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 1970 - 30 June 1971

VOLUME II

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

AD

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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 1970 - 30 June 1971

Volume II

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

SUMMARY

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

FOREWORD

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

TABLE OF CONTENTS

VOLUME II

PROJECT 3A061102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 01
Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6465	71 07 01	DD-DR&E-AN(A)6

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD/N INSTR'N	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
70 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NSC CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	611023	3A061102B71R	01	091
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Include with Security Classification Code)

(U) Metabolic Problems Associated with Disease and Injury (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002309 Biochemistry 003500 Clinical Medicine 012900 Physiology

13. STUDY CATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	Cont	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA — EXPIRA...

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 71	6	170
FISCAL YEAR CURRENT 72	6	170

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Enzymology; (U) Hemorrhage; (U) Trauma

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

23. (U) 1. To relate factors responsible for metabolic injury and tissue damage produced by trauma and disease to morbidity and mortality. 2. To evaluate therapeutic agents in the treatment and prevention of disease and injury in relation to altered biochemical and physiological parameters. 3. To investigate metabolic problems associated with oxygen and electron transport.

24. (U) 1. Establish and study animal models for use in experiments. 2. Study biochemical, histological, and physiologic systems for altered metabolic processes and/or tissue damage to include surgical removal or isolation of tissue for study purposes. 3. Evaluate pharmacological doses of various compounds in treatment. 4. Determine levels of various compounds and their effects on metabolic systems.

25 (U) 70 07 - 71 06 1. Effects of diaminodiphenylsulfone (DDS) on thyroid function and 2-3 diphosphoglyceric acid (2-3 DPG) content was studied in rats. At the doses used no thyroid effect was noted. However, 2-3 DPG levels in red cells were increased in animals treated with DDS and also in hamsters challenged with bacterial endotoxin and malaria. 2. Silastic bags were attached to biliary catheters, successfully implanted in rat abdomens and collections of bile made from them. 3. The effects of prozole on the drinking patterns of ETOH preferring rats was tested. Administration of pyrozole reduced consumption of alcohol in this trial study. Further work is in progress. 4. A comparative study of 4 different procedures for 2-3 DPG determination was conducted in conjunction with the Clinical Chemistry section. The Prins and Loos method was determined to be the most stable and most reliable. 5. A preliminary study of halothane toxicity was completed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

(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 68 AND 1498-1, 1 MAR 68 FOR ARMY USE ARE OBSOLETE

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 091, Metabolic problems associated with injury and disease

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC; John W. Diggs, M.S.; Ting-Kai Li, M.D.; Lawrence Lumeng, M.D.; Jean E. Matusik, B.S.; 2LT Philip E. Nino, MSC; MAJ James B. Powell, MC; H. Kenneth Sleeman, Ph.D.

DESCRIPTION.

The delineation of mechanism of action of a variety of agents continues to be of marked interest in biological and physiological chemistry. Methods of studying these mechanisms in animal models and the effects of these agents on the animal models are the goal of this work unit. In addition to development of model animal systems and observation, results are reported concerning halothane induced hyperexia.

1. DDS. Organic compounds containing the aminobenzene grouping have been shown to inhibit the synthesis of thyroid hormone. DDS is structurally related to other thyroid active agents and this activity was measured in terms of depression of thyroxine levels and 2,3-DPG levels. The depression of thyroid function and related effects on metabolic systems may be related to the antimalarial effects of DDS.

2. 2,3-DPG. It is known that a direct relationship exists between the organic phosphate content of erythrocytes and the oxygen affinity of hemoglobin. The major organic phosphate of erythrocytes and the one having the major influence on oxygen affinity is 2,3-diphosphoglycerate (2,3-DPG). Preliminary studies have been performed to determine the levels of 2,3-DPG in bacterial shock and malaria and to relate these results to the course of the disease.

3. Collection of bile from laboratory animals is important in studying excretions of hepatic origin. Many water insoluble drugs are metabolized in the liver and excreted in the bile. The development of a successful method of implanting an accessible plastic bag and biliary catheter into the abdomen of the rat will make studies of sequential bile samples much easier.

4. The biochemical mechanism which underlies alcohol preference in rats is unknown. Metronidazole (Flagyl), a weak inhibitor of liver alcohol dehydrogenase, has been found to be effective in decreasing alcohol consumption in rats with induced or natural preference for ethanol while the drug was given. We are evaluating the effect of

pyrazole (an analog) on the drinking pattern of rats with induced or natural preference for alcohol.

5. The clinical syndrome of anesthetic-triggered malignant hyperexia has been described with increasing frequency in the recent medical literature. The mortality in this condition is ~70%. The agents responsible have included halothane, chlorpform and succinyl choline. The mechanism of the production of the syndrome is unknown. The susceptible patients develop, shortly after exposure to these agents, unrelenting fever and muscle rigor, ending in death. There is a familial predisposition, suggesting an inheritable disorder of muscle metabolism or oxidative metabolism.

An animal model has been developed based on the observation that the South African Landrace pig exhibits the same reaction to anesthetics.

PROGRESS.

1. DDS. Experimental animals were divided into three groups (control, acute, chronic) and the acute and chronic groups received DDS (25 mg/rat suspended in 0.5% methyl cellulose containing 0.1% Tween 80). The dosage and sacrifice schedules were planned to provide a sampling of acute and chronic effects of treatment. The animals were sacrificed and thyroid gland excised and weighed. Blood samples were collected for 2,3-DPG and thyroxine assay. The study utilized 41 animals.

2. 2,3-DPG. A study of shock induced by bacterial endotoxins and of malaria was carried out and the erythrocyte organic phosphate levels were measured. Sprague-Dawley rats were used in the studies of bacterial shock and hamsters infected with P. berghei were used for the studies of 2,3-DPG levels in malaria. Anesthetized animals were bled from the abdominal aorta and hematocrit, pH and 2,3-DPG content determined.

3. Implantation. The implantation of a silastic bag in the abdomen was successful. Collection of bile introduced into the bag through a biliary connula can be carried out making possible serial collections of bile for further study.

4. A pilot study to evaluate the effect of pyrazole on the drinking patterns of rats with induced or natural preference for alcohol was completed. The situation was set up as a free choice system in which the animals choose between water and 10% w/v EtOH. Pyrazole was administered after establishment of baseline alcohol consumption and subsequent consumption was determined.

5. Landrace Pigs. A project was initiated and completed to determine whether or not the American Landrace pig showed the same response to anesthetic agents. Fourteen animals have been tested to date. They were exposed sequentially to anesthesia with thiopental, nitrous oxide

and halothane. Core temperatures were monitored and muscle biopsies for ATP levels were collected.

SUMMARY.

1. DDS. Prophylactic doses of DDS (25 mg) did not produce a significant hyperplasia of rat thyroid glands or change plase thyroxine levels compared to untreated or normal controls. These doses did produce depressed appetite and weight loss. The chromatograms of thyroid extracts showed qualitative decreases in T4 and T3 but not mono or diiodotyrosine. The discrepancy in the T4 assay cannot be explained at this time. Blood 2,3-DPG levels showed some increase in the DDS treated animals. If this elevation of 2,3-DPG can be confirmed it would indicate a hyperthyroid condition and an additional oxygen requirement by the tissues. The use of larger doses of DDS (250 mg/animal) should yield additional information on the effect of this compound on thyroid and related biochemical functions.

2. 2,3-DPG levels in the bacterial endotoxin shock animals were significantly lower ($p < .01$) than control animals. Dexamethasone treatment prevented changes in 2,3-DPG content of red cells and pH. A significant rise in red blood cell ATP ($p < .05$) and the increase in 2,3-DPG in malaria infected hamsters probably reflects a compensatory mechanism. The elevation of both ATP and 2,3-DPG would permit better oxygenation of the tissues.

3. Bile was successfully collected from the implanted silastic bag and cannula daily by insertion of a needle through the abdominal wall into the bag. One to 3 ml of bile were collected each day. This procedure provides a convenient reservoir for the serial collection of bile over several days. This system was utilized by other members of the biochemistry staff for the study of the metabolism and distribution of orally administered WR 33063.

4. The data indicate that pyrazole (25 mg/kg), given as a single intraperitoneal injection, decreased alcohol consumption 50% or more for 6-8 days in rats with either induced or natural preference. There are three possible explanations for this effect of pyrazole on rats: a) inhibition of hepatic metabolism of alcohol resulting in intolerable blood alcohol levels, b) the compound may produce an aversion reaction toward alcohol, and/or c) the compound may inhibit brain alcohol dehydrogenase, hence reducing acetaldehyde and tetrahydropapaveroline levels in the brain.

5. Fourteen American Landrace pigs were tested. Only one of the animals exhibited a hyperexia reaction, resulting in death. No muscle ATP changes were observed in non-reactive animals but the reactive animal demonstrated a marked fall in muscle ATP content. The fall in muscle ATP content exceeded that normally observed with exhaustive

muscular activity in man. Serum ionized and total calcium concentrations become abnormally high during the reaction. These studies indicate that the American Landrace pig also exhibit this reaction to anesthetic agents, but the incidence is probably lower than in the South African strain. Inbreeding of the trait (if possible) can be expected to increase their incidence making this animal model economically feasible for the study of this important disease entity.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 091, Metabolic problems associated with injury and disease

Literature Cited.

Publications:

1. Sleeman, H. K., Diggs, J. W., Solis, R. T., and Angel, C. R.: The effects of shock on 2,3-Diphosphoglycerate levels in the erytococyte. Abstract, 160th Annual Meeting, American Chemical Society, p. 129, 1970.

2. Soloway, H. B., Robinson, E. F., Sleeman, H. K., Hoyser, K. L., and Hufnagel, H. V.: Resolution of experimental fat embolism. Arch. Path. 90: 230, 1970.

3. Houston, S., Ousterhout, D. K., Sleeman, H. K., and Leonard, F.: The effects of n-butyl-2-cyanoacrylate on liver function. J. Biomed. Mater. Res. 4: 25, 1970.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6527	71 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
70 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	61102A	3A061102B71R	01	092
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Clinical evaluation of responses of the body to combat injury (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	C In-House

17. CONTRACT/GRANT 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 71	6	170
FISCAL YEAR CURRENT 72	5	140

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME: Fleming, MAJ W. H.
NAME: Bowen, MAJ J. C. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Aeromedical Evacuation; (U) Hypoxemia; (U) Mechanical Ventilators; (U) 2,3 DPG; (U) Compliance; (U) Diuretics; (U) Liver

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 and quantitate the physiologic and metabolic derangements following combat injury and resuscitation.

24 (U) Studies in Vietnam, Japan and at WRGH have been or are being conducted on acutely wounded soldiers including resuscitation, surgery, postoperative management and the effects of aeromedical evacuation.

25 (U) 70 07 - 71 06 In-flight studies demonstrated that cabin altitude is the most critical factor in preventing excess morbidity and mortality during air evacuation; patients are already maximally breathing and can respond to the decreased ambient oxygen principally by marked increases in cardiac output in order to maintain adequate tissue oxygenation. A comparison of pressure-cycled versus volume-cycled mechanical respirators clearly demonstrated the superiority of the latter in maintaining oxygenation of severely wounded combat casualties. The influence of 2,3 DPG on the oxygen-hemoglobin dissociation curve has been evaluated in massively transfused combat casualties. The use of strong diuretics in the treatment of early wet lung syndrome has been strikingly demonstrated. A dynamic compliance apparatus has been devised which is a simple but highly useful non-invasive guide to mechanical ventilator management. Management of 81 consecutive liver injuries has been analyzed. Inapparent hypoxemia has been shown to occur in infectious hepatitis patients, both on the ground and at altitude. The complications of long-term postoperative ventilatory support have been analyzed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

568

*Available to contractors upon originator's approval

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01 Surgery

Work Unit 092, Clinical evaluation of responses of the body to combat injury

Investigators.
Principal: COL Robert T. Cutting, MC
Associate: MAJ Phillip M. Levin, MC; MAJ Robert D. Croom, III, MC; CPT Neven A. Popovic, VC

1. Patency of Vein Grafts in the Venous System

a. Statement of the problem: Extremities have been lost as a result of venous hypertension despite adequate arterial repair indicating the need for a more aggressive approach to vein repair following trauma. Yet thrombosis is a nearly inevitable event following venous reconstruction.

b. Background: Ligation remains the usual procedure following trauma to the major venous supply of an extremity. Numerous techniques have been tried to improve patency of venous repairs but none have proved uniformly successful. The use of a distal arteriovenous shunt has improved the patency of venous autografts in dogs.

c. Approach to the problem: Clinical follow-up of the patients in the Vietnam Vascular Registry was made with special attention to those patients having sustained venous injury alone or combined venous and arterial injuries in the extremities.

d. Results and Conclusions: Extremities have been lost due to venous insufficiency despite adequate arterial repairs. The majority of lateral venous repairs remain patent. Autogenous vein grafts in the venous system were prone to thrombosis but provided time for collateral development and later underwent recanalization. Venous repairs can be performed with few complications; the potential benefits, particularly in the lower extremities, outweigh the risks.

2. The Role of Arteriovenous Shunts in Venous Reconstruction

a. Statement of the problem: Under combat conditions extremities have been lost as a result of venous hypertension despite adequate arterial repair indicating a need to improve venous drainage in the traumatized extremity if more limbs are to be saved.

b. Background: Numerous techniques have been tried to maintain patency of venous repairs but none of them have proved uniformly successful.

c. Approach to the problem: By constructing a distal arteriovenous fistula the patency of autogenous venous grafts can be improved. This hypothesis was tested in 20 dogs.

d. Results: The use of a distal arteriovenous shunt improved the patency of venous autografts as compared to controls. However, the control grafts showed a surprisingly high patency rate of 90% six weeks following operation. Because only 25% of the control grafts were patent 72 hours postoperatively, the six week figure indicates the high degree to which venous grafts undergo recanalization.

e. Conclusion and Recommendations: The distal AV shunt increases the patency of venous autografts. Venous autografts can frequently undergo recanalization. The advantage of the distal shunt appears to be in its ability to maintain patency of the venous graft during the early postoperative period.

3. Peripheral Venous Reconstruction Using Autogenous Connective Tissue Grafts Grown in Response to Implanted Silastic Rods

a. Statement of the problem: It is often difficult to find suitable veins which can be grafted, especially in patients with multiple extremity injuries.

b. Background: The importance of repairing acute venous injuries in the lower extremities is stressed. Early patency has been enhanced with the use of temporary distal AV fistula. The availability of suitable autogenous veins for repair of both acute and chronic venous injuries is limited. A possible source of suitable prosthetic is a connective tissue tube which forms secondary to implantation of silastic tubing.

c. Approach to the problem: Autogenous connective tissue tunnels grown in response to implanted silastic rods are being evaluated as replacement material for secondary peripheral venous reconstruction both with and without a distal AV fistula.

d. Results: Study is underway.

4. The Effects of Acepromazine on pH, Oxygen and Carbon Dioxide in Arterial Blood of Dogs

a. Statement of the problem: Research using dogs requires varying degrees of pharmacological control of CNS responses. Acepromazine (10-(3-Dimethylamino) propyl) phenothiazine-2-yl-methyl ketone) maleate is a neuroleptic agent used for tranquilization of dogs and cats and as a

pre-anesthetic medication.

b. Background: Acepromazine has been used in connection with various projects at the Walter Reed Army Institute of Research without knowledge of its effects on the respiratory and circulatory systems nor on biochemistry.

c. Approach to the problem: Measurements of pH, PaO_2 and $PaCO_2$ were selected as the most critical parameters of physiologic function following the administration of CNS depressants. In addition, heart rate, respiratory rate, minute ventilation and rectal temperature were obtained.

Under local anesthesia polyethylene catheters were placed in the femoral artery and vein. Arterial blood samples were collected before the drug administration and periodically after the drug injection (intramuscularly at 0.5 pound of body weight).

d. Results: There were no changes in pH, $PaCO_2$, PaO_2 and oxyhemoglobin saturation and minute ventilation. However, significant decreases were observed in the respiratory rate, temperature, heart rate, arterial pressure, and central venous pressure. These are consistent with other phenothiazine derivatives.

e. Conclusions: Acepromazine is a satisfactory pre-anesthetic agent in medica' studies involving the dog.

f. Recommendation: Further studies are not required.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01 Surgery

Work Unit 092, Clinical evaluation of responses of the body to combat injury

Literature Cited.

References:

1. Moseley, R.V., and Doty, D.B.: Hypoxemia during the first twelve hours after battle injury. Surgery 67(5): 765, May 1970.

2. Phillips, S.J. and Butner, A.N.: Percutaneous transvenous cardiac pacing initiated at bedside. Results in 40 cases. J. Thor. Cardiovasc. Surg. 59: 855, June 1970.

Publications:

1. McNamara, J.J., Molot, M.D., Stremple, J.F.: Screen filtration pressure in combat casualties. Ann. Surg. 172(3): 334, Sep 1970.

2. Gielchinsky, I. and McNamara, J.J.: Cardiac wounds at a military evacuation hospital in Vietnam. J. Thor. Cardiovasc. Surg. 60(4): 603, Oct. 1970.

3. McNamara, J.J., Messersmith, J.K., Dunn, R.A., Molot, M.D., and Stremple, J.F.: Thoracic injuries in combat casualties in Vietnam. Ann. Thor. Surg. 10(5): 389, Nov. 1970.

4. McNamara, J.J., and Stremple, J.F.: Major complications following combat injury. The contribution of surgical research in delineating the problems and improving therapy. Med. Ann. DC, 39(7): 349, Jul. 1970.

5. Fleming, W.H., Aaby, G.V., and Randolph, J.B.: A comparative study of arterio-arterial and intra-aortic balloon counterpulsation in the therapy of cardiogenic shock. J. Thorac. Cardiovasc. Surg. 60(6): 818, Dec. 1970.

6. Berman, I.R., Scheetz, W.L., Jenkins, E.B. and Hufnagel, H.V.: Transthoracic electrical impedance as a guide to intravascular overload. Arch. Surg. 102: 61, Jan. 1971.

