acid volatile fraction. Small amounts of succin te were isolated, but did not account for most of the glucose carbon utilized. Pyruvate C14 was utilized rapidly. The methyl carbon was incorporated into acetate but the carboxyl carbon was not. Plasmodium falciparum from Aotus monkeys had appreciable cytochrome oxidase activity. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval

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

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 129, Host responses to malaria

Investigators.
Principal: Elvio H. Sadun, Sc.D., Lib. Doc.
Associate: CPT L. W. Scheibel, MSC; SP4 W. K. Pflaum

1. Carbohydrate metabolism in Plasmodium knowlesi.

Early studies of the metabolism of the malaria parasites led Moulder to conclude "that the malarial parasite possesses the same mechanisms for the breakdown of glucose as does its vertebrate host ..." However, in 1961 this concept was questioned when Bowman et al., Bryant and Voller, and Bryant et al., could not substantiate the existence of a tricarboxylic acid cycle in the rodent malaria Plasmodium berghei and P. vinckei.

Scheibel and Miller reported that P. knowlesi parasites separated from host blood cells did not incorporate sufficient quantities of C^{14} labeled glucose into respiratory CO_2 to account for the occurrence of either a tricarboxylic acid cycle or a pentose phosphate pathway as quantitatively significant sequences. These findings are compatible with the studies of Bowman et al., who concluded that pentose phsophate shunt activity was insignificant in P. berghei. In addition, Fletcher and Maegraith could not demonstrate glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase activity in extracts of liberated P. knowlesi. Barnes and Polet suggested that the parasite may utilize the pentose phosphate pathway of the erythrocyte host. Scheibel and Miller reported the rate of glucose utilization remained constant under atmosphere of 100% N_2 or air, indicating the absence of a Pasteur effect. These authors were unable to detect the presence of glycogen within P. knowlesi after incubation in a glucose medium, and lactate formation alone could not account for the glucose utilized, suggesting that other products were accumulating. A similar observation had been made by Silverman et al, using P. gallinaceum infected chicken erythrocytes. Therefore, an attempt was made to account for all glucose used in terms of products formed.

Except where indicated, parasites were isolated and incubated essentially as described by Scheibel and Miller. In those experiments where pyruvate was employed as substrate (Table 9) glucose 0.008%) was present only in the first wash fluid. Incubations were carried out in either 95% N_2, 5% CO_2 or 95% air, 5% CO_2 as indicated, and contained Trager's high potassium medium with 0.002 M adenosine triphosphate (ATP, P-L Biochemicals Inc.), 0.005 M sodium pyruvate (Boehringer and Soehne), 0.0325 M reduced glutathione (GSH, Boehringer and Soehne), 0.013 M coenzyme A (P-L Biochemicals Inc.) as Trager has shown these components promote in vitro longevity of the parasite. Reactions were terminated, carbon dioxide was trapped, glucose, lactate and protein were assayed as previously described.

Neutral and acid volatile compounds were obtained by steam distillation under neutral conditions followed by acidification of the fermentation mixture with 1 M H_3PO_4 and further steam distillation. Radioactivity in aliquots of each fraction was determined in a scintillation spectrometer. The acid volatiles were neutralized, then concentrated by evaporation. Acetate and formate were separated chromatographically on buffered celite colums.

Succinate was isolated from the residue of the steam distillation after oxidizing the mixture with $KMnO_4$ to decompose contaminating lactate a major metabolic end product of the parasite. Succinate, which is resistant to $KMnO_4$ oxidation, was reisolated by continuous ether extraction, and chromatographed from acid celite columns.

Lipids were isolated and counted in a scintillation counter to determine the incorporation of radioactive glucose carbons into the lipid fraction. Keto acids were isolated by ether extraction and the 2, 4-dinitrophenyl hydrazone derivatives prepared. Unreacted 2, 4-dinitrophenylhydrazine was removed by ether extraction from the alkaline solution, and the keto-acid derivative reextracted with ether after acidification. The isolated compounds were separated by means of ascending paper chromatography using butanol saturated with water as solvent.

Glycogen was quantitated by the enzymatic micro-procedure of Bueding and Hawkins. Glycerol was determined according to the method of Wieland and alpha glycerol phosphate was assayed by the method of Hohorst.

Pyruvate was determined according to the method of Bücher, et al. except for the substitution of 0.5M glycyl glycine buffer in place of 0.4M triethanolamine buffer. The parasite pellet was washed repeatedly with water until there was an insignificant amount of radioactivity in the wash fluid. Then the pellet was digested in hyamine hydroxide at 60°C for 60 minutes prior to counting.

Glucose-6-C^{14} utilization and formation of lactate, keto acids, lipids and CO_2. Free parasites were suspended as described above and the following were determined: glucose disappearance, lactate formation and the incorporation of radioativity into respiratory CO_2, keto acids and lipids. Results are recorded in Table 7. Although experiments 1 and 2 can not be compared directly, results pertaining to glucose utilization and lactate formation are in conformity with previous findings of Schiebel and Miller. Glucose utilization varies with each parasite preparation, but lactate accumulation is consistently higher in an anaerobic environment. Unfortunately, the source of lactate in these experiments can not be determined, since large quantities of pyruvate as well as glucose were present, and radioisotope incorporation into lactate was not determined. On the other hand, the low levels of isotope incorporation from glucose-6-C^{14} into CO_2, keto acids and lipids indicate that these are not quantitatively significant end products of glucose utilization.

Table 7

Glucose-6-C^{14} utilization and formation of lactate, keto acids, lipids and CO_2 by Plasmodium knowlesi in Trager's medium

Expt	Atmosphere	mμ moles Glucose Utilized /mg Protein	mμ moles Lactate Produced /mg Protein	Glucose C^{14} Incorporation					
				CO_2		Keto Acids		Lipids	
				mμ moles /mg Protein	Percent	mμ moles /mg Protein	Percent	mμ moles /mg Protein	Percent
1	Anaerobic 95% N_2 5% CO_2	118.0	271.0	0.054	0.046	2.55	2.17	3.70	3.14
2	Aerobic 95% Air 5% CO_2	183.0	51.0	0.378	0.21	1.37	0.75	2.66	1.46

Each vessel contained an average of 30.0 mg of parasite protein in a total volume of 4 ml of incubation medium containing 0.25% glucose-6-C^{14}. Incubations were carried out at 38°C for one hour. Initial specific radioactivity of the substrate was 22,330 dpm/μ mole. Center wells contained 5 meq. of NaOH. Reactions were stopped by addition of perchloric acid to a final concentration of 3%. Atmospheres in experiments 1 and 2 were as indicated.

Table 7

Glucose-6-C^{14} utilization and formation of lactate, keto acids, lipids and CO_2 by Plasmodium knowlesi in Trager's medium

Expt	Atmosphere	mμ moles Glucose Utilized /mg Protein	mμ moles Lactate Produced /mg Protein	Glucose C^{14} Incorporation					
				CO_2		Keto Acids		Lipids	
				mμ moles /mg Protein	Percent	mμ moles /mg Protein	Percent	mμ moles /mg Protein	Percent
1	Anaerobic 95% N_2 5% CO_2	118.0	271.0	0.054	0.046	2.55	2.17	3.70	3.14
2	Aerobic 95% Air 5% CO_2	183.0	51.0	0.378	0.21	1.37	0.75	2.66	1.46

Each vessel contained an average of 30.0 mg of parasite protein in a total volume of 4 ml of incubation medium containing 0.25% glucose-6-C^{14}. Incubations were carried out at 38°C for one hour. Initial specific radioactivity of the substrate was 22,330 dpm/μ mole. Center wells contained 5 meq. of NaOH. Reactions were stopped by addition of perchloric acid to a final concentration of 3%. Atmospheres in experiments 1 and 2 were as indicated.

Table 8

Incorporation of glucose-C^{14} into end products by Plasmodium knowlesi

Glucose-C^{14} Incorporated	1		2		3		4	
	Glucose-6-C^{14} (95% Air; 5% CO_2)		Glucose-U-C^{14} (95% Air; 5% CO_2)		Glucose-6-C^{14} (95% N_2; 5% CO_2)		Glucose-U-C^{14} (95% N_2; 5% CO_2)	
	mμ moles /mg Protein	Percent Incorp.	mμ moles /mg Protein	Percent Incorp.	mμ moles /mg Protein	Percent Incorp.	mμ moles /mg Protein	Percent Incorp.
Parasite Pellet	5	2.05	--	--	5	0.70	--	--
Succinate	14	3.92	5	1.67	13	1.71	6	2.76
Neutral Volatiles	32	13.76	81	29.67	74	10.09	64	28.42
Acid Volatiles	124	53.30	78	28.67	162	22.10	92	41.00
(a) Formate	18	5.07	25	9.24	59	7.98	34	15.19
(b) Acetate	76	21.11	23	8.46	71	9.69	22	9.63
Glucose Utilized*	233	--	273	--	731	--	224	--
Lactate Produced*	405	--	260	--	1,052	--	366	--

*Determined enzymatically.

Incubations with glucose-6-C^{14} contained an average of 20.0 mg parasite protein in a total volume of 4 ml of 0.25% glucose Trager's medium, and had an initial specific radioactivity of 17,225 dpm/μ mole. Those experiments employing glucose-U-C^{14} contained 67.1 mg parasite protein in a total volume of 15 ml and had an initial specific radioactivity of 42,450 dpm/μ mole. All vessels were gently shaken at 38°C for 1 hour, at which time perchloric acid was added to a final concentration of 3%.

Table 9

Incorporation of C^{14} Pyruvate into end products by P. knowlesi

Pyruvate C^{14} Incorporated	Pyruvate-U-C^{14}		Pyruvate-1-C^{14}		Pyruvate-3-C^{14}	
	mμ mole/mg Protein	Percent Utilized	mμ mole/mg Protein	Percent Utilized	mμ mole/mg Protein	Percent Utilized
Succinate	0.73	0.36	0.04	0.02	0.37	0.19
Neutral Volatiles	19.40	9.43	3.80	2.43	33.80	17.03
Acid Volatiles	166.8	81.12	101.20	64.1	--	--
Formate	4.64	2.26	5.37	3.40	6.27	3.16
Acetate	63.80	31.05	1.10	0.69	92.9	46.80
Pyruvate Utilized*	206.0	--	158.0	--	199.0	--
Lactate Produced*	32.0	15.5	27.6	17.4	35.5	17.8

*Determined enzymatically

Incubations were conducted for one hour at 38°C in 4 ml Warburg vessels containing 26.8 mg parasite protein under atmospheres of 95% air, 5% CO_2. Initial specific radioactivities were 82,440 dpm/μmole sodium pyruvate C^{14} (U), 82,760 dpm/μmole sodium pyruvate-1-C^{14} and 84,890 dpm/μmole sodium pyruvate-3-C^{14} in an initial concentration of 0.005 M sodium pyruvate. Experiments were terminated by addition of perchloric acid to a final concentration of 3%.

contamination catabolizes carbohydrate via glycolysis. The physiological significance of a tricarboxylic acid cycle as an energy pathway, however, is seriously questioned, since (1) the incorporation of C^{14} glucose into CO_2 is negligible and the tricarboxylic acid cycle pathway requires the formation of CO_2 from this substrate, and (2) all of the glucose utilized can be accounted for in end products other than CO_2. In this regard, it would also appear that the hexose monophosphate shunt does not contribute significantly to the energy metabolism of the free P. knowlesi parasite. It has been suggested that the parasite depends upon the host red blood cell for the shunt pathway. Contrary to earlier studies, products other than lactate were found to accumulate in large amounts during the fermentation of carbohydrate by P. knowlesi. Of particular significance were the volatile acids and neutral volatile fractions. The constituents of the neutral volatile fraction have not been identified. Similarly, only acetate and formate have been separated from the volatile acid fraction. Although large quantities of lactate are formed in these experiments (Tables 7 and 8), the actual amount arising from glucose can not be determined, since high concentrations of pyruvate were also added to the media. It can be estimated, however, that most, if not all, of the glucose carbons dissimilated can be accounted for in these experiments, therefore adding additional evidence that the terminal respiratory pathway present in most tissues of the mammalian host is not functional in the parasite.

Although the mechanism of formation of acetate by the parasite is still not known, pyruvate may serve as a precursor. What one carbon precursor is liberated from pyruvate can not be determined from these experiments. However, acetate is formed from glucose without the liberation of significant quantities of respiratory CO_2. This finding would suggest formate as a possible one carbon fragment, by a phosphoroclastic type reaction similar to that found in some bacteria. Formate is formed during P. knowlesi fermentations, but has not been recovered in sufficient amounts to account for all the acetate formed. Whether this is a result of the high volatiligy of formate (and, therefore, difficult to recover quantitatively) can not be determined at this time. Formate and acetate were previously reported to be products of P. berghei metabolism (Fulton and Spooner, 1956). However, no quantitation was attempted in these earlier studies.

The lack of a highly efficient aerobic terminal respiratory pathway in terms of maximum ATP production per glucose utilized would be in accord with the findings of Trager who showed an in vitro dependence of free P. lophurae parasites for survival on externally added ATP. Further studies showed either adenosine triphosphate plus pyruvate or adenosine diphosphate plus phosphoenolpyruvate favor parasite development. It was concluded that the parasites might be deficient in ATP generating systems. Brewer and Powell (1965), Brewer and Coan and Eaton and Brewer have reported that P. falciparum, P. berghei and P. cynomolgi respectively develop more rapidly in hosts whose red blood cells had a high ATP content.

From these findings, it would appear that P. knowlesi, like all of the parasitic helminths studied and many other parasitic protozoans, is not capable of completely oxidizing substrates to CO_2, indicating again a limited terminal respiration. It has been demonstrated that the parasitic helminths Hymenolepis diminuta and Ascaris lumbricoides both lack a tricarboxylic acid cycle, but both are capable of synthesizing electron transport associated ATP by non-glycolytic pathways. The possibility arises that the oxidative conversion of pyruvate to acetate by P. knowlesi may be associated with a similar energy yielding pathway.

It has been demonstrated for the first time that a malarial parasite partially ferments carbohydrates to neutral volatile products. Although this was surprising, it is by no means unique. A broad spectrum of neutral volatile compounds are formed by bacteria, parasitic protozoa, such as trypanosomes and parasitic helminths.

It has been shown that the malaria parasite is capable of incorporating radioisotope from C^{14} glucose or $NaHC^{14}O_3$ into glutamic acid, alanine, and aspartic acid as well as citrate, malate and succinate. This should not be taken as evidence that these parasites have a Krebs cycle since incorporation in terms of μ moles of substrate into these products are very low. Many bacteria and parasitic helminths have "altered their metabolism" so that there is no terminal respiration of acetate and the tricharboxylic acid cycle no longer functions as a terminal respiratory pathway. In spite of this, the synthesis of oxaloacetate and alpha ketoglutarate, the precursors for aspartate and glutamate respectively, contines at a reduced rate. Under these conditiona the cycle is modifed to form a branched biosynthetic pathway from oxaloacetate. One branch leads to succinate by a reductive reversal of the usual pathway. These reductions accept hydrogens from oxaloacetate resulting in a dismutation and the oxidative formation of alpha ketoglutarate. Such a system would agree with the findings reported above and those of Polet et al. Polet et al. in which P. knowlesi did not take up significant amounts of aspartic or glutamic acid into protein but synthesized it from glucose-C^{14}.

Even though cytochrome oxidase has been found in P. knowlesi, P. berghei and P. cynomolgi and more recently in P. falciparum, it is not necessarily of physiological significance to the metabolism of the parasite in view of the absence of a Krebs' cycle. In support of this, Cohen and Butcher found increased multiplication rates in P. knowlesi in vitro using 90% N_2, 5% CO_2, 5% O_2 over that observed under 95% air, 5% CO_2. The canine whipworm, Trichuris vulpis contains cytochrome oxidase but survives for longer periods under anaerobic conditions; Schistosoma mansoni also contains cytochrome oxidase and cytochrome c but these enzymes account for less than 10 percent of the oxygen taken up by this trematode. It would be desirable, therefore, to determine if oxygen is truly significant to the overall energy metabolism of P. knowlesi. This, in turn, would allow for a more competent evaluation of the role of the cytochrome system in this parasite, if indeed, there is such a role.