7. Levin, P.M., Rich, N.M., and Hutton, J.E.: Collateral circulation in arterial injuries. Arch. Surg. 102: 392, Apr 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 08 6461	71 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
70 07 01	D. Change	U	U	NA	NA	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71R	01	093
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code) (U) Response of Cells Derived from Subjects Injured by Physical Trauma, Radiation or Infectious Agent Exposure (09).

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	Cont	DA	In-House

17. CONTRACT/GRANT
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	PREV STUDY 71	5	125
	CURRENT 72	5	125

19a. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]
ASSOCIATE INVESTIGATORS
NAME: Johnson, LTC. M.C.
NAME: Miller, MAJ. J. DA

PII Redacted

21. GENERAL USE
Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Wound healing; (U) Cytogenetics; (U) Radiobiology (U) Cellular hypersensitivity; (U) Host resistance; (U) Inflammatory response;

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

23. (U) To determine the reaction of cultured white blood cells (lymphocytes) as it pertains to antigenic, infectious, radiobiological, drug, biochemical and physical injuries encountered in military operations.

24. (U) Cytogenetic, histological, radiotracer, and serologic methods are used to examine proliferating lymphocytes cultured in the presence of well-characterized stimuli. This lymphocytic response, in a system where non-experimental variables are well-controlled, is used to test hypotheses concerning the nature and therapeutic modification of whole body responses.

25. (U) 70 07 - 71 06 A specific gamma globulin was found in the plasma of kidney transplanted dogs which inhibited the transformation of lymphocytes from the kidney donor for several months after transplantation. This is the first conclusive demonstration that an animal strongly stimulated to produce a cellular immune reaction produces (in the circumstances of this experiment) an antibody that interferes with a response which directly affects recovery from many infections. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 70 - 30 June 71.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01 Surgery

Work Unit 093, Response of cells derived from subjects injured by physical trauma, radiation, or infectious agent exposure.

Investigators.
Principal: COL M. C. Johnson, MC
Associate: LTC J. Miller, MC; M. M. Hill; M. H. Davis

Problem

To better understand the role of cellular interaction and immune responses with specific emphasis on radiation injury and histocompatibility studies.

Background

This department has since its inception been concerned with investigation of cellular phenomenon. While initially the study was restricted to radiation effects, more recently and following an international trend the emphasis has been shifted to problems of transplantation and wound healing.

Approach

The greatest emphasis has been placed on the study of cells in vitro systems using techniques of tissue culture, cellular immunology and radiobiology.

Results

The final data from the study of the effect of combined antibiotic therapy and bone marrow transplantation on wound healing in rats exposed to neutrons has been evaluated. The results were presented in preliminary form by M. M. (McLaughlin) Hill in July 1970 at the Fourth International Congress of Radiation Research, Evian, France. A manuscript entitled, "Combined Bone Marrow and Antibiotic Therapy in Rats Wounded After Exposure to Neutrons " has been prepared by M. C. Johnson, M. M. McLaughlin and M. H. Davis. It has been submitted for publication in Transplantation and is presently in the hands of the reviewers. As a result of the information gained from this investigation and others,[1,2,3] it has been postulated that the biphasic changes seen in the wound healing patterns of rats exposed to neutrons are due primarily to radiation induced changes in vascular permeability. An attempt is underway to correlate post-irradiation changes in wound closure with changes in vascular permeability. Radioiodinated serum albumin (RISA) is being used to detect changes both at the wound site and in whole body excretion. Preliminary data, obtained from animals

wounded on different post-irradiation days, indicates that both the amount of radioactivity which 'leaks' into the wound bed as well as the amount excreted by the animal varies with the time interval between radiation and wounding.

Immunogammaglobulin (IgG) has been isolated from the serum of patients by DEAE Sephadex ion exchange chromatography. The various fractions of the eluate were monitored by UV absorption at 280 mu and appropriate portions were lyophilized before storage at -70C. The concentrated material has subsequently been used in mixed lymphocyte cultures for cytotoxicity studies. This method of gel filtration has also been used to isolate the fraction containing late acting thyroid stimulating hormone (LATS) from the sera of patients whose thyroid status is being evaluated in the Nuclear Medicine Clinic, WRGH. In conjunction with this in vitro method a bioassay for thyroid stimulating hormone (TSH) has also been undertaken. Weanling mice maintained of a carefully formulated, iodine deficient diet are used in the assay of TSH levels in patient sera.

The studies concerned with the relationship between peroxidation and cation loss in liposomes, which have been carried out during the past two years, have been completed. They are described in a manuscript by M. Leibowitz and M. C. Johnson, entitled, "Relation of Increased Peroxidation to Cation Loss in Liposomes, which has been accepted for publication in the November 1971 issue of the Journal of Lipid Chemistry.

In the field of cellular immunology, the research can be divided into two broad categories:

1. The study of enhancement, i.e., the specific suppression of immunity by humoral antibody.

2. The study of the cellular mechanisms of immunity in vitro and in vivo, i. e., the central segment of the immune arc and its cellular makeup.

Progress is being made in each of these fields:

1. The mixed lymphocyte culture has been and is being studied in detail. This is considered to be an in vitro model of cellular immunity (transplantation as well as bacterial and viral).

Lymphocytes of the dog, human and inbred mouse are being studied.

A humoral factor isolated by column chromatography from dog serum and found to be Immunoglobulin G or 7S antibody, was discovered to occur in the serum of recipients during the course of unmodified allograft rejection. This factor inhibited mixed lymphocyte culture reactions by binding to the stimulated population of lymphocytes. Although it is not completely clear there is evidence that not only cytotoxic, but also enhancing mechanisms are involved. The factor arises as early as five

days after renal transplantation in the dog and can be eluted from the rejecting organs. These studies are being extended to humans after clinical renal transplantation.

2. Several studies have been performed using specific anti-lymphocyte sera in mice. Sera have been made to strain specific (H-2) antigens as well as lymphocyte cell specific antigens such as the thymic derived "T" as opposed to the bone marrow derived "B" type lymphocyte.

3. The role of the granulocyte in transplantation immunity is being investigated with the use of pure granulocyte preparations using an *in vitro* mixed lymphocyte culture system. A antigranulocyte serum has been prepared in rabbits and its role in this model is being studied.

4. Also in progress are studies on factors affecting tumor growth in the mouse. More specifically the role of enhancing antibody is being investigated.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01 Surgery

Work Unit 093, Response of cells derived from subjects injured by physical trauma, radiation, or infectious agent exposure.

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5. Spees, E. K., Miller, J., and Wistar, R.: Specificity of non-thymic antilymphocyte sera. National Meeting of the Federation of American Societies for Experimental Biology. Chicago, Illinois, May 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6402	71 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 CONTRA FOR 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	61102A	3A061102B71E	01	094
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Healing and Repair of Combat Inflicted Injury. (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

017100 Weapons Effects; 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 01	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 71	4	125
FISCAL YEAR CURRENT 72	4	125

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21. GENERAL USE

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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Weapons Energy; (U) Combat Injury; (U) Wound Healing; (U) Fibroblast Suspension Culture; (U) Growth Regulation; (U) Cell Division; (U) Collagen Synthesis

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

23.(U) Combat inflicted injury is due to the penetration of the energy released by various types of weapons into the body of the soldier. While the type of energy involved may vary, the result is invariably damage and loss of living tissue, which when compatible with life, is followed by tissue regeneration, healing and repair. As the restoration of the soldier's health and combat capability depends on these repair processes, this study aims to uncover the underlying mechanisms and to increase their effectiveness.

24.(U) Since living tissue is built from cells and these in turn from molecules, the attainment of this objective requires the analysis and mapping out of the sequence of molecular and cellular events which lead from the early development of tissue injury to its subsequent repair and healing. Model systems involving tissue cells under rigorously controlled conditions, are being developed and used for this analysis.

25.(U) 70 07 - 71 06 The essential cellular sequence of wound healing, i.e. early replication of DNA, active synthesis of cell protein, and mitosis, followed later by the establishment of a regulated steady state population of fibroblasts synthesizing collagen, has been successfully reproduced in the model system developed: a culture of fibroblasts kept in suspension. By minimizing interference by physical factors such as cell to cell contact, the system offers unique opportunities for investigating humoral regulation with all the potential that this entails for the eventual clinical control of wound healing through pharmacological means. Accordingly, current work aims at: a) identification of the active components of the humoral environment of the cells; b) definition of the role of the cell surface in the interaction between cells and environment; and c) analysis of the ensuing modification of intracellular synthesis and metabolism responsible for the wound healing sequence as reproduced in the model. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available in limited supply pending approval

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PII Redacted

3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 094, Healing and repair of combat inflicted injury

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: B. Taylor, Cpt. CmlC; E.M. Bartos, Ph.D.; J.M. Vail, Ph.D.; R.J. Werrlein, M.S.

Problem and Background

Combat inflicted injury is due to the penetration of the energy released by various types of weapons into the body of the soldier. While the type of energy involved may vary, the result is invariably damage and loss of living tissue which, when compatible with life, is followed by tissue regeneration, healing and repair. As the restoration of the soldier's health and combat capability depends on these repair processes, it is the objective of this study to uncover the underlying mechanisms and to develop means for increasing their effectiveness. Since living tissue is built from cells and these in turn from molecules, the attainment of this objective requires the analysis and mapping out of the sequence of cellular and molecular events which follow tissue injury and lead to its eventual repair and healing. It has been decided to proceed with this analysis by considering one of the most essential sequences following injury: the proliferation of fibroblasts in the early phase of the wound followed later by arrest of cell growth and elaboration of collagen. The significance of this sequence for the clinical course of wound healing in the injured soldier becomes readily apparent when it is considered that the first process, fibroblast proliferation, is an important determinant of the speed of healing, while the second, elaboration of collagen, is responsible for the development of the tensile strength of the wound. From this point of view the problem of wound healing can then be restated in the form of the following two questions: 1) What is the nature of the changes in the cellular environment which, following injury, first induce fibroblasts to proliferate and later limit cell division to the maintenance of a collagen producing steady state cell population? 2) What is the nature of intracellular molecular interactions which occur in response to these extracellular changes and result in the early phase of DNA replication and general protein synthesis followed later by a marked reduction of these activities while the synthesis of collagen continues at a relatively high rate?

Approach

The reason that a great number of clinical and experimental studies have

failed to provide answers to the two questions defined above, lies in the great complexities of the clinical situation in man and the experimental conditions in the whole animal. Even the discovery that cultures of fibroblasts attached on glass or plastic surfaces reproduce the wound healing sequence (i.e. pronounced decline of DNA replication, general protein synthesis and cell division while collagen synthesis is maintained at relatively high levels) as they proceed from low to high cell densities[1], has failed to provide the desired answers. This is due to the fact that in attached cultures, it is impossible to distinguish between possible physical growth regulatory mechanisms necessitating cell to cell contact and humoral regulation operating through decreased uptake of substances essential for growth by dense cultures.

We decided to approach this problem through the use of suspension cultures where cell to cell contact is minimized: reduction of DNA synthesis, of general protein synthesis and of mitosis with maintenance of collagen synthesis in such cultures would unequivocally indicate humoral regulation. An opening wedge would thus be provided for identifying the extracellular environmental changes and intracellular metabolic interactions responsible for the wound healing sequence.

The origin and quantitative karyotype of the L-929 cell subline and the culture methods used to obtain viable and structually intact high density populations have been described previously[2,3,4]. During the period covered by the report, DNA synthesis, mitosis, synthesis of cellular proteins and of collagenous and non-collagenous proteins released in the media of these high density cultures were investigated and compared with low density cultures as follows:

I. To determine the fraction of cells synthesizing DNA, 9 ml samples were removed from the experimental cultures at desired time intervals and pulse labeled for 10 minutes with ^{3}H thymidine added to the samples at a final concentration of 0.25 μCi/ml. The cells were then centrifuged, washed with cold Earle's balanced saline, fixed in a 1:3 cold mixture of glacial acetic acid-ethanol and streaked on slides. After air-drying, the slides were stained with Feulgen, coated with Kodak Nuclear Track Emulsion NT B2, exposed for 3-6 days and developed. Total and labeled cells were determined in the final autoradiographs by counting a least 2000 cells.

II. To determine the fraction of the cell population in mitosis at desired time intervals following medium renewal, appropriate samples of the cell suspension were added to equal volumes of a 1:2:3 mixture of 0.1 percent crystal violet in 0.1 M citric acid - 0.1 M citric acid - 0.25 percent methylcellulose in 0.1 M citric acid and allowed to stand for 30 minutes. Wet mounts were then prepared, and total and mitotic cells determined with the aid of an ocular grid. At least 2000

cells were counted for each determination. To determine the fraction of the cells entering mitosis over an extended period of time, colchicine at a final concentration of 6 µg/ml was added to cultures 2 hours after renewal of the medium when dispersal of the cells was almost complete. Eighteen hours later, i.e., 20 hours following medium renewal, wet mounts were prepared, and total and mitotic cells determined as just described.

III. To determine rates of synthesis of cellular proteins and of collagenous proteins released into the medium, L-proline 3,4-^{3}H with a specific activity of 5.86 Ci/mM was added to low and high density cultures at a final concentration of 2 µCi/ml. The cultures were allowed to incubate at 35° for desired periods of time. At the end of these times 5 ml samples of the suspensions were removed and the cells separated from the medium through centrifugation at 500g for 10 minutes. The cell pellets were washed twice with 5 ml freshly made medium and the washings added to the original medium bringing the total to 15 ml. The cell pellets were then treated with 5 ml 0.5N PCA in an ice bath for 20 minutes. The resulting precipitate, was removed by centrifugation, washed with cold PCA containing 1 mg/ml of cold proline and hydrolysed overnight in 6N HCl at 115°. The 15 ml samples of media and cell washings were placed back in the incubator and stirred for 15 minutes after addition of cold proline sufficient to yield a concentration ratio to the isotope of 10^{5}:1. The medium then was treated in the same way as the cell pellet except that an equal volume of 1N PCA was used to precipitate the high molecular weight components. Cell and medium hydrolysates were subsequently evaporated to dryness and dissolved in 0.2 M sodium citrate buffer at ph 3.42. Radioactive proline and hydroxyproline in the hydrolysates were then separated through cation exchange column chromatography and quantitated by means of liquid scintillation counting.

Results and Discussion

I. The fraction of cells synthesizing DNA at various times after medium renewal in cultures of varying densities is shown in Fig. 1. In the case of the low density exponentially growing cultures, this fraction increased from an initial mean value of 0.41 at 2 hours to 0.53 at 14 hours and then declined again to a value of 0.36 at 26 hours. This slight degree of synchrony is most probably due to interference of the mechanical manipulations and temperature changes associated with medium renewal, with the normal progression of the cells through the cycle. If, in order to facilitate comparisons, this slight degree of synchrony is ignored and the fraction of cells synthesizing DNA is averaged over the entire 26-hour observation period, a value of 0.45 is obtained.

Renewal of the medium had a strikingly different effect on high density cultures as shown by the immediate rise of the fraction of ^{3}H thymidine

labeled cells to a maximum value of 0.06 where it remained until 8 hours. After this time, the fraction of labeled cells declined progressively to a minimum value of 0.006 where it remained until the medium was again renewed (Fig. 1-II). Mechanical manipulations and cooling during media renewal obviously cannot account for the sudden rise of the fraction of labelled cells. Accordingly, synchrony in this case cannot be ignored and the values of the fraction of cells synthesizing DNA must be compared individually to the average rate characteristic of low density cultures. This comparison shows a reduction, ranging from 87 percent for the maximum rate between 2 and 8 hours, to 97 per cent for the minimum rate between 20 and 26 hours.

Following dilution of high density populations to the low levels characteristic of exponentially growing cultures (Fig. 1-III), the number of DNA synthesizing cells showed an increase which, minimal at 8 hours, reached a progressively higher rate resulting in a value of 0.60 at 20 hours with a subsequent decline to 0.40 at 26 hours.

II. The kinetics of the mitotic activity in cultures of varying cell densities as a function of time after medium renewal (Fig. 2) were found to parallel closely the kinetics of DNA synthesis just described. Thus, in the low density exponentially growing cultures, the curve of the fraction of cells in mitosis (Fig. 2-I), while qualitatively similar to the curve of the ^{3}H thymidine labeled cells (Fig. 1-I), appears to have been displaced to the right. Increments and decrements of mitotic activity followed regularly the corresponding changes in DNA synthesis confirming the slightly synchronizing effect of the manipulations involved in media renewal.

Although this synchronization was present in all populations examined, the ranges plotted around the means indicate that the fraction of cells undergoing mitosis at any given time fluctuated widely among the populations examined, with the greatest differences found at 14 and 20 hours when mitotic activity was highest. These differences were very nearly eliminated when the number of metaphases obtained by treating the cultures with colchicine for 18 hours was considered (Fig. 2-I, bar graph). This indicates that the main cause for the variation noted in these experiments was that, while the total number of cells entering mitosis over an extended period of time was nearly identical in all cultures, the fraction of cells undergoing mitosis at any given point in time varied considerably in different cultures. In this respect, cell populations growing in culture are no different from tissue cells growing *in vivo*, as is readily shown by a comparison of the ranges around the means of the time curves of Figs. 1 and 2 with the variation seen in similarly obtained time curves of DNA synthesis and mitosis in the regenerating rat liver[5].

It follows, that in order to obtain a reliable estimate of the true mitotic rate of such populations, the length of the exposure to colchicine should be such as to minimize fluctuations of the mitotic activity with time in individual cultures without allowing a significant number of cells to leave the metaphase block. The 18-hour exposure used in this work was chosen because preliminary determination of the kinetics of colchicine arrested metaphases showed that the number of cells leaving the block at 18 hours was negligible. while the choice of an earlier time interval would reintroduce the type of variation seen in the mitotic index curve.

The bar graph of Fig. 2-I indicates that at the end of an 18-hour exposure to colchicine, beginning 2 hours after renewal of the medium of low density exponentially growing cultures, the fraction of cells which accumulated at metaphase was 0.60, or, a mean rate of flow of cells into mitosis of 0.033 cells per hour. The average mitotic index over the same period of time, calculated as in the case of the ^{3}H thymidine labeled cells by ignoring the slight synchrony due to medium renewal manipulations, was 0.02. Standard calculations based on these data indicate that the mean mitotic duration of low density exponentially growing populations was 0.59 hours.

As with low density cultures, increments and decrements of the mitotic index in high density populations followed earlier changes in the fraction of ^{3}H thymidine labeled cells. A maximum value of 0.005 was reached at 14 hours and a minimum of 0.003 at 2 and at 26 hours (Fig. 2-II). This is a reduction of 75 and 85 percent, respectively, compared to the average mitotic index of the low density populations. On the other hand, the fraction of cells arrested in metaphase was 0.049, corresponding to a mitotic flow of 0.003 cells per hour, or, a reduction of over 90 percent, in comparison to low density populations. The discrepancy of the reduction between mitotic index and mitotic flow indicates that the duration of mitosis is prolonged in high density cultures. Another indication of this prolongation is provided by the greatly reduced amplitude of the fluctuations of the mitotic index of the high density cultures when compared with the earlier fluctuations of the fraction of DNA synthesizing cells (Fig. 1-II). The resulting relative constancy of the mitotic index made possible the calculation of an average value for the interval 2 to 20 hours, which in turn was used to obtain a first approximation of the duration of mitosis. The value thus obtained is 1.4 hours.

Following dilution of high density populations (Fig. 2-III), there was a slow gradual increase in the average mitotic index which, however, remained within the range characteristic for high density cultures until 20 hours. After this time, the increase of the mitotic index reached a very high rate paralleling a similar increase of the fraction of DNA synthesizing cells (Fig. 1-III), but with a considerable delay. This

delay was also reflected in the fraction of colchicine arrested metaphases which at 20 hours had risen bearly above the level characteristic for high density populations (Fig. 2-II and III, bar graphs). Consequently, it became necessary to extend the observations on the post-dilution flow of cells into mitosis beyond 20 hours. The mitotic degeneration known to occur in cultures continuously exposed to colchicine was no obstacle because it was found that with prolonged exposure to colchicine L cells pass through the easily identifiable micronuclear and ameboid-nuclear stages first described in the regenerating liver of the rat. By counting cells in these stages, in addition to metaphases, it has been possible to extend the observations to 32 hours as shown in Fig. 3. The completeness of mitotic accumulation between 20 and 32 hours is indicated by the near linearity of the curve. Qualitatively and in regard to timing, the curve is identical with the mitotic index curve (Fig. 2-III) and confirms the fact that the entry of cells into mitosis following dilution of a high density culture follows the kinetics of DNA synthesis.

The curve consists of three distinct linear or near linear segments rendering possible the calculation of three mitotic flow rates following dilution of a high density culture. These rates expressed as the fraction of cells entering mitosis per hour, are: for the first 8 hours following medium renewal, 0.001; for the interval 8 - 20 hours, 0.006; and for the interval 20 - 32 hours, 0.043. Thus, for the first 8 hours following dilution, the rate of mitotic flow remained within the limits characteristic for high density populations and showed only a slight rise from 8 to 20 hours; after this time it exhibited a dramatic 14-fold increase exceeding by approximately 30 percent the mitotic flow rate of low density exponentially growing cultures.

In Figs. 1-II and 2-II, it can be seen that at the end of the 26-hour period following renewal of the medium, the population was virtually depleted of cells in the S period and the mitotic index was low. Medium renewal caused an immediate rise of the fraction of cells in S to a maximum of 0.06 followed at 14 hours by a rise of the mitotic index. While the prolongation of the duration of mitosis previously discussed, makes a direct quantitative comparison of mitotic and labeling indices uncertain, it can be calculated that with a mitotic flow of 0.003 cells per hour and a mitotic duration of 1.4 hours the maximum fraction of the cells expected to complete mitosis per day is 0.048. There is near identity of the values for the fractions of DNA synthesizing cells and of those completing mitosis, with a value of approximately 0.05 for the fraction of nonviable cells which were found to occur in high as well as low density exponentially growing cultures, most probably because of the manipulations associated with medium renewal.

The abruptness of the rise of the number of DNA synthesizing cells to a maximum at 2 hours (Fig. 1-II) indicates that beside prolongation of

mitosis there is also delay of a small fraction of cells in the G_1 period. Medium renewal triggers the entry of these cells into the S period with differences of the duration of this period among different cells accounting for the gradualness of their depletion between 8 and 20 hours after medium renewal.

The same response from the G_1 delayed fraction is obtained at 2 hours after dilution of a high density population (Fig. 1-III). Beginning at 8 hours after dilution, however, there is a further marked increase of the number of DNA synthesizing cells, the cumulative kinetics of which suggest that, besides the G_1 delayed cells a much larger fraction of the population is triggered into the cell cycle from a G_0, or, early G_1 phase. This increase of DNA synthesizing cells is followed 12 hours later by an increase of mitoses. both, mitotic index (Fig. 2-III) and mitotic flow (Fig. 3) curves are of the same cumulative type, further supporting the concept that a large fraction of the cell population in high density cultures is in a G_0 or early G_1 state.

These findings represent the first demonstration that suspension cultures are capable of the type of steady state regulation of growth exhibited by attached cultures and tissue cells in the body: the cultures of L cells described, remained viable for prolonged periods, the large majority of their cells were arrested in a G_0 or early G_1 phase resuming growth readily upon dilution, and small daily losses due to culture manipulation were compenstaed by DNA synthesis and division limited to a correspondingly small number of cells. The significance of physical factors such as cell to cell contact is by necessity minimal in cell suspensions. Accordingly, the system offers unique opportunities for the study of humoral regulation in a sequence *in vitro*, closely resembling the orderly succession of the different phases of wound healing *in vivo*.

III. The kinetics of protein synthesis in low and high density suspension cultures are shown in Table 1. The longest labelling time used in these first experiments is 6 hours, corresponding to 7 hours following renewal of the medium. Despite this relatively short observation period, it is apparent that in high density cultures intracellular protein synthesis, indicated by the ^{3}H-proline counts in cells, is reduced by 73-79 percent, while the synthesis and release of collagen, indicated by the media ^{3}H-hydroxyproline counts, is reduced by only 33-40 percent. Thus, suspension cultures are shown to exhibit regulatory features of general protein and collagen synthesis characteristic of attached cultures[1] and of healing wounds. Since the reduction of collagen synthesis per cell is limited to a maximum of 40 percent, while the cell population of high density cultures is increased approximately 10 times, net collagen synthesis is increased over five-fold. It is probable that a similar mechanism accounts for the apparent increase of collagen elaboration in the late wound.

In addition to collagen, L cells were found to release other proteins, indicated by the media ^{3}H-proline counts. This is in good agreement with the recently reported release of non-collagen proteins by chicken fibroblasts[5]. The nature and function of these proteins is at present obscure. The table, however, shows that in contrast to the synthesis of intracellular protein and of collagen which is reduced by a constant percent in the high density cultures, these proteins, expressed by the media proline counts, show a reduction which becomes progressively greater with time. Properly exploited this finding might provide us with some interesting clues regarding their role.

Finally, the data indicate that the reduction of intracellular collagen, indicated by the ^{3}H-hydroxyproline counts in cells, is approximately midway between the reduction of cellular protein and of released collagen and, like them, remains invariant throughout the 6-hour observation period. Intracellular collagen levels represent a steady state between synthesis and release of this macromolecule and changes of the rates of these two processes cannot be expected to be the same. It is not, therefore, surprising that the reduction of intracellular and extracellular hydroxyproline counts in high density cultures is not identical. This interpretation is further supported by the finding that while in both low and high density cultures intracellular hydroxyproline increases during the 2- to 4-hour interval and decreases slightly thereafter, extracellular hydroxyproline continues to increase, albeit at a lesser rate, up to the end of the observation period.

Conclusions and Recommendations

The essential cellular sequence of wound healing: early replication of DNA, synthesis of cell protein and mitosis followed by the establishment of a steady state population of fibroblasts synthesizing collagen, has been reproduced for the first time in a model suspension culture system. By minimizing interference by physical factors, such as cell to cell contact, the system offers unique opportunities for investigating humoral regulation. This type of regulation has important potentialities for the eventual control of the clinical course of wound healing in the injured soldier through chemical means. Accordingly, it is highly recommended that these investigations be vigorously pursued along three main lines: a) identification of the components of the cellular microenvironment responsible for the type of regulation described in this report; b) exploration of the role of the cell surface in the interaction between cells and environmental factors; and c) analysis of the ensuing modification of intracellular syntheses and metabolism responsible for the orderly succession and completion of the different phases of the healing process.

LEGENDS OF FIGURES AND TABLES

FIG. 1 Effects of culture density on DNA synthesis.. At the indicated times, cell samples from (I) low density (4 - 8 X 10^5 cells/ml), (II) high density (6 - 10 X 10^6 cells/ml), and (III) high density cultures following dilution to the density level of (I) at 0 time, were pulse labeled with tritiated thymidine as described. Nuclei with more than 5 grains were considered labeled. Each point represents the mean of at least three cultures, and vertical lines indicate the range around each mean.

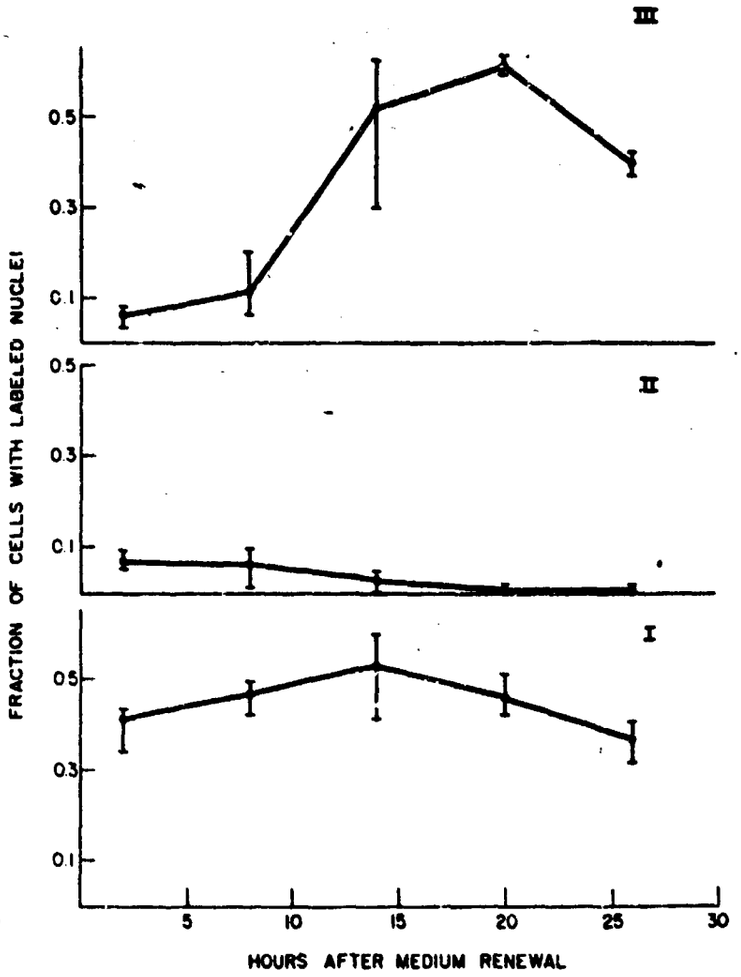
FIG. 2 Effects of culture density on mitosis. At the indicated times, cell samples from (I) low density (4 - 8 X 10^5 cells/ml), (II) high density (6 - 10 X 10^6 cells/ml), and (III) high density cultures following dilution to the density level of (I) at 0 time, were taken and the fraction of cells in mitosis determined as described. Each point represents the mean of at least four cultures and vertical lines indicate the range around each mean. The bar graphs on the right indicate the fraction of cells in metaphase, in samples obtained at 20 hours from a group of cultures which had received 6 µg/ml of colchicine 2 hours after medium renewal. Each bar graph represents the mean of at least 3 cultures and vertical lines indicate the range around each mean.

FIG. 3 Kinetics of the flow of cells into mitosis following dilution of high density population. Colchicine (6 µg/ml) was added to the culture two hours after its dilution from an initial concentration of 7.0 X 10^6 cells/ml to 4.3 X 10^5 cells/ml. In addition to metaphases and cells with scattered chromosomes, the fraction of cells in c-mitosis includes those with micronuclei and ameboid nuclei.

TABLE I. Kinetics of protein synthesis in cultures of varying cell densities.

In order to obtain proper dispersion of the cells and accurate cell counts, L proline-3,4-^{3}H was added to the cultures one hour after centrifugation and resuspension in new medium. Labeling times refer to the intervals between addition of the isotope and sampling. Accordingly, by adding one hour to the figures given in the table, the corresponding post medium renewal times are obtained. Low density refers to cultures with 4-8 X 10^{5} cells/ml; high density to those with 6-10 X 10^{6} cells/ml. All counts have been normalized in regard to cell number and are therefore expressed as disintegrations per minute of tritium (dpm-^{3}H) per million cells. Proline and hydroxyproline counts refer to chromatograms of hydrolysed, acid percipitable material obtained as described in the text; they are, therefore, expressions of general protein and of collagen synthesis, respectively. Distribution of the counts between cells and media expresses cellular retention and release, respectively, of these newly synthesized materials.

FIG 1


III
II
I
0.5
0.3
0.1
FRACTION OF CELLS WITH LABELED NUCLEI
5
10
15
20
25
30
HOURS AFTER MEDIUM RENEWAL

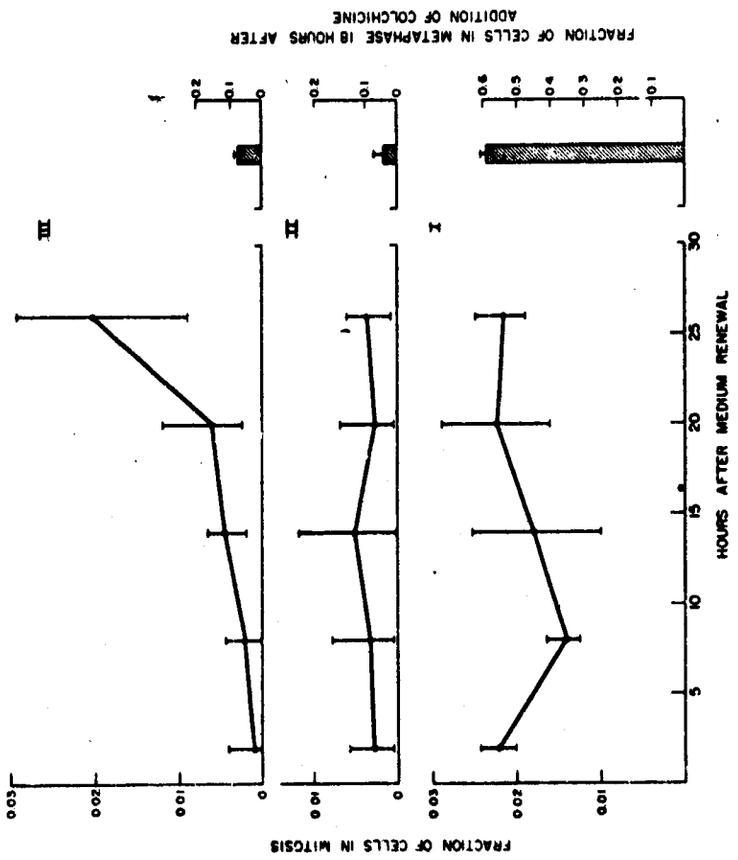

FRACTION OF CELLS IN METAPHASE 18 HOURS AFTER ADDITION OF COLCHICINE
FRACTION OF CELLS IN MITOSIS
HOURS AFTER MEDIUM RENEWAL
I
II
III

FIG 3

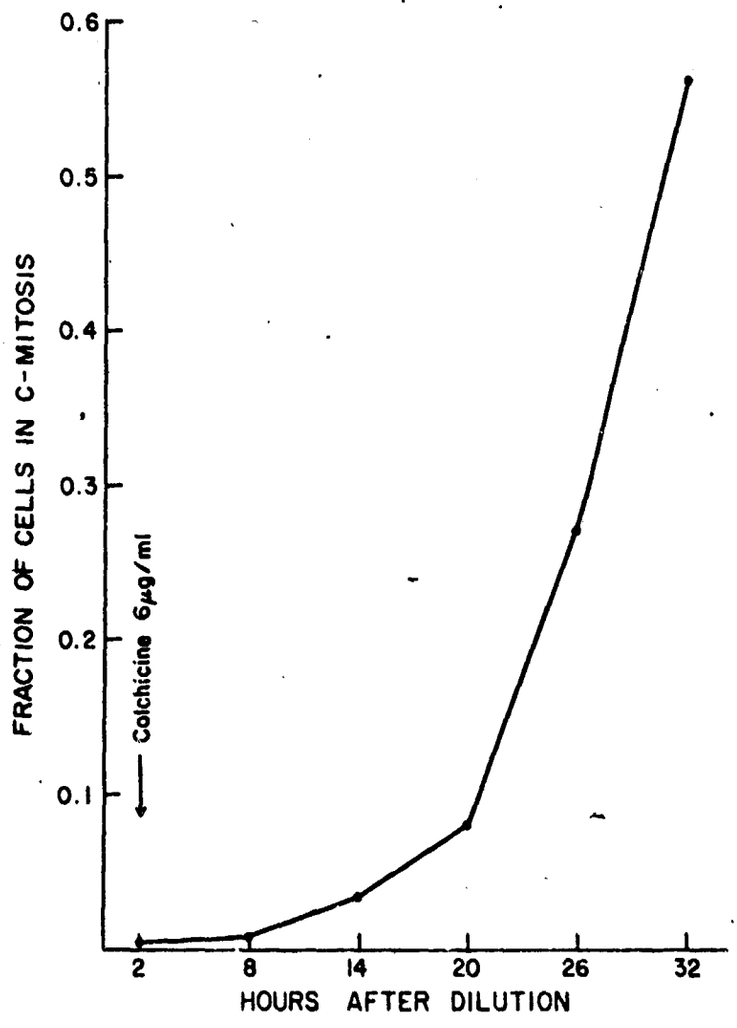

FRACTION OF CELLS IN C-MITOSIS
0.6
0.5
0.4
0.3
0.2
0.1
Colchicine 6μg/ml
2
8
14
20
26
32
HOURS AFTER DILUTION

TABLE I

LABELING TIME (Hours)	COMPARTMENT	PROLINE			HYDROXYPROLINE		
		LOW DENSITY CULTURES	HIGH DENSITY CULTURES		LOW DENSITY CULTURES	HIGH DENSITY CULTURES	
		dpm - ^3H / 10^6 cells	dpm - ^3H / 10^6 cells	% Reduction	dpm - ^3H / 10^6 cells	dpm - ^3H / 10^6 cells	% Reduction
2	CELLS	640,573	142,295	78	4,737	1,732	63
	MEDIA	23,905	11,978	50	1,078	573	47
4	CELLS	1,005,891	270,683	73	8,501	3,077	64
	MEDIA	59,155	21,194	64	3,346	2,228	33
6	CELLS	1,495,981	311,033	79	7,918	2,585	67
	MEDIA	107,966	26,028	76	6,310	3,793	40

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PROJECT 3A061102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 02
Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6451	71 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. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	61102A	3A061102B71R	02	085
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)
(U) The Heart Under Abnormal and Pathological Stresses (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
012900 Physiology 002400 Bioengineering 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 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 71	9	240
	CURRENT 72	11	275

19. RESPONSIBLE DOD ORGANIZATION
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NAME: Elliot, Dr. E. C. DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Cardiovascular system; (U) circulation; (U) heart; (U) blood; (U) coronary vessels; (U) myocardium; (U) oxygen.