2. Cytochrome oxidase activity in platelet-free preparations of Plasmodium falciparum.

Cytochrome oxidase activity has been observed in the trophozoites and schizonts of P. berghei, P. knowlesi and P. cynomolgi, by the sensitive and specific spectrophotometric assay of Smith. To determine possible differences between these and human plasmodia, trophozoites and schizonts of P. falciparum (Camp. strain) were isolated from the blood of an experimentally infected owl monkey (Aotus trivirgatus) and assayed for cytochrome oxidase as previously described. Table 10 shows that P. falciparum like other species of malaria studied previously contains appreciable amounts of cytochrome oxidase activity.

Table 10

Cytochrome Oxidase in P. falciparum

	Cytochrome Oxidase Activity		
	mμ Moles Cytochrome c oxidized/min/ml protein		mμ Moles Cytochrome c oxidized/min/ml blood
Percent Parasitemia	Supernatant	Residue	
40	1.21	9.24	1.77
0	-	-	0.67

The metabolic value of this enzyme in P. knowlesi was studied and reported elsewhere. It was shown that this parasite does not rely on a functional tricharboxylic acid cycle for ATP (adenosine triphosphate) synthesis and no Pasteur Effect is demonstrable. Very little incorporation of isotope from glucose C^{14} in CO_2 was found as would be expected if an active tricarboxylic acid cycle were operating. Essentially all of the glucose utilized was accounted for in products other than CO_2. Cohen and Butcher also showed evidence of increased multiplication rates in 90% N_2, 5% CO_2 and 5% O_2 in P. knowlesi over that observed under 95% air 5% CO_2. These findings are in line with results obtained with other parasites indicating that the mere presence of an ezyme such as cytochrome oxidase does not prove it is important to the over-all economy of the cell. Trichuris vulpis contains cytochrome oxidase but survives longer under anaerobic conditions.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 129, Host responses to malaria

Literature Cited

1. References

None

2. Publications

Scheibel, L. W. and Miller, J. W.: Glycolytic and cytochrome oxidase activity in plasmodia. Mil. Med. 134(No. 10):1074-1080, 1969.

Scheibel, L. W. and Miller, J. W.: Cytochrome oxidase activity in platelet-free preparations of Plasmodium knowlesi. J. Parasit. 55: 825-829, 1969.

Sadun, E. H., Wellde, B. T. and Hickman, R. L.: Resistance produced in owl monkeys (Aotus trivirgatus) by inoculation with irradiated Plasmodium falciparum. Mil. Med. 134(No. 10):1165-1175, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6523	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	H. Terminatinn	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	130
b. CONTRIBUTING				
XXXXXXXXXX CDOG	1L12A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Literature of Malarial Test Data (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	2	70
FISCAL YEAR CURRENT 70	2	70

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Medicinal Chemistry

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Eckermann, LTC E. H.

TELEPHONE: 202-576-2292

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Chemical; (U) Chemistry; (U) Pharmaceutical; (U) Literature

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To maintain in machineable form all biological information associated with the test programs supported by the malarial project. Test results are to be maintained in a manner to be readily available to individuals requiring this data to properly manage the program to develop drugs which are more effective against the resistant strains of malaria encountered in Southeast Asia.

24 (U) Preliminary machining of approximately 50 percent of data is accomplished at the source. The remainder of preliminary machining and final processing of all data are done at Walter Reed. Programs have been written to allow processing of both biological and chemical data into one output. This permits searching of the files on either biological or chemical parameters.

25 (U) 69 07 - 70 06 Modification of programs to interface biology and chemistry were completed. Programming to convert drug inventory system to the in-house computer was completed. Testing of interface and inventory program has begun. Study has been initiated to determine the most economical method of creating indices for chemistry and/or biology. Currently 10 hours of 1401 computer time, 27 hours of 7090/94 and 10 hours of CDC 3300 time are required per week to maintain and process new data. Records for 220,000 compounds and data from 2,250,000 biological tests are stored on 60 tapes. Files are updated weekly with 700 new compounds (or combinations) and 3000 biological tests. This work unit is being terminated and that portion not completed is being combined with another work unit, Test Systems Development. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Classification assigned upon originator's approval

DD FORM 1 MAR 68 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.

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 130, Collection and retrieval of malarial test data

Investigators
Principal: Edgar H. Eckermann, LTC, VC
Associate: Robert O. Pick, CPT, MSC; John F. Waters, June A. Schafer

Description

The antimalarial drug development program of the Division of Medicinal Chemistry is highly dependent upon computer programming for the data collection and retrieval which is an important aid in guiding this program. Maintenance of inventory records, accession of new compounds, distribution of samples and compound information are controlled through computer programming. The chemical synthesis program is highly dependent on data assembled through an interface of chemistry, biology and inventory files which permit searching of files by chemical structure, biological activity, submitter, inventory status, or any combination of these parameters.

Progress

A. Inventory Control

The computer system for control of chemical inventory was operated routinely on a commercial computer during this report period. Programming has been written under contract to Computer Usage Corporation to convert this system to the newly installed computer at the Walter Reed Army Institute of Research. A period of one month has been authorized to run this system in parallel on the two machines to eliminate programming and operational problems. During reprogramming, new features were added to increase the usefulness of this system. Under the new system, routine orders for shipments of newly accessioned chemicals is accomplished by machine. Reports on all newly accessioned chemicals were possible.

B. Biological Data

Programming was produced to process data not previously processed by computer. This included programming for the mosquito test system operated by L. A. Terzian at the National Naval Medical Center, The Insect Repellent Test System operated by the Department of Agriculture, and the sporozoite-mouse test system operated by Illinois Institute of Technology. These programs included necessary provisions for interfacing with chemistry and inventory. This work was accomplished through continuation of a contract with Advanced Computer Techniques, Inc. A study was begun to determine the feasibility of

converting to the WRAIR computer without modification, those biology data programs written in COBOL.

C. Interface of Files

Work was begun on carrying shortened records through interface with edit with expansion occurring post-merge. This was made necessary by the rapid growth of chemistry and biology files. Work has been completed but has not been tested. Modification in the interface was made to allow the interface of inventory files with chemistry and biology. A study was initiated to determine the most economical method of creating indexes for chemistry and/or biology files. Programming in this area was accomplished through continuation of a contract with the Service Bureau Corporation.

Summary and Conclusions

Programming for new biology test systems have been accomplished and provisions have been made to interface inventory records with chemistry and biology data. Conversion of the inventory system to the WRAIR computer is near completion and planning for conversion of biology systems is accomplished.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 130, Collection and retrieval of malarial test data

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6525	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	132
b. CONTRIBUTING				
c. ~~XXXXXXXXX~~	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) CLINICAL STUDIES OF HUMAN MALARIA (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 2600 BIOLOGY

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 11	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	2	70
	CURRENT 71	1	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W.H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Medicine

ADDRESS: Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Canfield, LTC C.J.

TELEPHONE: 202-576-3529

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) - Study pathophysiology of acute falciparum and vivax malaria, assess various modes of antimalarial therapy with respect to clinical responses and radical cure.

24. (U) - Document clinical features of acute disease, evaluate available therapeutic agents with respect to clinical response and radical cure, provide surveillance for toxicity and efficacy testing of new antimalarial agents by government contractors, provide expert consultation on treatment of resistant falciparum infections and secure new strains of malaria for introduction into the volunteer test program.

25. (U) 69 07 - 70 06. Since the previous report, 16 patients have been treated at Walter Reed General Hospital for acute vivax malaria with single C-P tablets. Twelve of these have been observed for three weeks and four for one week prior to routine curative doses of chloroquine and primaquine. There have been no relapses during the period of observation. Twelve patients with acute falciparum malaria have been treated with Trimethoprim and sulfalene with total doses of as great as 4.5 and 3 Gm respectively. Six patients had an R-1 response, and four did not recur during 30 day observation periods. Three resistant falciparum strains have been frozen for later introduction into the prison volunteer program. Three patients not responsive to standard antimalarials have been treated with WR 33063 and all have shown a sensitive response. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 132, Clinical studies of human malaria

Investigators.
Principal: LTC Craig J. Canfield, MC

Description.