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

23. (U) Research is devoted (1) to studies of the hemodynamic and biochemical controls of the normal heart and its coronary circulation under a variety of normal and abnormal stresses, and (2) to studies of the natural history of development of the coronary arterial collateral circulation in the presence of induced coronary insufficiency and of ways to improve such collateral compensation.

24. (U) The major research is based on two experimental models developed for long term study of the coronary normal and coronary collateral circulations in the conscious dog.

25. (U) 70 07 - 71 06. Adenosine uptake by the canine myocardium appears to occur by facilitated diffusion, and binding to the transport carrier is stereospecific. The effects of purine nucleosides on adenosine transport appear to be independent of their effect on coronary vascular tone. These results suggest that alterations in the properties of heart cell membranes may contribute to the cardiac deterioration seen in hemorrhagic and endotoxin shock. A semiconductor beta radiation detector was tested successfully in the dog, and a more sensitive detector is under development. In conscious postoperative dogs, excitement causes a plethora of coronary flow and a markedly elevated coronary sinus oxygen saturation. This response is not altered by total cardiac neural ablation. Detailed studies have conclusively established that a large collateral circulation develops very quickly following gradual or abrupt coronary occlusion, disappears rapidly upon relief of the occlusion, and is re-established within minutes if the vessel is reoccluded. Congenital obstruction of the outflow tract of either ventricle markedly restricts systolic coronary blood flow and imposes a serious impediment to myocardial oxygen supply during exercise, excitement and other stress states. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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 3A61102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, The heart under abnormal and pathological stresses

Investigators.

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Associates: COL Ray A. Olsson, MC; LTC George M. McGranahan, Jr., MC; MAJ Jerry A. Snow, MC; Eric C. Elliot, M.D., Ph.D.; Darrell W. Haas, Ph.D.; Edward M. Khouri; Howard S. Lowensohn; Charles E. Cain; R. Richard Gray, Mary K. Gentry; G. Peter Frick; Bertram Pitt, M.D.; Stanislaw Pasyk, M.D.; Colin M. Bloor, M.D.; Ellis L. Jones, M.D.; William B. Mitchell, R. Lee Pyle, Ph.D.; D. F. Patterson, M. D.

Description.

Development of standardized biological preparations for long term hemodynamic and biochemical studies of the controls of the circulation and of myocardial activity in the normal state and under the influence of abnormal and pathological stresses.

Progress and Results.

1. Development of Instruments and Methods for Cardiovascular Research.

The design and development of a lithium-drifted silicon semiconductor beta ray detector suitable for long-term studies of tissue clearance of diffusible indicators in conscious postoperative animals was successfully concluded. One such unit was implanted on the surface of a dog's heart and functioned satisfactorily for four weeks. Tissue reaction to the encapsulant was minimal. Because of the low efficiency of this type of detector, studies of avalanche diodes were begun. Preliminary results indicate that the latter is several times more efficient than the Li/Si type, and may be suitable for measurements of tissue clearance of Kr-85.

2. Metabolic Control of Left Coronary Blood Flow.

Adenosine kinase from dog heart has been separated from adenosine deaminase and its kinetic characteristics have been studied. This enzyme has a Km of 0.4 μM, and its activity in dog heart is on the order of 20-30 nmole/g LV·min. The enzyme is stimulated by Mg^{++} and although ATP is a substrate having optimal effects at 2 mM, it appears to be a competitive inhibitor at 10 mM. The low Km of this enzyme relative to that of adenosine deaminase affords a plausible

explanation for the observed predominance of phosphorylation over deamination of adenosine subsequent to its entry into the myocardial cell.

The estimates of the kinetic parameters of adenosine uptake by dog heart have been further refined, the Vmax being 4.9 ± 0.7 nmole/g LV·min and the Km being 12.0 ± 2.4 μM. Insulin appears to raise both Km and Vmax, as has been reported for arabinose and xylose transport. Several 6-substituted purine ribosides inhibit adenosine uptake, their relative effectiveness being: $-NH_2 > -NHCH_3 > -SCH_3 \approx S > O$. Adenosine uptake is also inhibited by a number of adenine furanosides whose effectiveness is in the order: 5'deoxyriboside > arabinoside > psicofuranoside; 2'deoxyadenosine was an inhibitor only at very high concentrations and adenosine xyloside was not inhibitory at all. These preliminary results suggest that the binding of adenosine to the myocardial membrane carrier which mediates its transport may depend upon the electron-donating capacity of the purine 6-substituent and on the orientation of the 2' and, to a great extent, the 3'hydroxyl of the ribosyl moiety.

The relative effectiveness of the 6-substituted purine ribosides as coronary vasodilators is in the same general order as their effectiveness as inhibitors of adenosine transport, except that neither inosine (=O) nor thioinosine (=S) is vasodilatory, and the effect of the methylthio analog is delayed in onset. Apparently, both the 2' and 3' hydroxyl groups oriented trans to the purine moiety is the conformation required for coronary vasodilatory activity. Only adenosine and its 5'deoxyriboside were strongly vasoactive. Psicofuramine, which has a $-CH_2OH$ group adjacent to the epimeric carbon (corresponding to the 2' carbon of the ribofuranose ring), is a relatively weak coronary vasodilator, and neither adenine arabinoside nor xyloside has any vasodilatory effect.

3. Myocardial Metabolism.

The studies on site-specific inhibitors of oxidative phosphorylation in heart sarcosomes have been almost completed and the work is being written for publication. The specificity of biguanides for the second phosphorylation site is dependent on the entire biguanide group being sterically unencumbered. The blocking out of one of the imine groups or the terminal amine groups will cause the resulting compound to act as a guanidine derivative and be specific for the first rather than the second phosphorylation site.

Work is now proceeding to study the mechanism of this inhibition in order to gain some insight into the normal coupling phenomenon involving inorganic phosphate, adenosine diphosphate and a possible aliphatic intermediate.

Work on the new submitochondrial particle prepared from beef heart is being continued. A stable preparation has been obtained which exhibits good oxidative phosphorylation properties. This particle contains about 30% of the pyridine nucleotide present in the intact mitochondrion while the cytochrome content is increased about threefold on a protein basis. The endogenous NAD^+ is reduced by 80% on the addition of succinate, and is rapidly reoxidized on the addition of an uncoupling agent. The morphological studies have been slow due to an overload of use on the available electron microscopes.

4. The Normal Coronary Circulation.

Using an experimental model set forth in the 1970 Annual Report, an unusual response has been found of the normal coronary circulation of the resting dog. Following excitement induced in various ways, a very large increase in left coronary inflow occurs within 10 to 15 seconds. This response is similar to that with exercise, and is accompanied by significant elevation of systemic dynamics. However, instead of increasing, the oxygen extraction across the heart decreases markedly with the result that the coronary sinus oxygen may rise to 60% saturation from a control of about 25% saturation. This marked rise in coronary sinus oxygen content also occurs in the dog with a heart chronically denervated by the stripping technique, and in such a dog it can occur without significant elevation of heart rate and aortic blood pressure. This indicates that there is a plethora of coronary blood flow. Excitement is the only known natural stress in which the oxygen supply exceeds the oxygen demand.

5. Regulation of the Coronary Collateral Circulation.

Using the experimental models outlined in the 1970 Annual Report, studies have been completed of the changing coronary collateral compensation in chronic conscious dogs, 1) in which the blood flow in the circumflex branch of the left coronary artery was partially removed, gradually reduced to zero, and abruptly reduced to zero; and 2) in which after artery closure, the vessel was reopened for a long period and then abruptly reclosed.

a. Progressive circumflex occlusion. Left circumflex flow has been gradually reduced to zero in 1.5 to 12 days in 13 chronic dogs by an Hg constrictor adjusted externally so that minimal to no changes occurred in the electrocardiogram and systemic dynamics. At autopsy, there was minimal to no infarction. All collateral indices increased markedly; left descending flow and its reactive hyperemia nearly doubled, the circumflex peripheral pressure rose 4 to 5 times, xenon clearance in the occluded circumflex bed rose to values approximating those before induction of coronary insufficiency. Retrograde collateral flow from the non-occluded coronary arteries, measured terminally, was greatly elevated even after an occlusion of only one and one-half days. It was found not necessary for coronary blood flow to be reduced

to zero to establish a significant collateral circulation. Sizeable increases in circumflex peripheral pressure and collateral flow were observed even when circumflex flow was reduced by about 60% over a 2½ day period.

b. Abrupt circumflex occlusion. Abrupt and complete closure of the circumflex branch for 1 to 4 days was carried out in 15 dogs. No dog succumbed or developed ventricular fibrillation despite classical changes in the electrocardiogram and massive myocardial infarction. Evidence for improved collateral function during the first day or so was the large elevation of circumflex peripheral pressure, a mild rise in xenon clearance and in retrograde collateral blood flow. These changes were greatly increased after the first day of occlusion. After 1 to 2 days of occlusion, the collateral indices were all greatly elevated, the collateral blood flow being many times greater than in control dogs.

c. Regression and reappearance of coronary collaterals. In eight dogs, after gradual or abrupt circumflex branch occlusion as described in 5b, the vessel was reopened for varying periods and then abruptly reoccluded. Following release, all collateral compensations dropped to near control levels within 3 to 24 hours. Following reocclusion of the circumflex 3 to 90 days later, the collateral indices rose to very high levels within less than an hour. Even the directly measured collateral flow was very high.

6. The Effects of Pulmonic Stenosis and Subaortic Stenosis on the Right and Left Coronary Circulations.

A previous study designed to test in the trained conscious dog the effects of pulmonic stenosis and aortic stenosis of the subvalvular type, on the right and left coronary circulations of the trained, conscious dog, has been expanded considerably.

a. Pulmonic stenosis. Progressive constriction of the pulmonary arteries was mechanically induced over a few weeks in a third dog (see 1970 Annual Report). Early and with only moderate elevation of right ventricular systolic pressure (about 45/3 mm Hg), right coronary blood flow increased throughout the cardiac cycle; later, both mean and systolic coronary flow decreased. These flow trends are similar to but of smaller magnitude than those found in previous dogs in which much higher right ventricular pressures were maintained.

In a joint project with the Comparative Cardiovascular Studies Section, University of Pennsylvania, two mature well trained dogs with congenital pulmonic stenosis of the fibrous ring type, have been studied in this laboratory; a third dog is presently being evaluated. Following recovery from implantation of electromagnetic flowmeters on the ascending aorta and right coronary artery, and a pressure tube in the aorta, the dogs were studied at rest and under the influence of

natural stresses. With right ventricular systolic pressure greatly exceeding aortic systolic pressure, the right coronary blood flow of 35 ml/100g/min at rest is about the same as in the dog with a normal right ventricular pressure. There is very little right coronary systolic forward flow and negative flow is generally seen during late systole. In exercise, excitement, and reactive hyperemia, mean right coronary flow rises largely because of vasodilatation during diastole, but coronary systolic flow increases scarcely at all. These findings contrast with those in the dog with a normal right ventricular pressure, in whom at rest the level of flow throughout systole is about as high as during diastole, while during stress states coronary blood flow increases greatly throughout the cardiac cycle.

b. Congenital subaortic stenosis. An additional dog with congenital subaortic stenosis of the fibrous ring type has been studied as part of a continuing collaborative investigation with the Cardiovascular Studies Section, School of Veterinary Medicine, University of Pennsylvania (see 1969 Annual Report). With left ventricular systolic pressure almost twice the aortic systolic pressure, mean circumflex flow is only 15 ml/min/100g, and the left circumflex systolic flow is almost all backflow. This confirms the previous work. The stresses of reactive hyperemia, excitement, and mild to moderate treadmill exercise, all increase markedly circumflex blood flow, but the increase is almost entirely in diastole, for there is scarcely any forward flow in the left coronary artery during left ventricular ejection. These findings contrast sharply with those in the dog with a normal left heart. In the latter at rest, systolic forward flow is considerable, and the left coronary flow per 100g/min is much larger. During the aforementioned stresses, circumflex coronary flow increases largely in systole as well as in diastole.

Conclusions.

Adenosine uptake by canine myocardium appears to occur by a process of facilitated diffusion, and binding to the carrier is stereospecific. The effectiveness of a purine nucleoside as an inhibitor of adenosine uptake appears to be independent of its action on coronary vascular tone. These preliminary results suggest that alterations in the properties of heart cell membranes may contribute to the cardiac deterioration seen in hemorrhagic and endotoxin shock.

The specificity of biguanides for blocking various phosphorylation sites is dependent on the presence or absence of interfering groups. A stable subsarcosomal particle of high phosphorylation efficiency tightly coupled to respiration has been developed.

Excitement in the normal heart or the heart with cardiac neural ablation causes a plethora of left coronary inflow so that coronary sinus oxygen saturation rises markedly.

Studies have been presented of the natural history of the development of coronary collateral function in the chronic conscious dog during gradually or abruptly induced left coronary insufficiency. Such development is very rapid and large, starting within the first day or so after total or partial coronary occlusion. Its magnitude is such that when coronary flow is gradually reduced to zero over a 2 to 3 day period, there can be minimal to no myocardial infarction, and no permanent alteration of the electrocardiogram occurs. These collaterals quickly become nonfunctional when the coronary insufficiency is released. However, up to 90 days after release, they are immediately available to resupply the myocardium following a subsequent reocclusion. Unfortunately, these observations do not provide evidence for the mechanisms controlling collateral development but they do point the way for future experimentation.

Studies have been made in chronic dogs concerning the effect on the right and left coronary circulations of high outflow tract impedance of the right and left ventricles, occurring naturally in congenital pulmonic stenosis and congenital subaortic stenosis. With the right and left ventricular pressures considerably in excess of the aortic blood pressure, forward flow is largely prevented during systole in the respective coronary arteries with the dog at rest and during various stress states. This imposes a serious impediment to the myocardial oxygen supply, presumably at a time of greatest need. In normal conscious dogs during gradual mechanical pulmonary artery constriction for 2 to 3 weeks, right coronary systolic flow diminished in relation to the extent of elevation of right ventricular systolic pressure.

Project 3A61102B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, The heart under abnormal and pathological stresses

Literature Cited.

Publications:

1. Gregg, D. E.: I. Coronary collateral circulation: Introduction to the problem. II. Recent advances in experimental methodology and their practical significance for clinical conditions. Acta Cardiologia Suppl. XIII, 1970, pp. 114-130.

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4. Khouri, E. M., Gregg, D. E., and McGranahan, G. M., Jr.: The regression and reappearance of coronary collaterals. Am. J. Physiol. 220:655, 1971.

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6. Gregg, D. E.: The role and functional significance of collateral vessels. In, "Myocardial Ischemia," proceedings of symposium, New York, N. Y., March 1970. Editors: Ross and Hoffman. Excerpta Medica, 1971, p. 44-50.

7. Gregg, D. E.: Physiological factors which determine coronary blood flow. In, "Coronary Heart Disease," proceedings of American College of Cardiology Symposium, New York, N. Y. Editors: Russek and Zohman. J. B. Lippincott Co., Phila., 1971; pp. 19-24.

8. Gregg, D. E.: Collateral circulation and myocardial infarction. In, "Coronary Heart Disease," proceedings of American College of Cardiology Symposium, New York, N. Y. Editors: Russek and Zohman. J. B. Lippincott Co., Phila., 1971; pp. 163-166.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6450	71 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
70 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	61102A	3A061102B71R	02	086
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Military Hematology (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

0800 Life Support 002600 Biology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 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 71	7	130
	CURRENT 72	7	130

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Coagulation; (U) Malaria; (U) Blood; (U) Blood Transfusion; (U) Anemia

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

23. (U) Studies of the pathophysiology of diseases with hematologic manifestations in soldiers and the investigation of those which occur because of their occupation.

24. (U) Studies of hematologic abnormalities produced by chemicals, drugs and infectious agents encountered primarily in military populations and in native of geographic areas of potential military operations. Studies of blood, blood products and blood substitutes used for the treatment of casualties and the prevention and diagnosis of diseases in soldiers.