The objective of this work unit is to assess the clinical state and therapeutic response of patients to acute falciparum and vivax malaria, provide surveillance for toxicity and efficacy testing of new antimalarial agents by contractors, provide expert consultation on treatment of resistant falciparum infections, and secure new strains of malaria for introduction into the volunteer test program. In addition, the various aspects of the pathophysiology of the disease have been studied.

Progress.

Admissions to Walter Reed General Hospital for acute malaria infections have continued in small numbers and a variety of therapeutic regimens have been employed to supplement prison volunteer drug evaluation.

a. P. vivax. During 1965-1966 vivax infections accounted for only 2 percent of acute malaria admissions in Vietnam. Subsequently, this ratio increased and during a 9-month interval (May 1968 - January 1969) 58 percent of infections in U. S. Army personnel were due to P. vivax. During 1966 DDS became available and was added to the weekly C-P prophylaxis. DDS is an effective suppressant of P. falciparum infections but is not considered active against P. vivax. Although this change in chemoprophylaxis could explain a relative increase in vivax infections, a study was undertaken to evaluate the possibility that strains of P. vivax were present from Vietnam which were not responsive to the suppressive effect of the weekly C-P tablet. Thirty U. S. Army military personnel with acute vivax malaria were treated with a single C-P tablet in Vietnam and are reported elsewhere. Sixteen patients with acute vivax malaria were treated at Walter Reed General Hospital with single C-P tablets and observed for 3 to 4 weeks for evidence of therapeutic failure. All patients studied responded promptly to this therapy and did not recur during the periods of observation. Thus, the presently recommended weekly C-P tablet appears to be an adequate suppressant to naturally acquired P. vivax from Vietnam.

b. P. falciparum. Occasional patients with falciparum malaria have been seen who have had multiple relapses following standard treatment with combinations of quinine, pyrimethamine, DDS, chloroquine, and sulfisoxazole. Although the number is small only four were seen prior to January 1970 and seven have been seen since January at Walter Reed. Investigational drug regimens have been employed in these patients.

Under supervision of the principal investigator two additional patients were treated at Letterman General Hospital and two at Fitzsimons General Hospital.

Twelve patients were treated with trimethoprime and sulfalene with total doses as great as 4.5 gm and 3.0 gm respectively. Six patients had an R-1 response, four had a sensitive response, and results are pending on two.

Six patients were treated with WR 33063, two in combination with sulfanomides and four without additional drugs. All patients responded and thus far have no evidence of recurrence.

Blood from three of this latter group was preserved by freezing in glycerol for later introduction into the prison volunteer program. This is in addition to the Smith strain which was already distributed and a Panama strain which showed a R-1 response to chloroquine.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 132, Clinical studies of human malaria

Literature Cited.

1. Canfield, C.J.: Renal and hematologic complications of acute falciparum malaria in Vietnam. Bull. of the N.Y. Acad. of Med. 45: 1043, 1969.

2. Brooks, M.H., Barry, K.G., Cirksena, W.J., Malloy, J.P., Bruton, J., and Gilliland, P.F.: Pituitary-adrenal function in acute falciparum malaria. Amer. J. Trop. Med. & Hyg. 18:872, 1969.

3. Klainer, A.S., Gilliland, P.F., Cirksena, W.J., Bartelloni, P.J., and Beisel, W.R.: Serum glycoproteins in naturally acquired malaria in man. Arch. Int. Med. 123:620, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA 0A 6483	2. DATE OF SUMMARY* 70 06 30	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 60 07 01	4. KIND OF SUMMARY H. TERM.	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	133
b. CONTRIBUTING				
c. CONTRIBUTING	CDOC 1412A(2)			

11. TITLE (Precede with Security Classification Code)*
(U) ACUTE RENAL INJURY AND FAILURE IN MALARIA (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
012900 PHYSIOLOGY 012600 PHARMACOLOGY

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 07	CONT	DA	C. IN-HOUSE

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:*
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 69	3	100
	CURRENT 70	0.5	18

19. RESPONSIBLE DOD ORGANIZATION
NAME:* Walter Reed Army Institute of Research
ADDRESS:* Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME:* Walter Reed Army Institute of Research
Division of Medicine
ADDRESS:* Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Olsson, R. A., LTC, MC
TELEPHONE: 202-576-5121
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Anuria; (U) Kidney; (U) Shock; (U) Mannitol; (U) Renal function; (U) Renal tubule; (U) Malaria endocrine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) - To establish a rational approach in the prevention and treatment of acute renal failure associated with malaria.

24. (U) - Animal models are being used to study the metabolic, hemodynamic and hormonal alterations which lead to kidney damage and failure in malarial infections. Chronically implanted blood sampling tubes and physiological sensors have been used to study monkeys with malaria, thus avoiding the physiological disturbances caused by anesthesia and surgery. Completed work in this project showed that kidney failure in malarious monkeys occurs without a change in total kidney blood flow, suggesting that blood is shunted inside the kidney. Special sensors able to detect blood flow changes in localized areas within an organ are being developed in order to study this possibility.

25. (U) - 69 07 - 70 06. Satisfactory plastics for encapsulation of radiation sensors have been found, and prototype sensors have been tested in experimental animals. Improvement in signal to noise ratio of these implanted sensors has been achieved by changes in connector and cable design and use of Krypton 85 having high specific activity. This work is closely related to the activities of the Department of Cardiorespiratory Diseases and so is being carried out entirely within that department. Accordingly, this project is terminated and further progress will be reported under Work Unit 85. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 133, Acute renal injury and failure in malaria

Investigators.
Principal: LTC Ray A. Olsson, MC
Associate: Edward M. Khouri; Billy G. Bass

Description.

Animal models are being used to study the metabolic, hemodynamic and hormonal alterations which lead to kidney damage and failure in malarial infections; chronically implanted blood sampling tubes and physiological sensors have been used to study monkeys with malaria, thus avoiding the physiological disturbances caused by anesthesia and surgery. Special sensors, able to detect blood flow changes in localized areas within an organ, are being developed in order to study intra-organ distribution of blood flow.

Progress and Results.

Lithium-drifted semiconductor devices for beta ray detection are being evaluated for the possible study of intrarenal and renal insufficiency. Encapsulation techniques have been developed and a cable of adequate flexibility, low noise, low capacitance has been achieved. Two prototype units have been made and have helped to overcome further difficulties. To reduce the noise level further, a special stainless steel implantable coaxial connector has been designed and tested. The use of a bias amplifier has been found to help in further rejecting artifact noises.

Conclusions.

Ongoing work in this project is being incorporated into Project 3A061102B71R, RESEARCH IN BIOMEDICAL SCIENCES, Task 02, Internal Medicine, Work Unit 085, The heart under abnormal and pathological stresses. This document therefore constitutes a final report.

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 133, Acute renal injury and failure in malaria

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6531	70 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA: CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	H. Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	134
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Malaria Screening Systems (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 008800 Life Support

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	Cont	DA	C. In-House

17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 69	5	150
FISCAL YEAR CURRENT 70	5	150

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W. H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Biochemistry
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Angel, LTC C. R.
TELEPHONE: 202-576-2211
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria Plasmod SPP; (U) Mass Screening Techniques; (U) Automation

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The measurement of antimalarial drug effectiveness against the malaria parasite in the erythrocytic phase in vitro.

24. (U) Automatic wet chemical analysis subdivided into an incubation phase and an analytical phase is being employed to define the ability of the malaria parasite within the red cell to utilize nutrients or produce metabolites before and after incubation with chemical compounds under test for antimalarial activity.

25. (U) 69 07 - 70 03 The automated analytical chemistry program of the Division of Biochemistry has absorbed this work unit. Project has been combined with work unit 3A66371D829, 00, 108 and will be reported under DA 0A 6506. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

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

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 134, Malaria screening systems

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: R. L. Beaudoin, Ph.D. (NMRI); N. D. Brown, B.S.; J. I. Davis, B.S.; S. Garson, Ph.D.; R. T. Lofberg, Ph.D.