25. (U) 70 07 - 71 06 Dapsone, an important prophylactic antimalarial drug, can cause both methemoglobinemia and hemolysis. Hydroxylaminodapsone was shown to be primarily responsible and the mechanisms of action were delineated. This derivative is not primarily responsible for the antimalarial effect of dapsone. Disseminated intravascular coagulation was demonstrated in patients with scrub typhus and Korean hemorrhagic fever and aotus monkeys with falciparum malaria. Platelet abnormalities were observed in dogs with canine hemorrhagic fever but not disseminated intravascular coagulation. Protein-like substances were found in sera which act as inhibitors of chemotactic activity. Basic studies of the importance of charge on the surface of the red blood cells in the causation of hemolysis were undertaken. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon originator's approval

DD FORM 1 MAR 84 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, NOV 85 AND 1498-1, MAR 88, FOR ARMY USE, ARE OBSOLETE

PII Redacted

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 086, Military Hematology

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Associate: LTC Donald E. Dillon, MC; LTC George M. Bernier, MC; MAJ Ronald O. Gilcher, MC; MAJ M. Jeffrey Maisels, MC; MAJ Dennis S. O'Leary, MC; MAJ Michael E. Leibowitz, MC; MAJ Bertil E. Glader, MC; MAJ Phillip P. Toskes, MC; MAJ Jeffrey Berenberg, MC; Mr. Harold L. Williams; Mrs. Ruth G. Brennan; Miss Donna J. Wicker; Mr. Charles F. Barr

Description.

Basic and clinical studies to investigate the functions and disorders of blood and blood forming organs.

Progress and Results.

Dapsone has become an important chemoprophylactic drug in the prevention of chloroquine resistant Plasmodium falciparum malaria in many geographic areas of the world. This drug produces clinically apparent methemoglobinemia in people with a heterozygous deficiency of NADH methemoglobin reductase deficiency and a measurable increase in methemoglobin blood levels in normal individuals. NADH methemoglobin reductase deficiency occurs in about one per cent of the normal population. Although methemoglobinemia in small quantities does not produce gross clinical symptoms, it involves respiratory enzymes as well as hemoglobin and may cause minor decrements in visual and audio Perception at low levels. Hydroxylamine dapsone was shown to be the only identifiable derivative of dapsone which produces methemoglobin formation. Additional studies with this derivative have suggested the mechanisms of action. The hexose monophosphate shunt which is vital to protecting the red blood cell from oxidative stresses is paradoxically responsible for the methemoglobin formation observed with hydroxylamine dapsone. The reducing equivalents produced by this pathway are responsible for further activation of the drug, resulting in additional methemoglobin formation. This was substantiated by studying methemoglobin formation in red blood cells from G6PD deficient subjects. Red blood cells from G6PD deficient patients contained less methemoglobin than erythrocytes from normal subjects; this observation is consistent with the observation that the hexose monophosphate pathway is less effective in affected red blood cells.

Dapsone is synergistic to primaquine in causing enhanced hemolysis which is most pronounced in subjects with G6PD deficiency. Studies of a number of derivatives of dapsone showed that hydroxylamine dapsone

appears to be the active hemolytic agent. In contrast to methemoglobin formation which is enhanced when the hexomonophosphate shunt is functioning normally, hemolysis occurs when there is impairment of this glucose pathway. In the normal red blood cell with adequate amounts of available glucose, hemolysis does not occur in the presence of physiologic quantities of hydroxylamine dapsone. However, in red blood cells with diminished glutathione, there is a marked reduction in red blood cell survival following exposure to the drug in therapeutic concentrations. Studies are in process to ascertain if this is caused by the excessive generation of peroxides within erythrocytes.

Basic studies to investigate the etiology of hemolysis were undertaken because this is an important problem in diseases such as malaria. Studies were undertaken to examine whether red blood cells were destroyed because of loss of negative surface charge upon their surface or deformability or both. It was shown that older red blood cells had a diminished negative charge on their surface than young erythrocytes. Similarly, other investigators have shown that older red blood cells were treated with neuraminadase in order to reduce the capability of erythrocytes to maintain a negative charge on their surface. Chemical treatment with neuraminadase markedly diminished the survival of these cells when they were transfused. Preliminary studies show that this method of treatment of erythrocytes decreases both the negative surface charge on erythrocytes and also decreases the capability of these cells to undergo deformability.

Studies of neutrophil function and leukocyte chemotaxis were initiated during the year in collaboration with Dr. Peter Ward at the Armed Forces Institute of Pathology. Inflammation has both destructive and beneficial effects. Damage following a foreign insult is often host mediated such as is observed in acute immunologic vasculitis and glomerulonephritis. Further, polymorphonuclear leukocyte depletion eliminates the necrosis of the tick feeding lesion. Tick salivary gland extracts contain a potent enzyme which cleaves C5 to produce the chemotactic factor C5A. This was confirmed by use of C5 deficient mouse sera, sucrose gradient ultracentrifugal analysis and specific antibody suppression. Furthermore, complement depletion of host rats by cobra venom factor also eliminated the characteristic necrosis following a tick bite.

The potential damage to host tissues resulting from the accumulation of neutrophils is substantial. Restraints of the inflammatory process must exist. The C1 esterase inhibitor and anaphylatoxin inhibitor are two examples. Two protein-like substances were found in serum which block the activity of both complement and noncomplement chemotactic factors. They do not have characteristics of antibodies and seem to be important in the homeostasis of inflammation. Purification of these compounds is being pursued because of the possibility

that excess production of these inhibitors could be associated with increased infection. Preliminary studies have shown the necessity for glycolysis in the polymorphonuclear leukocyte to respond to chemotactic stimuli.

Measurement of carbon monoxide production in the body provides an accurate means of quantifying the rate of red blood cell destruction. It is preferable to methods in which red blood cells are labeled with a radioisotope because it permits measurements to be made within hours rather than weeks and it includes hemoglobin degradation from ineffective erythropoiesis. Using this method three groups of patients have been studied: soldiers with G6PD deficiency with and without the ingestion of primaquine, patients with sickle cell disease before and after treatment with urea and patients with chronic unconjugated bilirubinemia before and following caloric deprivation. Hemolysis occurs within hours after the ingestion of primaquine and accelerated destruction persists for days in patients with G6PD deficiency. The use of small doses of primaquine (5 mg daily) which are gradually increased to 15 mg daily provide a method of avoiding moderately severe anemia in the treatment of patients with malaria who have G6PD deficiency. The total number of red blood cells hemolyzed remains the same, but the rate of hemolysis is decreased. Urea in invert sugar has been advocated as a method for treatment of sickle cell crises. Studies of red blood cell destruction showed that this treatment significantly increases red blood cell destruction. Differentiation of patients with hemolytic disease and ineffective erythropoiesis from patients with Gilbert's disease has been a time consuming clinical problem Use of CO production and the increase in serum bilirubin values following a one day fast have proven valuable in decreasing the period of medical evaluation from one month to several days.

Disseminated intravascular coagulation was observed in a soldier with scrub typhus, two soldiers with Korean hemorrhagic fever and occurs in Aotus monkeys infected with Plasmodium falciparum malaria. In the soldier with scrub typhus, coagulation abnormalities were marked and persisted for one week despite prompt recovery of the patient following tetracycline therapy. Similar coagulation abnormalities were observed in serial blood specimens obtained from patients with hemorrhagic nephroso-nephritis in Korea. These findings make it important to examine specimens from other patients with these diseases to ascertain if the coagulation abnormalities are only an occurrence in an occasional patient or are an important manifestation of the disease which requires appropriate therapy.

Studies of a patient with severe Factor XI (PTA) deficiency and Type I dysgammaglobulinemia became important because she had severe anaphylactic reactions following blood or plasma transfusions. This patient was shown to have a deficiency of IgA and had developed an

antibody to IgA following transfusions. Transfusions of plasma from normal donors with a congenital absence of IgA were unassociated with any reaction. Since approximately 0.2 per cent of the population are IgA deficient, this problem requires scrutiny in both the blood transfusion field and in the administration of serum and serum components (gamma globulin) for immunization against diseases.

Coagulation studies of dogs with tropical canine pancytopenia failed to show abnormalities in coagulation. The bleeding abnormality is believed to be caused by a diminished production of platelets in association with an increased peripheral destruction of platelets. Preliminary studies of platelets in these animals show excessive clumping of thrombocytes in the peripheral blood. Platelet survival studies are planned in affected animals to provide quantitative information about this aspect of the problem.

In addition, the Department of Hematology, WRAIR, provides laboratory space and facilities for eight Fellows assigned to the Hematology Service, WRGH, in order that they may devote a portion of their training to clinical investigation. The laboratories establish and maintain standards for distribution to military laboratories and act as a reference laboratory for clinical and area laboratories and contractual agencies. Laboratory evaluation of patients is provided for difficult diagnostic problems in the areas of coagulation, hemoglobin abnormalities, disorders of immunoglobulins, red blood cell enzymes and abnormalities of iron, B_{12} and folic acid metabolism.

Recommendations.

Further studies of the etiology of the adverse reactions of the antimalarial drugs are required because it may be possible to prevent the untoward effects and preserve the antimalarial effects. Additional studies of the mechanism of hemolysis of red blood cells, the coagulation abnormalities which occur in various infections tropical diseases and of host responses to infection promise to provide basic information of value in the treatment of these diseases.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 086, Military Hematology

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA OA 6452	2. DATE OF SUMMARY: 71 07 01	REPORT CONTROL SYMBOL: DD-DR&E(AR)636
3. DATE PREV SUMRY: 70 07 01	4. KIND OF SUMMARY: D. Change	5. SEC REG: U	6. WORK SECURITY: U	7. REGRADE: NA	8. DOWN GRADE: NI	9. SPECIFIC DATA: YES NO ACCESS: YES NO	10. LEVEL OF SUM: A. WORK UNIT
11. NO. CODES: PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	61102A	3A061102871R		02		087	
b. CONTRIBUTING							
c. EXECUTIVE	CDOC 3512A(2)						

12. TITLE (Precede with security classification code)

(U) GASTROINTESTINAL DISEASE (80)

13. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 063500 Clinical Medicine

14. START DATE	15. ESTIMATED COMPLETION DATE	16. PERFORMING AGENCY?	17. PERFORMANCE METHOD
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18. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA
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f. CUM. AMT.:

19. RESOURCES ESTIMATE

	PROFESSIONAL MAN YRS	FUNDS (In thousands)
FISCAL YEAR 71	3	100
CURRENT 72	8	

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21. GENERAL USE

Foreign Intelligence Not Consider.d

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23. KEYWORDS (Precede each with Security Classification Code)

(U) Diarrheal Disease; (U) Intestinal Blood Flow; (U) Intestinal Reactivity; (U) Intestinal Absorption; (U) Intestinal Enzymes

24. TECHNICAL OBJECTIVE. 25. APPROACH. 26. PROGRESS (Precede individual paragraphs identified by number. Precede each with Security Classification Code.)

23. (U) The incidence of diarrheal disease in combat troops has been shown to detrimentally affect field operations. This department's mission relates to the pathophysiology of diarrheal disease in order to better approach treatment and control of these conditions in the field.

24. (U) A team studied the etiology of the prevalent diarrheal diseases in Vietnam. Animal models are being used to assess the effect of diarrheal disease on intestinal neuromuscular mechanisms, absorption and secretion, blood flow and enzyme activity.

25. (U) 70 07 - 71 06. A large part of the eighty percent of diarrheas of unknown etiology in Vietnam appear to be due to pathogenic E. coli and a hybrid E. coli/Shigella organism. Diarrheal disease is associated with a loss of inhibitory and an increase of excitatory nerve/muscle mechanisms in the intestine. The fluid lost in these diseases results from a reversal of water and salt absorption to secretion; this condition is benefited by providing the gut with a solution containing increased glucose. Intestinal blood flow in the subhuman primate has been shown to be effected by experimental shock in a manner different from classical concepts. Adrenergic stimulation causes mesenteric vasoconstriction which when prolonged is accompanied by autoregulatory escape. Intestinal blood flow has been shown to be independent of motility. Disaccharidase enzyme activity is reduced in several malabsorption diarrheas. These animal studies have provided specific information concerning absorption and motility changes in diarrhea; a corresponding study of diarrhea in the human is currently being organized. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1970 - 30 June 1971.

*Available to contractors upon originator's approval

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

Investigators.

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Kenneth G. Swan, LTC, MC;
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Description.

The research activity in the Depar ment of Gastroenterology this year was directed towar three areas of gastrointestinal pathophysiology. The first area continued our multidisciplined approach of studying the pathophysiology of diarrheal disease. In this area, studies were continued on intestinal transport mechanisms, disaccharidase activity, microvascular architecture and mesenteric blood flow. The second area of activity resulted from work done in the blood flow laboratory that was established last year. This work specifically evaluated the role of the gut in the pathogenesis of shock. The third area being pursued is a continuation of a study of the pathogenesis of gastric stress ulceration.

In addition to the above areas of activity, one investigator in this department has developed an interest in and has become active in the Institute's program studying narcotic addiction. The investigator has established a liason position with The District of Columbia Narcotic Treatment Administration and is active in both military and civilian populations concerned with drug abuse.

Progress and Results.

1. Pathophysiology of Diarrheal Disease

a. Intestinal Transport. Three papers dealing with mucosal transport in diarrheal disease are currently in press in Gastroenterology. In the first, the ilia of Salmonella infected rats were shown to secrete water, Na, K, and Cl. The second paper was concerned with glucose stimulated intestinal absorption. A perfusion solution containing 118 mM Na^+ and 56 mM of glucose caused maximum jejunal and ilial absorption in control rats. In Salmonella infected rats, this solution did not reverse

the ilial secretion and in a like manner suggested a jejunal defect since it failed to stimulate Na absorption in that segment of the gut. This data suggests that Salmonellosis differs from cholera in that oral fluids will not therapeutically rehydrate an individual. In the third study control and infected rats were perfused with either HCO_3^- free or HCO_3^- buffered solutions. The study tested the hypothesis that the rat jejunum possesses a Na^+/H^+ exchange mechanism that is similar to the one described in the human jejunal mucosa. The data suggest that H^+ secretion and Na^+ absorption are associated with the mechanism of HCO_3^- absorption in both control rat jejunum and infected ileum.

The luminal accumulation of fluid during experimental intestinal obstruction was studied in dogs. The secretion that occurred in the obstructed gut segment was characterized by active HCO_3^- transport into the lumen. This study has been published in abstract form (1)

Unidirectional mucosal fluxes of Na^+ and Cl^- were studied with radioisotopes in control and infected rats. The data indicate that the net accumulation of luminal fluid in diarrhea is due to a defect in absorptive mechanisms rather than to overactivity of secretory mechanisms. This work is being prepared for publication.

The turbidometric analysis of polyethylene glycol as used to determine water movement in these transport studies is effective but is a complex and cumbersome analysis. Studies are currently in progress using ^{14}C labeled PEG as the non-absorbable water marker. Early data are encouraging and suggest that the radioisotope technique will facilitate laboratory procedures.

The clinical investigation of intestinal transport in human volunteers that started last year is continuing. The study is being done in conjunction with personnel of the Department of Infectious Disease, University of Maryland Medical School. Several pilot studies have been done in order to establish the definitive protocol and the actual study of human diarrheal disease is scheduled for an early start.

b Mesenteric Blood Flow The adrenergic mechanisms in the mesenteric vascular bed were studied with electromagnetic flowmeter techniques. The effects of intra-arterial and intravenous injection of adrenergic agents on mesenteric blood flow showed epinephrine, norepinephrine and phenylephrine to be vasoconstrictors while

isoproterenol was a vasodilator. Alpha adrenergic blockade reversed the epinephrine and phenylephrine responses and beta blockade reversed the isoproterenol response. Thus, norepinephrine appears to be the only pure adrenergic agonist in the mesenteric circulation. This study was presented at the American Physiological Society Meeting (2), the catecholamine reversal data abstracted (3), and is in press in the American Journal of Physiology. These data provide base-line information for a study, currently in progress, on the effects of diarrheal disease on mesenteric blood flow.

Intravenous injection of epinephrine causes a large increase in portal pressure in the dog. In a study employing catecholamine injections, differential adrenergic blockade and splenectomy the response was shown to be primarily due to splenic contraction and secondarily due to portal venous constriction. This work has been abstracted (4) and submitted for publication.

The relationship between mesenteric blood flow and intestinal motility was studied in anesthetized dogs. Acetylcholine, when infused into the superior mesenteric artery, increased both motor activity and blood flow. Methacholine infusion had no effect on blood flow but caused a significant increase in motility. The data suggest that these parameters are independent of each other. This study has been submitted for publication.

c. Intestinal Disaccharidase Activity. A manuscript describing the distribution of disaccharidases in human fetal intestine is in press in the American Journal of Diseases in Children. The study demonstrated that maltase and sucrase levels reached adult value by four month of fetal development. However, lactase activity remained low beyond six months of development.

In order to test the possibility that fecal disaccharidase enzyme activity might be employed for screening malabsorption conditions, these enzymes have been analyzed in feces and compared to enzyme activity of the corresponding small intestinal mucosa. The data indicate that a good correlation exists between fecal and intestinal enzyme activities. This study is being prepared for publication.

d. Microvascular Architecture. A study was published in which the silicone rubber injection technique was used to describe the microvascular architecture of the normal colonic mucosa of man and compare it to vascular changes seen in ulcerative colitis (5). The vascular patterns seen support the concept of mucosal ischemia in ulcerative colitis.

e Etiology of Diarrehal Disease in Vietnam. Departmental personnel conducted an etiological analysis of diarrheal disease this year in Vietnam. Of the 150 patients studied, approximately 35% were demonstrated to be due to Shigella infection and 20-25% were due to previously undescribed strains of pathogenic E. coli. Of those cases of diarrhea due to pathogenic E. coli, 75% were the result of infection by a toxigenic organism and the remainder by a tissue invasive, dysentery-like organism. The pathogenicity of these "hybrid" E. coli organisms is being subjected to continued investigation in laboratory animals and ultimately clinical studies in human volunteers will be conducted.