Description.

The objective of this work unit has been to provide automated or semiautomated screening systems to study the effects of chemical compounds on the metabolism of Plasmodium species in vitro. This objective has been modified to include the study of the biochemical effects and the mechanism of action of chemotherapeutic agents. Activities within this work unit are subdivided into methodology development, studies utilizing Plasmodium berghei, Plasmodium fallax and Shistosoma mansoni and the chemical definition of 4,4'-diamino diphenylsulfone and diformyl-amino diphenylsulfone.

Progress.

1. Methodology development.

As previously reported in the WRAIR Annual Reports of 1966-67, 1967-68 and 1968-69, the Technicon Autoanalyzer has been utilized in a modular configuration to test the effect of chemical compounds on the metabolism of Plasmodium species, in vitro. This system has been under continuous evaluation and has been terminated. Emphasis has been shifted to development of procedures that allow for the screening of metabolic variables that result from taking specific drugs or methods that define the particular agent itself.

During the reporting period, procedures for the determination of 4,4'diamino diphenylsulfone (DDS), methemoglobin and methemoglobin reductase activity have been adapted from manual methods and standardized. Each method has been placed directly into use on research protocols. The amount of DDS appearing in serum after ingestion of standard tablets is quite low and pushes the detection of the compound by means of the automatic method to the limit. In order to counter this low concentration, development of simple spot tests have been undertaken with promising results.

2. Studies utilizing Plasmodium berghei, Plasmodium fallax and Shistosoma mansoni.

The full utilization of automated methods must have a developmental basis before they can be used with profit. The interplay of species of parasites with species of mammals coupled with the physiological application of certain standard stresses to cause change in the desired biochemical variables are pertinent considerations. This section is devoted to such studies.

a. Biochemistry of exoerythrocytic Plasmodium fallax in tissue culture. (In collaboration with Dr. R. L. Beaudoin and his associates, Naval Medical Research Institute.) The present study deals particularly with the amino acid requirements of exoerythrocytic P. fallax grown in tissue culture. Data showing changes occurring in the culture medium as a result of parasite growth are currently available. Free amino acid analyses of normal host cells, parasitized host cells and free floating merozoites have not been completed. Pending completion of this preliminary study, it is anticipated that subsequent work will include the use of radiotracer methodology and relate to protein synthesis in the parasitized host cell. Future studies, currently in the planning stage, will deal with aspects of carbohydrate and nucleic acid metabolism. A formal proposal has been submitted in this regard wherein it was noted that this type of study may contribute to the development of rational chemoprophylaxis, and, further, provide some understanding of the prophylactic mechanisms associated with currently available drugs. Moreover, such studies should permit certain biochemical comparisons of the exoerythrocytic and erythrocytic forms of the malarial parasite.

b. Physiological alterations in hamsters infected with Plasmodium berghei. Infections with P. berghei in mice and in young rats are almost invariably fulminant and rapidly fatal; however, a more prolonged clinical course of infection occurs in hamsters. The present study was undertaken to determine if hamsters infected with P. berghei would provide a suitable and convenient model for the investigation of malarial pathophysiology.

The course of infection with P. berghei in male hamsters was studied sequentially in terms of parasitemia, hematology, blood chemistry, relative organ weights, tissue enzyme levels and selected urinary constituents. This study is nearing completion. Additional observations have substantiated a significant and progressive rise in blood lactate concentrations and a concomitant decrease in levels of glucose previously indicated in P. berghei infected hamsters. A more recent aspect of this study deals with the pathophysiology of the infection subsequent to prolonged serial passage of P. berghei in the hamster.

c. Hyperbaric oxygen in experimental infections with Plasmodium berghei or Schistosoma mansoni. It is thought by some investigators that tissue anoxia leading to degenerative changes, as in centrilobular hepatic necrosis, plays a prominent role in the pathogenesis of malaria. In this regard, hyperbaric oxygenation or oxygen under high pressure (OHP) has received considerable attention in the treatment of diseases involving anoxia. Accordingly, a study was undertaken to determine the effects of OHP on the course of P. berghei infection in mice and in hamsters. Various regimens of exposure to OHP, including repeated short applications, were incorporated at different intervals in the course of infection. Significant alterations in survival time or in parasitemias were not observed with the regimens of OHP treatment employed. These results seemingly conflict with those of Brewer and Coan (Mil Med 134: 1056-1067, 1969) who observed small but apparently significant reductions in the parasitemias of P. berghei infected rats following hyperoxia treatment.

In collaboration with LTC D. Erickson (Dept. of Medical Zoology, WRAIR) the effects of OHP administered in various regimens were also examined in mice infected with Schistosoma mansoni. Again, OHP appeared to exert little significant influence on the course of infection as indicated by worm recoveries, organ egg counts and host survival times.

3. Chemical definition of 4,4'-diamino-diphenylsulfone acid diformyl-diamino diphenylsulfone.

The efficacy of administering sulfones in conjunction with other antimalarial agents has been established. The use of 4,4'-diamino-phenylsulfone in Vietnam with potential observed clinical side effects has led to a program of chemical definition of this agent and the testing of pharmaceutical preparations before and after delivery to the field. In addition an effort has been initiated to study diformyl-amino diphenyl-sulfone before it is actually procured and used in the field.

a. 4,4'-diamino-diphenylsulfone (DDS). A number of lots of commercially prepared tablets of DDS were returned from Vietnam for testing relative to potency. Samples (5 individual tablets) were dissolved in one normal HCl and centrifuged to settle the filler. An aliquot of the supernatant diluted to a final dilution of 1:2500 was introduced into an autoanalyzer analytical chain and measured. The method of Simpson (Int. Journ. Leprosy 17: 197, 1949) which entails diazotization of the amino groups followed by coupling with N-methyl napthylethylenediamine to give a purple chromogen. Results of this initial screen led to the conclusion that lots of materials manufactured in 1966 and early 1967 had in some way lost a portion of available DDS. Each lot of material that showed a repeated low value for DDS was evaluated for conformance

to the standards prescribed in VOL XVII, United States Pharmacopeia. All of the lots of material examined conformed to the standards except in concentration where the assay was the nitrite titration as prescribed in the Pharmacopeia. An experiment to determine the cause of this apparent deterioration was initiated. Heating a sample of DDS that had not been in Vietnam to 60°C at 100 percent relative humidity for several weeks and comparing it to a sample of the same lot of material that had been returned from Vietnam showed a comparable reduction in available DDS (24 percent).

Thin layer chromatography has been employed to determine the purity of DDS. Samples of a standard obtained from the United States Pharmacopeia, WR 448G and individual lots of commercially prepared tablets were examined. Various numbers of apparent impurities have been determined ranging from three in the Pharmacopeia standard to as many as nine in DDS tablets. One point of commonality in these studies arises in that three of the impurities are always present suggesting components that are closely related to DDS. The major impurities in DDS are aromatic amines of one or more configurations. Characterization of two of the impurities strongly suggest the presence of a monoamino-diphenylsulfone and monoamino-phenylsulfophenol. Additional characterization is being accomplished.

It would be desirable to have a quantity of DDS that is chromatographically pure. A procedure that yields pure DDS has been established. The separation entails placing DDS in hot benzene, dissolving the DDS in ethyl acetate and reprecipitating the DDS by adding a large volume of benzene followed by cooling to 5-6°C. The precipitated DDS, after a second recrystalization, was found to be chromatographically pure. Sample of this pure compound are to be characterized by UV spectrophotometry, infrared spectroscopy and nuclear magnetic resonance spectroscopy. This characterized pure chemical will serve as a basis for studies of the basic chemistry of DDS.

b. Diformyl-diamino-diphenylsulfone (DFD). DFD is to replace DDS as the sulfone of choice in malarial therapy. Based upon experience with DDS in terms of purity, studies have been initiated to characterize WR6798AM. After examining several thin layer chromatographic systems the optimum system that gives the best separation consists of the following components:

Support: Aluminum oxide (pH9 with a 254 mu indicator)

Solvent System: 15% methycellosolve, 85% ethyl acetate

Spot development by chemical means has not been achieved as yet but exposing to ultraviolet at 254 mu and 360 mu produces a characteristic fluorescence. Examination of sample WR6798AM shows a total of five

defineable fluorescent spots at RF values of 0.70, 0.52, 0.43, 0.35 and 0.22. Spot number 4 has been assigned to DFD. Comparison studies are being pursued to identify the contaminants.