2 Pathogenesis of Gastric Stress Ulcer.

A paper was published that described the rotational device employed to quantitatively stress rats and induce gastric ulceration (6). The stressed animals that developed gastric ulcers were shown to have elevated blood levels of catecholamines and corticosterone. Further experimentation demonstrated these hormonal blood levels to be sensitively dependent upon the animals' individual experiences, such as handling, environmental noise, and light/dark cycle. These factors render routine blood analyses questionable and therefore current efforts are aimed at correlating stress with urinary excretion of catecholamines and corticosteroids. In order to do these studies, a method is being developed using an implanted artificial bladder to collect 24 hour urine specimens which will then be subjected to chemical analyses. Early results indicate that an effective artificial bladder can be made and that urine collections can be accomplished under standardized conditions.

3 Shock

Current concepts describe the gut as a "target organ" in shock. The endogenous catecholamines released by hypotension reputedly cause intense constriction of the mesenteric vascular bed. This event leads to ischemia,

ultimately tissue necrosis, and the irreversible phase of the shock syndrome. A series of studies have been completed that question these traditional concepts.

a. Catecholamines were infused into the superior mesenteric artery of anesthetized dogs. Both epinephrine and norepinephrine, when infused for a ten minute period, caused vasoconstriction that was accompanied by autoregulatory escape and blood flow returned toward control within minutes. Phenylephrine and isoproterenol infusions caused mesenteric constriction and dilation, respectively, without autoregulatory escape. The occurrence of autoregulatory escape with infusion of the endogenous catecholamines suggests that the intestinal vasculature is incapable of maintaining a state of constriction and, thus, questions the validity of the target organ theory. This study was presented at the American Gastroenterological Association (7) and has been submitted for publication.

b. A study of the mesenteric hemodynamic events during endotoxemia in baboons has been abstracted (8) and submitted for publication. The intravenous injection of endotoxin, LD_{80}, reduced blood pressure to a third of control within two hours. Superior mesenteric artery blood flow and portal pressure were unchanged over a four hour observation period and, thus, mesenteric vascular resistance was reduced. The observation is opposed to the classical canine response to endotoxin and therefore questions the validity of the target organ theory.

c. A study of the mesenteric vascular responses during hemorrhagic shock in baboons has been submitted for publication. In this study the development of hypotension was controlled to parallel that observed during endotoxemia. Mesenteric arterial blood flow fell in parallel with blood pressure. Vascular resistance was therefore elevated and remained thus for the four hour observation period. Upon reinfusion of the shed blood, all hemodynamic parameters returned to control values and all animals survived. The canine model responds to this procedure in a similar manner, except the animal does not survive.

d. The new alpha adrenergic antagonist, WR-2823, was evaluated as a blocking agent in the mesenteric vascular bed. The new drug did not protect the gut against the vasoconstrictor effects of catecholamines as well as phenoxybenzamine. Work done in the Department of Pharmacology, WRAIR, has shown that WR-2823 protects

against the lethal effects of shock better than phenoxybenzamine. Our data then question both the site of action of the new drug in shock protection and the validity of the concept that the gut is involved in the pathogenesis of irreversible shock. This work has been abstracted (9), presented at the Federation of American Societies of Experimental Biology (10), and accepted for publication in Proceedings of the Society for Experimental Biology and Medicine.

Several projects are currently underway to further evaluate mesenteric involvement in the pathophysiology of shock. These include: 1) Mesenteric vascular responses to catecholamine injections and infusions in the baboon. 2) Hepatic and splenic artery blood flows are being measured during endotoxemia in baboons in order to determine if the other major divisions of the splanchnic vascular bed respond in a manner similar to the region served by the superior mesenteric artery. The data indicate that endotoxemia has no effect on hepatic artery blood flow but eventually lowers splenic artery flow. 3) The effects of sub-lethal doses of endotoxin on mesenteric catecholamine responses are being studied in both dog and baboon. 4) The silicone rubber injection technique is being used to assess the possibility of architectural change accompanying endotoxemia. Initial observations have revealed basic differences between dog and monkey as well as in the dog before and after endotoxin.

4. Drug Abuse.

Work in this area has centered on the following problems: 1) clinical examination and identification of the addict; 2) patterns of drug abuse within a population; 3) use of methadone and other modalities in treating addicts; and 4) the essentials of treatment and preventive programs. A report is currently being prepared that characterizes the addiction history and findings in a group of patients who have all been discharged from military service within the past two years.

These clinical studies have raised questions concerning the definition of addiction. This question is being studied under an approved protocol entitled "Development of an Immunoassay for Determination of Narcotics in Urine". An effort that is in an early stage of development is concerned with establishing a laboratory model of addiction to include the phenomena of tolerance and physical dependence.

Conclusions and Recommendations

Work done on animal models of diarrheal disease suggests that lumenal accumulation of fluid is a result of an absorption defect rather than excessive secretion. This information will be incorporated into the clinical protocol to evaluate mucosal transport activity in human volunteers. It is anticipated that the initial clinical study will employ Salmonella as the pathogenic organism and will be followed by a similar study of the pathogenic strains of E. coli. The effect of diarrheal disease on intestinal blood flow will be studied in monkeys infected with Salmonella and Shigella.

Several studies have been completed that question the validity of current concepts of intestinal involvement in irreversible shock. Further studies will be continued to define the role of the splanchnic vasculature in these hypotension syndromes.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				DA 0A 6453	71 07 01	
70 07 01	D. Change	U	U	NA	NL	
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(U) Military Nursing (09)

003500 Internal Medicine

58 03	CONT	DA	C. In-house

		71	4	250
		72	4	200

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(U) Military Nursing; (U) Guidelines for Procedure; (U) COMPSY; (U) Diabetes; (U) Operative Care

23 (U) Develop rationale underlying military nursing and guidelines for nursing procedures; the use of computers in military psychiatry (COMPSY); evaluation of the health teaching of the military diabetic patient; and the effect of pre-operative preparation of war wounded on post-operative recovery.

24 (U) Assessment of thermometer placement time and warming of post-operative patients; testing of the use of nurses' notes; teaching and testing of diabetic patients; interviews of soldiers pre-operatively.

25 (U) 70 07 - 71 06 Guidelines for adult oral temperatures derived; and pilot for warming patients begun; assessment of nurses' notes; definitive diabetic study completed and data analysis begun; pilot study completed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1970-30 June 1971.

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PII Redacted

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing

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Description.

Research in Military Nursing is concerned with both direct and indirect patient care, and with identifying and testing principles underlying nursing care. The ten areas being reported are concerned with: a comparison of three methods of diabetic teaching; an evaluation of two types of warming post-operative patients; a content analysis of queries of patients having major surgery; evaluation of sterile supplies; three studies regard thermometer placement times for adults' rectal temperatures, and for children's oral and rectal temperature measurements; two studies deal with a secondary analysis of data gathered for adult oral and adult rectal thermometer placement times; and computer support in military psychiatry.

1. Diabetic Study.

Diabetes mellitus, a major health problem in the U.S., ranks as one of the leading causes of death and of blindness. Because individuals with the diagnosis of diabetes must assume the major responsibility for their own care, it is imperative that health care personnel provide patients with information they need for their care. This study, regarding the teaching of adults attending diabetic clinic, has the purpose of ascertaining the most effective of three methods of health teaching. The study design included pre-testing, presenting three methods of instruction to three designated groups, and post-testing. The required attendance for the three methods of instruction was: (1) one-hour didactic-demonstration classes for three consecutive weeks and with several instructors for the conventional method; (2) one hour of programmed instruction for two consecutive weeks with one instructor; and (3) a combination of the two. Post-testing included a test at the completion of the instruction, and if patients were still available after one month, a retention test was administered.

2. Hypothermic Study.

Of the patients arriving in the recovery room from the operating room daily, many are hypothermic. The contributing causes may include: decreased body metabolism from anesthetic agents, depressed thermoregulating mechanism, peripheral vasodilitation, prolonged operation time, and transfusion of cold blood. Prolonged hypothermia is not a desirable state for patients to continue in, and therefore, various methods of warming them have been used. In this study these two methods of warming patients will be used: (1) three blankets covering the patient and (2) Emerson Mobile Heat Lamp. As soon as the warming method was instituted in the recovery room on each patient, both skin and rectal temperatures were taken every five minutes until a specified period of time after normothermia was reached.

3. Pre-operative Preparation Study.

Health care personnel traditionally provide patients with information they think patients should have. There have been few studies dealing with asking patients themselves what they would like to know. This study was designed to elicit patients' queries about their surgical event and immediate post-operative recovery. Soldiers were interviewed the evening prior to surgery the following morning and they were interviewed post-operatively.

4. Sterile Items Study.

"Sterilized" items for patient care were cultured to assess their sterility after being sealed in plastic for four years.

5. Thermometer Placement Time Studies.

Nursing and medical texts do not agree on the amount of time required to measure a person's body temperature accurately by clinical thermometers. Recommended oral thermometer placement times vary from 3-5 minutes, and from 1-5 minutes is recommended for rectal thermometer placement. These times were not derived from systematic investigations, but they have been used traditionally in nursing practice. Also, the amounts of time recommended are usually recommended for all individuals regardless of the variables of age, sex, and room temperature. In all of the thermometer placement studies, the purpose was to

ascertain the maximum and the optimum placement times. The following were the operational definitions: (1) maximum temperature - the highest registration on a clinical thermometer during the testing period; (2) optimum temperature - a temperature reading 0.2° Fahrenheit (F) lower than the maximum temperature; (3) maximum placement time - the time required for 90% of the subjects to register their maximum temperatures; (4) optimum placement time - the time required for 90% of the subjects to register their optimum temperatures.

One investigation deals with rectal thermometer placement times for adults with rectal temperature readings of at least 100.6° Fahrenheit (F). Fifty hospitalized patients, 25 men and 25 women ranging in age from 18 to 65, were the intended subjects. The procedure included taking six one-minute readings with five seconds intervening between each reading. Another study, also regarding rectal thermometer placement times, had Pediatric Clinic patients, ranging in age from one to six, as subjects. Forty boys and girls, with rectal temperature readings of 101°F or more, had their temperatures measured if they and their parents consented. Each child had a rectal thermometer inserted for one minute, removed five seconds for reading, and then re-inserted. The procedure was continued until eight readings were recorded. Also, fifty boys and girls, ages 7-13, had 12 one-minute oral temperature readings if they had a reading of at least 100°F. Two studies utilize a secondary analysis of data; one is for data collected for 6 different studies on adult oral thermometer placement times and the other is for different studies on adult rectal thermometer placement times.

6. Computer Support in Military Psychiatry.

The Computer Support in Military Psychiatry (COMPSY) study is a project in Walter Reed General Hospital's Department of Psychiatry and Neurology with full-time nurse support from the Division of Nursing, Walter Reed Army Institute of Research. It is being reported under Project No. 6215601A-3A0 25601A823 Task No.: 00-048.

Progress.

1. The data collection is completed on the diabetic study. Automatic data processing equipment was utilized for the analysis of data. Programmed instruction was far superior to the other methods of teaching for the comprehension of

knowledge. The conventional teaching method was somewhat better for the retention of subjects' knowledge. Overall, it was concluded that programmed instruction should be utilized much more than at present for health teaching.

2. The pilot phase of this study was recently finished. The data collection sheet and the method for the study were revised and data were collected for one subject in the definitive study. However, due to the high humidity and high ambient temperature in the recovery room during the summer months, a sufficient sample of patients with hypothermia are not presently available. This study is postponed until the winter months.

3. The pilot phase is completed. Male subjects were interviewed late in the evening prior to surgery the following morning. These same subjects were interviewed postoperatively after returning to the parent ward. Hopefully, the queries patients have can be categorized into areas of information patients still want after they have received what health care personnel think they should have. At present a content analysis of the interview data is being done to ascertain whether or not the data collected is suitable for categorization. It is questionable as to whether or not it would be profitable to continue with the definitive study.

4. The bacteriological testing which was done on 27 items showed the following results: twenty items were sterile; seven items were not sterile when cultured. Because of the type of organisms isolated, it is felt that four items were accidentally contaminated, but that possibly three items never had been sterile.

5. Data collection is almost completed on the adult rectal thermometer placement time study. The data for the two children's studies has been collected and analyzed. The findings for these studies were as follows: The overall maximum and optimum placement times were 11 and 7 minutes for oral thermometers. The maximum and optimum placement time for rectal thermometers was 7 and 4 minutes, respectively. There were too few subjects in each sex and room temperature groups, in both the oral and rectal study, to make definite statements regarding these variables separately. Both sets of data do show, however, that the thermometer placement time was less in a warmer, as compared to a cool, room. The secondary analysis for adult oral thermometer placement times, with 390 subjects, is

completed. It requires 7 minutes for optimum oral readings for all adults in warm rooms, and 8 and 9 minutes, respectively, for men and women in cooler rooms. The secondary analysis of adult rectal thermometer placement times, with 403 subjects, will be done as soon as the first study mentioned above is completed.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing

Publications.

Glor, Beverly A.K. and Estes, Zane E. "Moist Soaks: A Survey of Clinical Practices." Nursing Research, 19: 463-465, September/October 1970.

Estes, Zane E. "Moist Soaks: An Evaluation of Time, Temperature, and Solution." Presented at the Sixth Nursing Research Conference in San Diego, April 1970, Published 1971, pp. 139-164.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	3. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6464	71 07 01	DD-DR&E(AR)636

2. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTR/ACTOR ACCESS	9. LEVEL OF SUM
70 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	61102A	3A061102B71R	02	C89
b. CONTRIBUTING				
XXXXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) BODY FLUID AND SOLUTE AND RENAL HOMEOSTASIS (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012 900 Physiology 003500 Clinical Medicine 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 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)
FISCAL YEAR	PRECEDING 71	2	50
	CURRENT 72	2	50

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunopathology (U) Heat Stress; (U) Renal Failure; (U) Dialysis; (UA) Acid-Base; (U) Electron Microscopy (U) Kidney Function; (U) Renal Hemodynamics; (U) Fluid & Solute Homeostasis; (U) Shock

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

23. (U) - To investigate mechanisms for maintaining fluid, electrolyte and hemodynamic homeostasis in reponse to disease, injury and environmental stresses of military significance, such as shock, heat stress, infectious disease, gastrointestinal disorders, and renal failure in order to provide rational basis for prevention and treatment.

24. (U) - Clearance methods, externally monitored isotope methods, isotope dilutions, experimental models, in vivo renal micropuncture, in vitro renal microperfusion, membrane transport, light and electron microscopy and immunopathology.

25. (U) 70 07 - 71 06 Renal cell sodium localization studies were extended to show alterations induced by injury and transport inhibitors. The role of physical composition of blood was found to be as or more important than the volume status of the animal in regulating renal salt excretion. A study was conducted attempting to correlate renal functional and histologic changes in combat casualties with acute renal failure. Methods for physiologic maintenance of the isolated perfused whole kidney were investigated. Optimum protein intake, nitrogen balance, red blood cell metabolism and survival, glucose metabolism and insulin production were studied in renal failure patients. New methods of dialysis were explored in these patients. Heat stress studies assessed factors predisposing to this disorder as well as methods for prevention. Renal concentrating mechanisms are being evaluated in a variety of primates. Effects of propranolol on renal hemodynamics in dogs showed alterations in renal function were due to extrarenal influences. Splenectomy and combination immunosuppressive regimens are being evaluated in murine lupus. Evidence for a primary alteration in renal hemodynamics in acute renal failure has been demonstrated. A direct influence of potassium on renal renin content has been found. Cyclic 3'5' AMP stimulates fluid reabsorption in the isolated perfused proximal tubule of the rabbit. For technical reports , see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

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Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089, Body fluid and solute and renal homeostasis

Investigators:
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Associates: MAJ Charles B. Carter, MC; LTC Jordan J. Cohen, MC; MAJ Andrew Saladino, MC; MAJ Charles Wallas, MC; MAJ Vincent Dennis, MC; John A. Gagnon, Natalie Lawson, James McNeil, Amy Lee, Martha Huddleston and Doug Grove

Description: Studies are directed at investigation of mechanisms for maintaining body fluid, electrolyte and hemodynamic homeostasis or their correction in response to disease, injury and environmental stress of military significance, including shock, heat stress, infectious disease, gastrointestinal disorders, and renal failure. A variety of methods have been developed and utilized, including externally monitored isotope techniques, isotope dilution, experimental models of acute renal failure and shock, in vivo renal micropuncture, in vitro microperfusion, membrane transport, dialysis systems, light and electron microscopy, and immunopathology. The role of adaptive homeostatic mechanisms, including renal and extrarenal mechanisms, whereby body fluid and solute balance is achieved and maintained in the face of stress has been emphasized in order to provide a rational basis and develop improved methods for prevention and treatment of altered fluid, electrolyte and hemodynamic states and acute and chronic renal failure induced by these stresses.

Progress:

1. Renal Solute and Water Handling:

a. The relationship of volume expansion per se to alterations in renal sodium excretion and proximal tubule sodium rejection was studied in dogs using micropuncture methods. Volume expansion was produced by using normal saline, homologous plasma or homolgous blood. Renal perfusion pressure was kept constant so that only alterations in blood hematocrit and/or plasma concentration in association with volume

were studied. Results indicate that proximal tubule sodium reabsorption is decreased when volume expansion is associated with a decrease in hematocrit or a decrease in hematocrit and plasma protein concentration. When these changes are avoided by expansion with homolgous whole blood, no change in proximal sodium reabsorption was observed early after infusion (1,2). A late effect of volume expansion per se, consistent with formation and release of a circulating humoral substance affecting late proximal tubules could not be ruled out; current studies will compare early and late effects of blood expansion without change in physical composition of the blood. Further studies will investigate the effects of volume contraction without alteration in renal perfusion pressure on proximal sodium reabosrption.

b. The concentrating ability related to renal morphology of wide variety of primates was evaluated in order to determine the structural requirements vital to formation of a concentrated urine. Results indicate that in these species, unlike man, a direct relationship between concentrating ability and length of the renal medulla does not exist and that factors other than overall length of the loop of Henle must be important in the attainment of a concentrated urine in these animals (3). A few more species remain to be studied.

c. A study of dogs has been completed directed at defining the mechanism(s) responsible for the pharmacologic effect of propranolol on sodium metabolism noted in human subjects. Findings suggest that effects of propranolol on renal hemodynamics and sodium excretion are not attributable to an intrarenal effect, but are likely mediated by effects of the drug on cardiac output and total peripheral resistance (4).

d. Using the fully operational techniques of perfusing isolated segments of rabbit nephrons, the physiologic and toxic factors influencing the reabsorption of salt and water by the proximal tubule are being determined with current activity centered on the observed stimulation of fluid transport by cyclic 3'5'-adenosine monophosphate, a major intermediary of cellular metabolic and hormonally regulated processes.