Summary and Conclusions.

The original technical objective of the work unit has been shifted to emphasize the biochemical effects and mechanisms of action of chemotherapeutic agents. Automated methodology has been developed and standardized to measure 4,4'-diamino-diphenylsulfone, methemoglobin and methemoglobin reductase. Biological studies have concentrated on the biochemistry of exoerythrocytic Plasmodium fallax in tissue culture, physiological alterations in hamsters infected with Plasmodium berghei and Shistosoma mansoni. Studies on DDS have indicated that the chemical purity is questionable. Identification studies have shown the presence of a monamino-diphenylsulfone as well as a substituted phenol. Samples of DDS have been treated and recrystallized to produce a pure sample of DDS. Studies of the diformyl derivative DDS have suggested that impurities are present in the current compound in use. Thin layer chromatographic techniques have been developed to separate the visible impurities.

Project 3A663713D829 MALARIA PROPHLAXIS

Task 00, Malaria Investigations

Work Unit 134, Malaria screening systems

Literature Cited.

1. Polet, H_4, Brown, N. D., and Angel, C. R.: Biosynthesis of Amino Acids from ^{14}C-U Glucose, Pyruvate and Acetate by Erythrocytic Forms of P. knowlesi, in vitro. Proc. Soc. Exptl. Biol. and Med., Vol 131, Nr 4: 1215-1218, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6534	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	135
b. CONTRIBUTING				
XXXXXXXXXX	CDOG 1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Experimental Pathology and Metabolism of Plasmodia (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 26 00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: Not Applicable d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 70	2	30
	CURRENT 71	2	30

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Meroney, COL W. H.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Experimental Pathology

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Sprinz, COL H.

TELEPHONE: 202-576-2677

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Rock, MAJ Robert C.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Plasmidial Prote (U) Plasmodial Ribosomes; (U) Plasmodial Microsimes; (U) Plasmodial Lipids

23. TECHNICAL OBJECTIVE. 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) To study metabolic pathways in malarial parasites maintained in vitro. To isolate, purify and identify by chemical, physical and morphologic means the subcellular components of malarial parasites, including cellular localization of anti-malarial drugs.

24. (U) Biophysical, biochemical and morphologic techniques are employed.

25. (U) 69 07-70 06. Our major studies involve analysis lipid composition and biosynthesis in Plasmodium knowlesi grown in vitro. A manuscript entitled "Lipid Composition of Plasmodium knowlesi" by Rock et al has been submitted for publication. Work done in our department by Dr. R. A. DeZeeuw, a visiting scientist from the State University, Groningen, The Netherlands, will be published in Pharmaceutish Weekblad, as a paper entitled "The Use of Vapor-programmed Thin-layer Chromatography in the Phospholipid Analysis of Malarial Parasites." This very promising technique will be pursued in further collaborative studies with Dr. DeZeeuw. Results of current investigations of lipid biosynthesis by Plasmodium knowlesi, will be presented by Rock at the Second International Congress of Parasitology, Washington, D.C., in September 1970, in a paper entitled "Membrane Metabolism and Phospholipid Biosynthesis in Plasmodium knowlesi." Two manuscripts dealing with characterizations of the ribosomes of Plasmodium knowlesi are in preparation; the first by Cook et al, presents biophysical measurements, and the second, by Aikawa et al, shows ultrastructural morphology. A manuscript is in preparation dealing with the pathology of Plasmodium falciparum in the owl monkey, Actus trivirgatus. The ultrastructure of malarial parasites is the topic of several manuscripts in preparation by Ladda et al, including 1. cytoplasmic microtubules and their relationship to parasite nucleic acid, 2. ultrastructure of Plasmodium vivax, 3. ultrastructural effects of chloroquine and freeze etching electron microscopy. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69-30 Jun 70.

Available to contractors upon originator's approval.

DD FORM 1 MAR 66 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

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 135, Experimental pathology and metabolism of plasmodia

Investigators.
Principal: COL Helmuth Sprinz, MC
Associate: MAJ Roger Ladda, MC; MAJ Robert Rock, MC

Description

Both in vitro and in vivo studies have been carried out (or are in progress), utilizing ultrastructural, biochemical and radioisotopic tracer methods in following growth, development and metabolic pathways of malarial parasites, and the effects of antimalarial compounds upon parasite metabolism and morphology.

Progress

1. The fractionation of the primate malarial parasite, Plasmodium knowlesi, was achieved by Dr. Cook (now at Case Western Reserve University) by a method of discontinouous sucrose gradient ultracentrifugation. This method was presented at the Third International Workshop on Malaria, and is in published form in a special supplemental issue of Military Medicine (September 1969).

2. This fractionation proceedure has been applied in the characterization of the lipid composition of Plasmodium knowlesi, by a comparison of a parasite light microsomal fraction (free of hosted red cell material) with the membranes of host red cell. Several major quantitative and qualitative differences in phospholipid and cholerterol composition have been found by combining this fractionation technique with thin-layer chromatography. (A manuscript reporting these findings has been submitted for publication by R. C. Rock, J. Standefer, R. T. Cook, W. Little and H. Sprinz.)

3. Further studies of the phospholipid composition of Plasmodium knowlesi has been carried out in collaboration with Dr. Rokus deZeeuw, a visiting scientist from the State University, Groningen, The Netherlands. By application of the advanced analytical technique ov vapor-programmed thin-layer chromatography (developed by Dr. deZeeuw), separation of large quantities of malarial parasite phospholipid has been achieved. (An article entitled "The Use of Vapor-Programmed Thin-Layer Chromatography in the Phospholipid Analysis of Malarial Parasites," by R. A. deZeeuw, R. C. Rock and H. Sprinz, has been accepted for publication in Pharmaceutish Weeklad.) This novel technique

of thin-layer chromatography results in separation of amounts of phospholipid sufficient for further characterization (i.e. by infrared spectroscopy and mass spectrometry), and further studies with Dr. de Zeeuw are in progress.

4. The intraerythrocytic growth of malarial parasites is accompanied by a marked increase in the membranes of the parasite (especially when compared to the membrane of the mature mammalian red cell). This synthesis of parasite membrane can be followed both in vitro and in vivo using radioactive precursors, including inorganic ^{33}P as well as ^{14}C-labelled glucose, acetate, glycerol, choline and ethanolamine. The active incorporation of these precursors into parasite phospholipid contrasts markedly with the minimal uptake by the mature mammalian host red cell. Of great interest are the differential effects of various metabolic inhibitors upon membrane biosynthesis of the parasite. These results will be presented by R. C. Rock at the Second International Congress of Parasitology, Washington, D.C., in September 1970, in a paper entitled "Membrane Metabolism and Phospholipid Biosynthesis in Plasmodium knowlesi."

5. Dr. R. T. Cook has continued his studies on the ribosomes of Plasmodium knowlesi and in vitro protein synthesis by parasite ribosomes. A manuscript by R. T. Cook, R. C. Rock, W. Little and H. Sprinz is in preparation, reporting the ultracentrifugal characterization of Plasmodium knowlesi ribosomes and the effects of various antibiotic and antimalarial compounds upon in vitro protein synthesis by isolated parasite ribosomes. A second manuscript in preparation by R. T. Cook and M. Aikawa reports the ultrastructure of parasite ribosomal subunits.

6. Dr. R. L. Ladda and co-workers are completing several manuscripts concerning the ultrastructure of malarial parasites and morphologic effects of antimalarial compounds (formerly included in Work Unit 112, Fine Structure - Malarial Parasites). These manuscripts include: (1) "The ultrastructure of Plasmodium vivax" by R. Ladda, G. Olson and H. Sprinz, (2) "Cytoplasmic microtubules in malarial parasites: structural direction in morphogenesis" by R. Ladda and H. Sprinz, (3) "Freeze-etching of malarial parasites" by R. Ladda, R. Steere and H. Sprinz, (4) "Association of microtubules and DNA strands with nuclear membranes of malarial parasites" by R. Ladda, R. Beaudoin and H. Sprinz, and (5) "Effect of chloroquine on human fibroblasts in culture" by R. Ladda, P. Kramer and H. Sprinz. Additional applications of tissue culture to cell fusion were presented by R. Ladda at the FASEB meetings in April 1970.

7. DNA has been isolated from several species of avian, rodent, and primate malarial parasites, and has been characterized biochemically (by hybridization and base-ratio determination) and morphologically (by ultrastructure of isolated DNA strands). The manuscript in preparation is entitled "Characterization of malarial parasite DNA" by

R. Ladda, J. Wohlheiter, R. Estensen and H. Sprinz.

Summary and Conclusions

During the past year, the work in progress on the fine structure of malarial parasites has been completed, with additional observations on parasite microtubules and their interaction with parasite DNA, ultrastructure of freeze-etched parasites, and morphologic effects of chloroquine. Work in our department has extended to include investigations of parasite protein, lipid, and nucleic acid metabolism. The work on malarial parasite nucleic acid has been completed by Maj. Ladda who is leaving at the end of fiscal year 1970. During the next fiscal year, Maj. Rock will continue the studies of cell membrane synthesis and lipid metabolism of the parasites.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 135, Experimental pathology and metabolism of plasmodia

Literature Cited

1. Ladda, R.L., Aikawa, M., and Sprinz, H.: Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. J. Parasit. 55:633, 1969.

2. Ladda, R.L.: New insights into the fine structure of rodent malarial parasites. Milit Med. 134 (Supplement): 825, 1969.

3. Cook, R.T., Aikawa, M., Rock, R.C., Little, W., and Sprinz, H: The isolation and fractionation of Plasmodium knowlesi. Milit Med. 134 (Supplement): 866, 1969.

4. Sprinz, H.: Comments on non-human Plasmodia. Milit Med. 134 (Supplement): 925, 1969.

5. Sprinz, H.: Comments on "Studies on the red cell-parasite relationship." Milit Med. 134 Supplement): 976, 1969.

6. Aikawa, M. and Beaudoin, R.L.: Morphological effects of 8-aminoquinolines on the exoerythrocytic stages of Plasmodium fallax. Milit Med. 134 (Supplement): 986, 1969.

7. Aikawa, M., Cook, R.T., and Sprinz, H.: Fine structure of erythrocytic stages of Plasmodium knowlesi. Zeitschrft. Zellforsch. v. mikrosk. Anatomie. 100: 271, 1969.

8. Barnes, M.L. and Polet, H.: The influence of methylene blue on the pentose phosphate pathway in erythrocytes of monkeys infected with Plasmodium knowlesi. J. Lab. Clin. Med. 74: 1, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6535	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A663713D829	00	136
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with security classification code)

(U) METABOLIC AND ENZYMATIC STUDIES OF NORMAL AND MALARIA INFECTED RED CELLS (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 BIOLOGY

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 12	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 70	1	35
FISCAL YEAR — CURRENT 71	2	60

19a. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL, W.H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Medicine
ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Canfield, LTC, C.J.
TELEPHONE: 202-576-3529
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number, precede text of each with security classification code.)

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 develop new drugs effective against resistant falciparum malaria.

24. (U) - Study the uptake of certain amino acids by infected red blood cells, measure folic acid reductase in parasite suspensions, to measure the effect of antimalarial drugs on morphologic growth, lactate production, 14-C methionine or 14-C orotic acid incorporation on in vitro schizogony.

25. (U) 69 07 - 70 06. In vitro culture of P. knowlesi parasites has continued. Incorporation of C-14 methionine lactic acid production and morphologic maturation have been shown to be sensitive indicators for evaluating potential antimalarials, exclusive of antifols, C-14 orotic acid incorporation into DNA has been shown to be an equally sensitive indicator for those compounds.

Studies of the mode of action of sulfalene and pyrimethamine have shown that a 90% inhibitory concentration of sulfalene may be completely reversed with PABA, folic acid or folinic acid in concentrations 1/100 of the sulfalene whereas only folinic acid produces minimal reversal of pyrimethamine efficacy. This suggests concurrent treatment with an antifol and folinic acid may be possible to obviate the thrombocytopenia, leucopenia and anemia attributed to antifol toxicity in malaria patients. Biochemical pathways for nucleic acid synthesis are being determined as an aid to rational development of new antimalarial drugs. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 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.

BLANK PAGE

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 136, Metabolic and enzymatic studies of normal and malaria infected red cells

Investigators.
Principal: LTC Craig J. Canfield, MC
Associates: Gerald J. McCormick, Ph.D.; Esther P. Jorolan, Ph.D.; MAJ Michael J. Dunn, MC

Description.

The objective of this work unit is to study metabolic pathways of the host red blood cell - parasite complex and to assess the effect of antimalarial drugs on these pathways in order to develop new drugs effective against resistant falciparum malaria.

Progress.

Investigations on intracellular sodium concentrations and sodium flux studies in P. knowlesi infected monkeys was completed and published (1,2).

Investigations of amino acid transport in infected red blood cells was also completed (3). Distribution ratios for isoleucine and methionine were increased. Incorporation into protein by malaria blood was greatest for isoleucine (150 times control), methionine was 30 times control, leucine and histidine 16 times control, and cystine 4 times control after 2 hours incubation.

Investigation of RBC 2-3 DPG during acute infections with P. knowlesi in Rhesus monkeys was undertaken. The concentrations of 2-3 DPG during infection increased to a degree similar to that observed in monkeys made anemic by blood-letting.

Work has continued on the in vitro P. knowlesi malaria culture system. In this system young ring forms of the parasite predictably develop into mature schizonts during 18-20 hours incubation in a nutrient medium. Also lactic acid is produced, 14-C methionine is incorporated into protein and 14-C orotic acid is incorporated into DNA and RNA. Dose response curves have been obtained using the above measurements of growth with known antimalarial drugs which correlate well with clinical experience.

Using a variety of labeled nucleic acid bases and nucleosides it has been concluded that the parasite red cell complex does not perform salvage synthesis of pyrimidine nucleic acids but must rely on de nova synthesis through the ortic acid pathway. This has led to the study of five substituted orotic acid compounds as potential antimalarials.

Thus far two have been found that were effective (5 fluoro and 5 bromo). These drugs inhibit parasite DNA formation and theoretically have the added advantage of not inhibiting host DNA synthesis by the salvage pathway.

Studies of the mode of action of sulfalene and pyrimethamine have shown that a 90 percent inhibitory concentration of sulfalene may be completely reversed with PABA, folic acid or folinic acid in concentrations 1/100 of the sulfalene whereas only higher concentrations of folinic acid produces minimal reversal of pyrimethamine effect. This suggests concurrent treatment with an antifol and folinic acid may be possible to obviate the thrombocytopenia and anemia attributed to antifol toxicity in malaria patients.

Further studies are underway on single carbon donors for nucleic acid synthesis by the malaria parasite. Thus far results have shown that serine histidine and methionine can serve as methyl donors whereas choline betaine sarcosine and formic acid cannot. Studies are planned to elucidate the pathways involved.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 136, Metabolic and enzymatic studies of normal and malaria infected red cells

Literature Cited.

1. Dunn, M.J.: Alterations of red blood cell metabolism in simian malaria: Evidence for abnormalities of non parasitized cells. Mil. Med. 134:1100, 1969.

2. Boehm, T.M., and Dunn, M.J.: The effect of experimental malaria on intracellular concentrations of sodium and potassium in muscle and liver. Proc. Soc. Exptl. Biol. & Med. 133:370, 1970.

3. McCormick, G.: Amino acid transport and incorporation in red blood cells of normal and Plasmodium knowlesi - infected Rhesus monkeys. Exp. Parasit. 27:143, 1970.