2. Acute Renal Failure:

a. Studies directed at pathogenesis and prevention of acute renal failure in an experimental animal model have allowed postulation of certain mechanisms for its development. Clearance and micropuncture studies in the methemoglobin-ferrocyanide model of acute renal failure in the rat have yielded evidence favoring an early renal vascular event

consisting of afferent arteriolar constriction and relative efferent arteriolar dilatation as the central alteration leading to oliguria and azotemia. Studies using B2 microglobulin have supported such interpretation and further suggest an absence of any significant obstructive element early in the course of development of the lesion. Studies wherein lissamine green dye is injected in tubule lumina offer strong evidence against an increase in passive back diffusion. Micropuncture studies utilizing intratubule pressure measurements and intravenous lissamine green dye infusion suggest the transepithelial movement of fluid (and solute) in a direction from interstitium toward lumen early in the course of the lesion. Such vascular alterations and pressure relationships would explain the consistent finding of tubule dilatation in this lesion in the presence of documented oliguria, azotemia, and low intratubular pressure. Further studies will require new and improved methods in order to 1) discern the critical stimulus for the initial vascular alteration 2) confirm the suspected pressure gradient relationship between lumen and interstitium.

Further evaluation of the possible role of renin release in initiating the vascular response will be possible only through contract with the laboratory of Dr. K. Thurau (University of Munich) which has been proposed as a joint protocol study under DA contract. Such studies would allow assessment of individual nephron renin release and vascular response in shock and acute renal failure, and are strongly urged as the single most important avenue of scientific exploration from the standpoint of military significance in this area of hemodynamic homeostatic mechanisms.

New methods are being developed for pressure measurements which should allow confirmation and denial of proposed intrarenal pressure relationships which may be intimately tied to pathogenesis and prevention of this lesion. Results of these studies to date have been presented to an international symposium on acute renal failure and has been published in book form (5).

b. Few investigations of the renal morphologic changes in ARI have been performed (6-10) and in these studies the techniques of collection and preservation of material may themselves have produced alterations in renal morphology. A recent study in Vietnam combat casualties was designed to obviate these artifactual changes and has been completed in patients both in the oliguric and diuretic phases of ARI (11). Light microscopy revealed collapse of a large number of proximal tubule lumina and mild to moderate dilatation of distal tubules, with focal degeneration

of tubule cells. Hyaline or pigmented casts were often contained in the distal tubule. Glomeruli appeared normal, but dilatation of peritubular vasculature was present. Interstitial changes, although occasionally present, were not prominent. These changes consisted of mild to moderate interstitial edema and round cell infiltration. Communications between distal tubules and peritubular capillaries were seen. Electronmicroscopy confirmed light microscopic findings and in addition showed normal proximal and distal tubules except for a cell or cluster of cells undergoing degeneration. Casts varied from homogeneous material to cellular debris. Tubule basement membranes were preserved. Glomerular changes were minimal and nonspecific. The only distinguishing feature between the oliguric and diuretic phases of ARI in these patients was the finding of distal tubule cellular regeneration in the diuretic phase. This study thus demonstrated that the renal cellular morphology does not reflect the severity of functional impairment in ARI.

c. The effect of heat stress and exercise on renal hemodynamics, distal tubular function (as estimated by urinary concentrating ability), urinary protein, cellular and cast excretion, body temperature, blood fibrinogen, and serum uric acid levels was studied in military recruits during summer training in an attempt to define the cause of acute renal failure in heat stress injuries. The high prevalence of dehydration and maximally concentrated urine in these recruits provide the most likely explanations for the summer occurrence of this form of acute renal failure. In the face of these predisposing factors any added insult of hypotension or myoglobinuria, or both, may precipitate acute oliguric renal failure. The improved systemic and renal hemodynamic responses to exercise, as well as the lower blood concentrations of fibrinogen, provide a possible explanation for the lower occurrence of acute renal failure in the later phases of basic training. This pathogenetic schema is obviously inferential and speculative but nevertheless provides a framework for considering such a potentially multifactorial problem. Additional studies are certainly necessary, and the well-defined physical and environmental stress of the recruit undergoing summer basic training provides a unique opportunity for such investigations (12).

d. A prospective study was conducted to evaluate the incidence and pathogenesis of rhabdomyolysis and renal failure in military recruits undergoing basic training. Within a two-year period, thirty-seven patients who developed severe muscle pain with laboratory evidence of muscle breakdown and dark urine during the first week of basic training

were evaluated. Data obtained so far suggests that this entity occurs year round with a higher incidence during hot, humid weather. Poor physical conditioning, dehydration, electrolyte depletion and inadequate acclimatization are commonly associated conditions. Continued muscle breakdown as evidenced by elevated serum muscle enzymes has been noted. Acute renal failure is probably caused by a combination of dehydration with hypotension, myoglobinuria, hypokalemia and/or other unrecognized factors.

e. The pathogenesis of acute renal failure has been investigated in the rat, using glycerol induced myohemoglobinuria, and dog, using parenteral uranium trinitrate (13,14). Renal renin depletion by chronic saline loading prevents the development of acute renal failure in both models. Depression of peripheral renin activity by renin immunization in the rat does not diminish renal renin content and is not associated with prevention of acute renal failure. Methods of functional inactivation of renal renin synthesis are being evaluated for their possible role in treating acute renal insufficiency.

f. A scientific exhibit which included a movie entitled, "Acute Renal Insufficiency in Viet Nam" was completed and shown at the Annual Convention of American College of Physicians in 1971 and is being presented at the American Medical Association Convention in June 1971 (15).

g. Renewed interest in the prevention and management of <u>falciparum</u> malaria has developed since the deployment of large numbers of U. S. military and civilian personnel in Southeast Asia. Malaria now represents the most significant cause of medical disability in this population. The high attack rate of falciparum malaria in Viet Nam has enabled us to collect the largest known series of cases of acute renal insufficiency secondary to this infection. During the four years between July 1965 and June 1969, forty patients with acute renal insufficiency due to falciparum malaria were cared for in three dialysis centers in the Asian War Theater. The pathogenesis, clinical features and management of these cases were reviewed. In addition two cases from the civilian community in the United States were studied. Twelve (29%) died, usually of respiratory failure or cerebral malaria. Delays in diagnosis and treatment were frequent in fatal cases. The establishment of a dialysis unit in Viet Nam and the development of an acceptable chemotherapeutic regimen decreased the initial mortality of 50% to the present 14%. The two patients from the civilian community died because of an inordinate delay in establishing the diagnosis of falciparum malaria.

h. The use of potentially nephrotoxic drugs, particularly antibiotics, in the post-surgical patient increases the risk of developing acute renal insufficiency with its excessive morbidity and mortality. In 1970 the Renal-Dialysis Service, Walter Reed General Hospital, treated eleven surgical patients with ARI requiring hemodialysis. In five patients (45%) the ARI followed the administration of nephrotoxic antibiotics during the perioperative period. These drugs included streptomycin, gentomycin, and kanamycin. In every instance the dosage of antibiotics was excessive for the existing state of renal function. Complications occurring after the development of ARI were uremia, sepsis, coagulation disorders, congestive heart failure, pericarditis, gastrointestinal bleeding, pulmonary embolism, decubitus ulcers, and nerve palsy. Despite the excessive morbidity, no deaths occured in this group. The average hospital stay was 83 days. Hemodialysis alone for this small number of patients cost in excess of $25,000. In conclusion, potentially nephrotoxic drugs should be employed only when necessary and in appropriate dosage. This retrospective study was presented to a major military surgeons meeting by the WRAIR Nephrology staff.

3. Acid-Base Homeostasis:

a. Studies were performed in Rhesus monkeys which determined the effect on chloride and extracellular acid-base parameters of selective potassium depletion. Previous work in dogs, however, suggest that chemical potassium depletion does not produce chloride resistant metabolic alkalosis. Clinical observations suggest that chronic potassium depletion does promote metabolic alkalosis in humans which is resistant to correction by the administration of chloride. Results of long term balance in four Rhesus monkeys support the contention that severe potassium depletion does initiate a sustained metabolic alkalosis in the face of adequate chloride intake.

b. Two chapters were prepared for a new Textbook of Medicine to be published by Grune and Stratton. The chapters were entitled "Physiology and Disorders of Hydrogen Ion Metabolism" and "Physiology and Disorders of Potassium Metabolism."

c. In addition, a section in Current Therapy, 1971, was prepared entitled "The Management of Electrolyte Disturbances in the Adult" (16).

4. Renal-Hemodynamic Studies:

a. An isolated perfused dog kidney has been developed for the purpose of studying a number of problems of particular interest to the Department of Nephrology.

Using difibrinated blood, to which is added various exogenous substrates, normal to supranormal blood flows are readily attained and maintained for periods of four or more hours. Low normal GFR values are achieved during the first two hours then gradually decline during the subsequent two hours to approximately 50% of control. Addition of a non-ionic emulsifier (Pluronic F68) to the perfusate has tended to increase GFR and maintain it at higher levels for a period of four hours. Presumably this emulsifier prevents or lessens glomerular microenbolization which is known to occur during extracorporeal circulation. In spite of the addition of substrates, aldosterone and vasopressin, osmotic U/P ratios rapidly decline after the first hour to below unity. Infusion of norepinephrine (102 mg/min) has prevented this ratio from falling below one (essentially isothermic) for up to four hours. More than 99% of the filtered Na+ load is usually reabsorbed during the first three hours, falling to 97 or 98% by the fourth hour. Arterial pCO_2 is maintained at approximately 28-32 mmHg; arterial blood pH ranges from 7.30 - 7.50. The energy cost of Na+ reabsorption in this isolated preparation is close to normal, 1 um O_2 being utilized for each 23 uEq Na+ reabsorbed. Occasionally, a small amount of glucose appears in the urine during the third or fourth hour.

In the study designed to examine the role of the renin-angiotensin system on renal hemodynamics and tubular function, plasma renin activity (PRA) was found to increase while renin substrate (angiotensin) usually declines over a period of several hours. Continuous administration of renin substrate does not alter hemodynamic or tubular function. Preliminary results also show a four-fold increase in angiotensin I suggesting depletion of converting enzyme. Studies are presently in progress to determine whether the addition of converting enzyme to the system will increase the plasma level of angiotensin II (reducing angiotensin I) thereby altering hemodynamic or tubular functions. The effect of catechalamines on PRA, in this denervated kidney preparation will also be examined.

b. Control data have been collected on the normal dog kidney through as long as six hours of in vitro perfusion. These data have indicated that although there is a slow diuresis in the absorption of sodium and a gradual loss of the ability to concentrate after four hours there is no morphological evidence of cellular degeneration. Preliminary studies have begun on the effects of anoxia in this system. After three hours of low blood O_2 saturation there is a shuntage of proximal tubular epithelium which correlate with a loss in the capacity to absorb sodium. Again evidence of frank necrosis is surprisingly lacking indicating a high degree of resistance to lethal injury secondary to inhibition of oxidative phosphorylation.

c. Renal hemodynamics have been evaluated using radioxenon washout and radioactive microspheres. Using these techniques, the pattern of renal blood flow and intrarenal distribution has been characterized. In a model of reproducible experimental acute renal failure in the dog, we have characterized the renal hemodynamics. Results indicate a primary alteration in blood flow in acute renal failure. Studies of vasoactive compounds capable of treating acute renal failure by normalizing renal hemodynamics are planned.

5. Cellular Transport:

a. The cerebrovascular response to malarial infection in the syrian hamster has been studied. The brains of 18 infected hamsters have been reviewed by light and electron microscopy similarities and differences between the hamster and the human lesion have been delineated. Both lesions have the same fusiform to spherical shape and distribution in the cortical white matter. The human lesion is characterized by more thrombosis and the presence of clear perivascular areas. Thrombosis in the hamster is infrequent and perivascular clear areas are absent. The hamster lesion is characterized by the presence of numerous circulating mononuclear cells many of which became adherent to endotehlial walls. Granular leukocytes do not particpate in the development of the hamster lesion, there is no evidence of necrosis and only moderate glial reaction. The findings are compatible with the pressure-extrusion hypothesis of development of the perivascular hemorrhages, and suggest a difference between the anatomical location of the blood brain barrier in humans and hamster. These findings were presented at the annual meeting of the American Association of Pathologists and Bacteriologists (17). At present, the effects of immunosuppressive agents on the hamster lesion are being considered for study, to determine the relationship between antibodies and both endothelial adherence and increased capillary permeability.

b. Studies have been performed evaluating the effects of uremia on erythrocyte metabolism. Measurements have been made of substrate levels, rates of lactate production, enzyme activity and kinetics in erythrocyte from patients with chronic renal disease and compared to control cells. Results have shown that the erythrocytes from patients with chronic renal disease are hypermetabolic with respect to lactate production. Enzyme activities of hexokinase, phosphofructokinase and pyruvic kinase are measured and found to be consistently elevated in the uremic erythrocytes. Kinetic studies have revealed that only the Vmax but not the Km were increased for these enzymes in the uremic cells. Levels of 2-3 diphosphoglyceric acid and ATP were elevated in the erythrocytes from patients with renal disease. These studies suggest that the average age of the erythrocyte is younger than would be expected in a control population and radioactive chromium and iron studies are currently underway to evaluate this as a possible explanation for the biochemical data.

6. Chronic Renal Disease:

a. The efficacy of protein restriction in patients with impaired renal function (creatinine clearance 5-15 cc/min.) is well known. However, when patients require hemodialysis for maintenance, the proper amount of protein required is less clear. Using nitrogen balance techniques, three diets, 0.75 g, 1.0 g and 1.25 g protein per kg of body weight were evaluated on patients undergoing three times weekly hemodialysis with the twin coil artificial kidney. Results to date show that 1.25 g of protein/kg of body weight is the optimum amount of protein producing positive nitrogen balance without significant increases in uremic symptoms and predialysis chemistries.

b. Clinical records and renal biopsy findings by light, electron, and fluorescent microscopy from twenty-six patients with so-called "benign" or "idiopathic recurrent hematuria" have been reviewed. Fifteen of these patients had electron microscopic evaluations of their renal biopsies and three had fluorescence microscopic evaluations.

This study was performed to observe if significant pathology would be demonstrable in these patients glomeruli and if any further information as to etiology of this disorder could be obtainable from these renal specimens examined by fluorescence and electron microscopy. Preliminary findings indicate that renal morphologic changes were most often not present or of a focal, non-specific nature by light microscopy. However, in a small percentage of patients moderately severe or significant renal lesions were present by light microscopy. Electron microscopy and fluorescent microscopy did not provide evidence for an etiology of this syndrome.

7. Dialysis:

a. Experimental evaluation of various oils for use in lipid dialysis of pentobarbital was performed. The lipid:water partition coefficient for corn oil was established to be 5.5. With the cellophane membrane, it was 5.6. These studies indicate that the lipid:water partition coefficient for pentobarbital was far less than the usual cited value of 39 and that a cellophane membrane is not a barrier to diffusion of pentobarbital. Further studies comparing aqueous and lipid dialysis of pentobarbital and glutethemide showed no advantage for the use of lipid dialysis (18).

b. In vivo and in vitro clearance measurements were performed to study the efficacy of plasma hemodialysis utilizing the Cell Separator and a Twin-Coil Artificial Kidney. Plasma dialysis showed a markedly increased ultrafiltration rate but no increased dialysance when compared to standard hemodialysis. Methods to improve dialysance are being sought.

c. Several patients with uremia complicated by glucose dysmetabolism were studied in an attempt to elicit the cause of their altered glucose metabolism. The data accumulated to date suggests that the type of circulating insulin in these individuals may be ineffective in promoting glucose transport across the cell membrane. Intensive dialysis reverses this metabolic abnormality.

8. Immunopathology:

a. The effect of splenectomy upon suppression of tetanus antitoxin production induced by methylprednisolone, chlorambucil, azathioprine and radiation was evaluated. Splenectomy was found to potentiate the immunosuppressive agents listed.

b. Allograft rejection and humoral antibody production in intact and splenectomized mice was evaluated. Immunosuppressive agents administered in combinations suppressed antibody synthesis and prolonged allograft survival in similar proportions of intact and splenectomized mice; depression of the two immune responses being positively correlated in individual mice.

c. Effect of high dose combination immunosuppressive agents upon the progression of murine lupus of NZB/W mice was evaluated. Proteinuria, anti-DNA antibody levels, and renal involvement were significantly less in single-agent-treated vs controls, double-agent-treated vs single-agent-treated, and triple-agent-treated vs double-agent-treated NZB/W mice; however, toxic deaths were significantly more numerous in triple-agent-treated mice.

d. Effect of low-dose combination immunosuppressive agents upon progression of murine lupus of NZB/W mice is being evaluated. In this study, toxicity has been averted and at 7 months (after 2 months of treatment) all controls (untreated) have proteinuria; however, 60% of single-treated, 47% of double-treated and 30% of triple-treated mice have proteinuria. These mice will be followed for survival.

e. The brains of NZB/W mice after 2 months of treatment with none, one, two or three immunosuppressive agents are being examined to evaluate the degree of immune complex deposition in the choroid plexes.

f. Young and old control Swiss or DBA/2N mice demonstrate prolongation of skin allograft survival after splenectomy. Young NZB/W (1 month old) mice also demonstrate prolonged graft survival, however, 8 month old NZB/W mice (after the development of murine lupus) were found no longer to demonstrate skin graft prolongation after splenectomy. The basis of this effect is being investigated by repopulation of old NZB/W mice with spleen cells from young NAB/W mice.

g. The sera from patients with renal cortical necrosis (RCN) were collected and examined for cytotoxic antibodies. Anti-platelet antibodies were demonstrated by a Factor III liberation technique in 9 of 11 sera from patients with RCN, and 0 of 9 matched control sera.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089, Body fluid and solute and renal homeostasis

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PROJECT 3A061102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 03
Psychiatry

642

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6454	71 07 01	DD-DR&E(AR)R 16

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
70 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	61102A	3A061102B71R	03	025
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code): (U) Analysis and Management of Behavior and Stress in Military Environments (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 013400 Psychology 012600 Pharmacology 012900 Physiology 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
63 08	Cont.	DA		C. In-house

17. CONTRACT/GRANT: Not Applicable
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 71	6.5	185
CURRENT 72	7.5	

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Division of Neuropsychiatry
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
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21. GENERAL USE
Foreign Intelligence Not Considered.