PROJECT 3A062110A830
BIOSENSOR SYSTEMS

Task 00
Biosensor Systems

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6441	70 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
69 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A830	00	055
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Development and Evaluation of Improved Biological Sensor Systems (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

001700 Animal Husbandry 011800 Operations

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 09	Cont	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 70	4	300
CURRENT FISCAL YEAR 71	3	225

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL
NAME: Meroney, COL W.H.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Department of Bio Sensor Research
ADDRESS: Edgewood Arsenal
Maryland 21010

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Castleberry, COL M.W.
TELEPHONE: 301-584-3312
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Wilke, CPT W.L.
NAME: Watkins, 2LT L.E. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Detector System; (U) Dogs; (U) Genetics; (U) Selective Breeding

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To develop a more intelligent and sensually acute dog which is physically and tempermentally better suited for military purposes than is now generally available.

24. (U) This study is being made in response to the approved (11 Dec 68) US Army QMDO, "Detector System Military Dog" (USACDC Action Control Number 12527). Critically evaluated AKC registered dogs were purchased as foundation stock. The progeny of these are closely evaluated by recognized tests designed to reveal the superior individual. These in turn are selectively bred and their offspring similarily tested, evaluated and bred. Linebreeding combined with continued evaluation of each generation will accomplish the objective.

25. (U) 69 07 - 70 06 Forty three whelpings produced 280 weaned puppies. Present kennel population of 222 dogs includes 177 German Shepherd Dogs, 31 German Shorthaired Pointers, 12 Pointer-Shepherd crosses, and 2 Drahthaars. An additional 217 dogs were transferred to other activities. Puppy evaluation procedures were revised slightly. The following studies were completed: hormonal control of the canine estrous cycle; use of a bird dog for scouting purposes; possibility of a dog locating a hidden hand gun. A computerized information retrieval system was effected with the AFIP. A stress machine for very young puppies was constructed. A loud speaker system was installed to familiarize the dogs with battle sounds and street noises. Consultant visits to this facility were made by national authorities. Construction of permanent facilities was completed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 69 - 30 Jun 70.

* Available to contractors upon originator's approval.

BLANK PAGE

Project 3A062110A830 BIO SENSOR SYSTEMS

Task 00, Biosensor Systems

Work Unit 055 Development and evaluation of improved biological sensor systems

Investigators
Principal: COL Merida W. Castleberry, VC
Associates: CPT Willard L. Wilke, VC; 2LT Leland E. Watkins, MSC

OBJECTIVE: To develop a more intelligent and sensually acute dog which is physically and tempermentally better suited for military purposes than is now generally available.

BACKGROUND: This study is being made in response to the requirements of the recently approved US Army QMDO, "Detector System, Military Dog", (USACDC Action Control Number 12527). Seven breeds of dogs, including crosses, were studied by the University of Maryland for behavioral evaluation and selection for army breeding and training (Army Contract No. DADA 17-68-C-8015). As recommended in the final report of that study, and because of the years of military experience gained with the German Shepherd Dog, this breed was selected for primary breeding emphasis.

APPROACH: Critically evaluated AKC registered breeding stock purchased especially for this purpose are selectively bred to produce superior progeny. These are in turn closely evaluated by recognized tests designed to reveal the superior individual. Line breeding combined with progeny testing of each generation is being used to accomplish the objective. Evaluation of other breeds, including crosses, continues on a limited scale.

PROGRESS

A. Breeding Program

1. Forty three whelpings produced 280 weaned puppies.

2. Kennel population: Present kennel population is as follows:

German Shepherd Dog	177
German Shorthaired Pointer	31
Pointer-Shepherd Cross	12
German Wirehaired Pointer (Drahthaar)	2
	222

3. Two hundred and one dogs were transferred to other activities:

Walter Reed Army Institute of Research	145
Fort Benning, Georgia	31
Fitzsimmons General Hospital	17
Operation Nitefox	8
	201

4. Puppy evaluation - Our puppy testing procedures have proven, in the main, to be quite effective. The sight, sound, and body sensitivity tests, however, were eliminated as non-contributory. A new test, rag agitation, was recently added.

B. Special Projects:

1. The initial study of the use of a field dog for scouting purposes was completed with a demonstration of the German Shorthaired Pointer at Fort Benning, Georgia. One of the two dogs demonstrated was subsequently returned and trained by the Scout Dog Platoon in off-leash scouting. This dog and his handler scored 100% in the class ORT (Operational Readiness Test). The class average was 89.9%. Having kept the better dog for our own purposes, additional matings were accomplished including two crosses with the German Shepherd Dog. This latter was made in hopes of obtaining the typical bird dog "point" and the German Shepherd intelligence and boldness. A few of the purebreds and crosses will be sent to Fort Benning in the late fall for training.

2. A study was completed and a report submitted concerning the possibility of regulating the onset of estrous and ovulation in the anestrous bitch by hormonal means. Two intramuscular injections of Pregnant Mares Serum, 1500 International Units per dose given to 6-month old female Beagles at 7-day intervals failed to consistently induce follicular development and growth. Subsequent administration of 1500 to 10,000 International Units Human Chorionic Gonadotropin or 1 to 15mg Luteinizing Hormone did not induce ovulation in the limited number of bitches which did exhibit ovulatory size follicles.

3. A computerized information retrieval system was programmed in conjunction with the Veterinary Pathology retrieval system at the Armed Forces Institute of Pathology. Work sheet coding of all eligible dogs has been completed.

4. An attempt was made to train a dog to locate a hidden pistol. If freshly fired, and not too well hidden, the dog could quickly locate the weapon. It is believed that intense, expert training of an adept dog would produce a highly valuable detector.

C. Consultant Visits: In addition to Mr. L. Wilson Davis, our continuing consultant on dog behavior and training, the Department received the benefit of a two day consultant visit by Mr. William Koehler, Animal Behaviorist and trainer for Walt Disney Productions. Dr. M.W. Fox, DVM, formerly of the Jackson Memorial Laboratory and presently with the Department of Psychology Laboratory, Washington University, St. Louis, Missouri, visited as a special consultant in animal psychology. Dr. Wayne Riser, DVM, University of Pennsylvania, made frequent visits in monitoring our hip and elbow dysplasia control program.

D. Facilities: A permanent facility was constructed with MCA funding. It provides adequate kennel space for 225 dogs, an appropriate whelping area, and suitable administrative quarters. Beneficial occupancy was taken on 24 March 1970.

E. Equipment

1. Stress machine - during a recent consultant visit, Dr. M.W. Fox described the benefits of mildly stressing the newborn puppy. As a result, certain of our puppies are now subjected for 13 days to 3 minutes per day of a combined teeter-totter and circular motion. To accomplish this, an electrically operated, flanged, circular, tilting platform 36 inches in diameter was constructed. This turns at 30 rpm and has an eight inch tilt. It is compartmentalized to hold ten puppies. The puppies are subsequently subjected to 1 minute of hypothermia (30° F) and are then petted for two minutes before being returned to the mother.

2. Loud speaker system - to better orient our kennel raised dogs, a loud speaker system with tape recorder was installed to provide battle sounds, march music and street noises.

DISCUSSION: Accomplishment of our mission is dependent upon our ability to successfully test and breed for superior intelligence, temperment, and physical normality (freedom from hip and elbow dysplasia). The principles of selective breeding have been used to obtain more milk, wool, meat, eggs, etc. No effort has been made, apparently, to breed for intelligence in a domestic animal. Carefully selected matings based on high puppy and subsequent evaluation scores is expected to produce a more intelligent and trainable dog. Breeding for a more stable temperment and dysplastic free hips and elbows is also a tedious but rewarding task of observation, testing, and records keeping.

CONCLUSION: The principles of applied selective breeding have been advantageously used by man for centuries. Of recent years the study of heredity has become a science. Based upon previous accomplishments in this field, it can be assumed that this project will produce a remarkably improved military dog.

RECOMMENDATIONS: None

PROJECT 3A062110A830 BIOSENSOR SYSTEMS

Task 00 Biosensor Systems

Work Unit 055 Development and evaluation of improved biological sensor systems

Literature Cited: None.