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Operant Behavior; (U) Military Psychiatry; (U) Drug Abuse; (U) Motivation; (U) Conditioning; (U) Performance Decrement; (U) Reinforcement.

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

23. (U) Complex behavioral models are developed simulating conditions likely to lead to ineffective performance or psychiatric decompensation in the military environment. The interaction of physiological and psychological variables relevant to the individual environment interaction are studied (e.g. the behavioral toxicity of therapeutic and non-therapeutic drugs, the effects of stress and fatigue upon alertness and performance, the interaction of autonomic and environmental variables, and updating of operant technology as it applies to the modification of behavior).

24. (U) The techniques of contemporary experimental psychology, particularly operant conditioning, are combined with those of neuroendocrinology, pharmacology, physiology, and anatomy to precisely define the variables that maintain and control both adaptive performance and behavioral dysfunction.

25. (U) 70 07-71 06 Animal studies of the behavioral toxicity of synthetic marihuana have demonstrated reductions in work output, decrements in tasks requiring timing behavior, decreased aggression, decrements in avoidance performance, and if administered daily, weight loss and attenuation of growth. Effects lasting up to 72 hours following a single administration have been documented. Studies of long transmeridian air deployment indicated a decrement in work rate and accuracy of performance. Studies of cardiovascular effects of presentation of stress-related stimuli indicated that on-going performance changes are correlated with changes in autonomic function. Improved techniques for evaluating behavioral effects of therapeutic and non-therapeutic drug use are under development. Pilot research on patterns of drug abuse at a major Army post has been completed and more systematic studies are being implemented. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

643

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

Project 3A0611D2871R RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 025, Analysis and management of behavior and stress

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Research during the reporting period has focused on four relatively distinct areas of concern: (1) The development and utilization of laboratory models of stress and psychosomatic disease; (2) Application of behavioral technology to problems in military medicine, especially military psychiatry; (3) Evaluation of the behavioral toxicity of non-therapeutic drug use with objective laboratory models; (4) Biological effects of microwave exposures.

LABORATORY MODELS OF STRESS AND PSYCHOSOMATIC DISEASE.

Systematic environmental perturbations have been shown to produce disruptions of on-going behavior as well as changes in patterns of visceral function. With repeated exposure to environmental perturbations, these disruptions of both behavior and physiology can be elicited by more subtle components of the original eliciting events and may represent the kinds of changes which take place early in the etiology of pathological responses to stress as well as psychosomatic disorders. The military environment, of necessity, presents the organism with the kinds of environmental contingencies which can lead to the relatively permanent behavioral and visceral changes representative of those involved in psychiatric decompensation and psychosomatic disorders. The studies reported in this section are directed at identifying classes of relevant variables and developing treatment models that can capitalize on the same behavioral principles responsible for the disorder.

Concomitant modifications of cardiovascular variables and behavior. Several experiments directed at the effect of environmental manipulations, known to produce perturbations in on-going behavior, on cardiovascular dynamics are in progress. Primates have been instrumented for continuous long-term monitoring of arterial blood pressure and heart rate. Pavlovian conditioning procedures have been used to elicit cardiovascular changes. Because the Pavlovian conditioning has been superimposed upon on-going operant performance it has been possible to simultaneously identify both disruptions in behavior and perturbations in cardiovascular dynamics. Reliable changes in arterial blood pressure

up to 40 mm Hg as well as 20-30 BPM changes in heart rate have been produced by conditioning procedures alone. By manipulating the character of the on-going behavior, that is, the behavioral context in which the cardiovascular changes are elicited, it has been possible to modify the outcome of the cardiovascular conditioning. It has been possible to obtain increases and decreases in arterial blood pressure, both with and without changes in heart rate, simply by changing the on-going behavioral context. These data point up the complexity of the visceral changes that accompany overt behavior and represent an initial step toward an objective analysis of the interaction between these two broad classes of events.

Omission of reward as a frustrative stress. Laboratory models of stress have produced advances in understanding the neuronal and hormonal mechanisms at work in the individual under stress. Most of these models have employed such potent stressors as fatigue, cold, heat, or electric shock. While none of these are without psychological consequences, other forms of stress, more psychological in nature, have seldom been employed with a view toward characterizing their physiological consequences for the organism. One such stressor, frustration, operationally defined as the omission of an expected reward, is being studied. The model being used requires that subjects be trained on schedules of reinforcement which lead the organism to behaviorally anticipate the delivery of reward and then to omit the expected reward. The lesion method has been used to document differences in the neuronal systems mediating the behavioral changes which take place in response to this frustrative stress. As with other stressors, the limbic system is involved in mediating such responses; however, it has been demonstrated that this more psychological stress is not handled by the central nervous system in the same way as are the more physiological stressors. These data appear to suggest that therapeutic measures designed for coping with stress-related behavioral phenomena may not be universally effective for all forms of stress. It may be necessary to consider the nature of the stressor as well as the overt symptoms of stress-related behavior disorders before prescribing courses of treatment involving drugs with CNS effects.

APPLICATION OF BEHAVIORAL TECHNOLOGY TO PROBLEMS IN MILITARY MEDICINE AND PSYCHIATRY.

The behavioral technology developed within the framework of the work unit has been applied to several problems of military medicine and psychiatry.

Effects of transmeridian air-deployment upon the performance of military personnel. A study is underway to confirm pilot data indicating that air deployment of personnel across long distances in an East-West direction produces psychological and physiological changes, known as transmeridian desynchronization. These changes result in temporary perfor-

mance decrements in tasks requiring sustained attention. Pilot data are now being collected from human subjects following direct flights from Washington, D.C., to Thailand and Japan and return. Initial plans call for selected WRAIR personnel, already required to travel long distances to complete assigned missions, to also serve as volunteer subjects in the study. Portable laboratory equipment has been developed for this purpose. Although the study has relevance to the performance of all military personnel after geographical relocation, assessment of performance decrements in medical personnel (e.g. surgeons) is of special interest. The potential relevance of these data to the geographical relocation of patients is also being evaluated.

The effects of behavioral stress upon the morphology of the autonomic nervous system of non-human primates. Following completion of a study that demonstrated changes in the morphology of the autonomic nervous system of rats as a result of exposure to an acute stress procedure, an attempt was made to identify similar changes in non-human primates. Rhesus monkeys have been exposed to 72-hour avoidance and sacrificed following administration of radioactive-labelled thymidine. The data on the first two groups of animals indicate that acute stress does modify the structure of the autonomic nervous system and that the effect is most pronounced in those animals required to emit responses to avoid electric shocks. Electric shock alone, with no avoidance requirement, produces a less pronounced effect. Preliminary analysis of urine and serum endocrine measures suggests that the morphological response may be mediated by hormonal changes resulting from exposure to the stress procedure. These data bear directly on the problem of identifying the physiological consequences of exposure to stress and to the development of a model for interpreting the failure of individuals to successfully adapt to the acute stresses encountered by military personnel. The identification of morphological changes in the autonomic nervous system of non-human primates exposed to acute psychological stress raised questions as to the effect of prolonged chronic stress on the structure and function of the nervous system. Rhesus monkeys have now been exposed to repeated 72-hour avoidance sessions. Following ten weeks of stress exposure, the morphology of their nervous systems is being examined using autoradiographic techniques. Data resulting from these studies may contribute substantially to our understanding of the anatomical and physiological mechanisms underlying the behavioral breakdown of military personnel exposed to chronic psychological stress.

Effects of anti-malarial compounds on visual vigilance performance. Because certain anti-malarial compounds are known to produce significant retinopathy, a procedure was developed to assess whether these compounds produce decrements in visual performance, which might appear even before any retinopathic changes were observable. A combination of three anti-malarial drugs (chloroquine, primaquine, and DFO) were administered to two rhesus monkeys via intubation to produce elevated methemoglobin

levels. The purpose of the study was to investigate the possible deleterious effects that these anti-malarial drugs might have on a visual vigilance task due to elevated methemoglobin levels. Methemoglobin levels were raised to between 5-10% in the two monkeys with no systematic change in vigilance performance. This study suggests that elevations of methemoglobin levels to 10% above baseline levels induced through chronic administration of a combination of three anti-malarial drugs have no effect on visual vigilance performance. These data strongly suggest that no significant reduction in visual performance is associated with the routine use of these compounds.

Effects of anti-malarial compounds on performance decrements produced by alcohol. Because certain anti-malarial compounds are known to interfere with enzymes important in the metabolism of alcohol, pilot work has been initiated to evaluate the duration and magnitude of behavioral deficits produced by alcohol. This baseline will then be used to determine whether anti-malarials alter the effect of alcohol on behavior. These data may be useful in extending the notion of the pharmacologic toxicity of compounds to include behavioral toxicity as well.

Delivery of mental health services: The behavior of a role innovator. Behavioral technology is being applied to problems of describing the behavior of an innovative chief of a Mental Hygiene Consultation Service and to assessing the concomitant changes in the behavior of the clinical staff, command elements on the post, and personnel responsible for delivering mental health services. The purpose of a behavioral approach to these problems is to develop an objective set of measures for improving the delivery of mental health care. Analysis of the data is not yet complete.

Development of the methodology for determining illegal drug use patterns at a large Army post. The purpose of this study is to find reliable, efficient, and objective behavioral methods for determining prevalence rates of drug use and abuse. Methods being evaluated currently include questionnaire administration in large groups, questionnaire administration in small groups combined with group discussion, anonymous ballot questionnaires, and observers' ratings based on relevant variables. Preliminary indications are that the small group questionnaire, combined with discussion, offers the greatest validity, but at prohibitive costs in terms of personnel.

Development of the methodology to determine the variables that initiate and maintain drug-taking behavior in the Army. All currently available measures for determining prevalence rates for drug abuse are being evaluated in terms of their suitability as instruments for obtaining data on the variables underlying the problem. The study is in the exploratory stage; however, preliminary data indicate that techniques for measuring only prevalence will be inadequate for this purpose.

The use of enlisted mental health personnel as drug abuse counselors. The study was designed to develop an objective data base related to the suitability of enlisted mental health personnel as drug abuse counselors. Primary focus has been on knowledge of drugs and their effects, personal history of drug abuse, and interest in functioning as drug abuse counselors. The entire enlisted staff of the MHCS at Ft. Meade has been interviewed for this purpose, as well as all of the senior NCO personnel being used to establish drug counselling facilities in RVN for returnees to CONUS. Although very preliminary, the data appear to indicate that special intensive training will be required before MHCS personnel can deal with problems of drug abuse on a routine basis.

EVALUATION OF THE BEHAVIORAL TOXICITY OF NON-THERAPEUTIC DRUG USE WITH OBJECTIVE LABORATORY MODELS.

Primary emphasis has been focused upon the behavioral toxicity of marihuana use. This decision was made because reports of the widespread use of this drug, both in the military and in that segment of the civilian sector likely to be inducted into the military, and the lack of a technology for the detection of marihuana use by biochemical means, appeared to indicate that marihuana abuse is likely to be a continuing source of concern for the Army. In summary, the studies in progress have all employed delta-9-tetrahydrocannabinol (THC), the principal active agent in marihuana. At effective dose levels, all studies have demonstrated a drop in work output, even on very simple tasks requiring very little effort. No evidence has been found of a reverse tolerance of the kind reported by human users. All laboratory studies have indicated that behavioral impairments were most severe with the initial administration and appeared to attenuate with repeated administration. The exception to this was a study in which food intake was found to decrease, and was sufficiently reduced to prevent any weight gain, throughout a 30 day chronic administration period. These data were obtained from young rats that should have gained substantial weight during the administration period. The other generally confirmed observation was disruption of timing behavior, with the subjects overestimating the passage of time. Individual studies in progress or completed are briefly described below.

Effects of THC on behavior maintained by behavioral stress. Shock avoidance procedures have been used with both primates and rats to determine the effects of THC on performance maintained by this stress procedure. The data are in agreement that moderate doses of THC impair shock avoidance performance. This decrement in avoidance performance is unequivocal in primates, but some rats actually show improved performance. The rat data are not definitive because the intraperitoneal route of administration was used. In this same study, it was found that rats administered THC intraperitoneally developed an acute irritative peritonitis of sufficient severity to result in death. The use of appropriate controls indicates that it was the combination of stress and THC that resulted in death, THC alone producing a less severe peritonitis from which non-

stressed rats recover. The presence of peritonitis served to confound the performance data in the rat studies; however, additional studies using oral administration, appear to confirm the primate data.

Conditioned fear following administration of THC. A conditioned suppression procedure, widely accepted as a laboratory model for the study of the effects of psychoactive compounds on fear, has indicated the possibility of increased fear in primates administered THC. These data are to be regarded cautiously, however, since no confirmation could be obtained in the rat and since the suppression of on-going behavior used as an index of fear may have been the direct result of the THC alone. In this study, as well as others, THC has proven extremely difficult to study with traditional psychopharmacological methods because the drug interferes with on-going performance in such a way as to make observations of more specific effects very difficult.

THC as a discriminatory cue. The dose levels of THC required to produce behavioral effects in animals, with the exception of the chimpanzee, are high, relative to those used by humans. The question has arisen as to the dose level at which an animal can recognize the presence of THC. The present study was designed to answer the question of what minimum dose could be discriminated from a placebo. When a monkey is working for food and a stimulus is presented indicating that the animal is about to receive an electric shock, there is an abrupt disruption of on-going performance. In the present study, placebo, as well as varying doses of THC, is administered intrvenously via chronic venous catheters to monkeys. Placebos never signal that shock is to be presented, but an administration of THC is always followed two minutes later by an electric shock. If an animal can discriminate the difference between placebo and a given dose of THC, then it can anticipate the presentation of shock, and the characteristic anticipatory disruption of on-going behavior can be observed. To date, data from two animals indicate that doses lower than those capable of producing decrements in performance are not discriminated, suggesting a close correspondence between the doses perceived by an organism and those that impair its performance. These data suggest that the higher doses of THC required to produce behavioral effects in laboratory animals are similar to those required to produce any sort of experiential effect.

THC and response effort requirement. Because the most typical finding following THC administration has been a failure of animals to work, a study was undertaken to determine whether the amount of work was a factor in determining the effect of the drug. THC was given by mouth to monkeys trained on a multiple ratio schedule requiring different numbers of responses to obtain food in the presence of different visual stimuli. In a series of five daily administrations of 4 mg/kg, no differential drug effects were associated with the different effort requirements.

Effect of THC on work rate, accuracy of performance, and timing behavior in chimpanzees. Three chimpanzees were trained on a task that required precise timing behavior as well as sustained performance for nearly 20 hours each day in order to obtain their daily ration of food. A dose response curve for orally administered THC was obtained for each animal. The data indicated that doses of THC comparable to those reported effective in humans resulted in overestimation of time and reductions in work output. At higher doses, effects lasted nearly three days, and at lower doses, nearly 24 hours were required to recover performance. It was also noted that after several administrations of THC given in a volume of .2cc injected into several orange slices, the animals refused to accept orange slices and would not eat oranges even when they contained no THC. This observation suggested that THC might have aversive properties and a study, described below, was conducted to verify this hypothesis.

THC and conditioned saccharine aversion. The fact that chimpanzees learned to refuse a preferred food when used to administer THC suggested that THC might have unpleasant or even toxic properties for the animal. To confirm this, advantage was taken of a naturally occurring phenomenon in rats. If rats are administered a treatment, such as poison or heavy dose of radiation, which makes them ill, they subsequently avoid any novel substance they may have been eating before becoming sick. In the present experiments rats were given a single dose of THC immediately after their first exposure to a saccharine solution, a solution usually preferred over tap water. In tests given 47 hours after THC administration, a dose-related reversal of the rats' normal preference for saccharine was found. These data support earlier informal observations, that THC may have unpleasant, or toxic properties for laboratory animals.

THC and duration discrimination in monkeys. Because of the accumulating evidence of an effect on temporal discrimination, THC was given by mouth both acutely in a wide dose range, or chronically, to monkeys working on a procedure requiring different responses to stimuli of different durations. In the acute experiment, all measures of behavior including accuracy, rate of work, and response bias showed dose-related decrements, with the lowest effective dose being about 4 mg/kg. In the chronic experiment, rate of work recovered after several daily administrations of high doses, and accuracy recovered much more slowly or not at all. The effect of high doses was characterized by a transient decrement in accuracy followed by complete cessation of response.

THC and flash rate discrimination in rats. Rats have been trained to respond differentially to lights flashing at different frequencies. Within the next month, several chronic drug administrations at several different dose levels will be begun. The data from this experiment will determine the generality of previous experiments with monkeys and pigeons, and different modalities on the effect of THC on various measures of timing behavior.

THC and timing behavior in rats. In this study, rats are being trained on a procedure conceptually similar to that in the chimpanzee study cited above. That study showed effects of THC well within the human dose range, while other studies with rats and monkeys have required much higher doses to show any effect. This study, with a baseline similar to that in the chimp study, should provide a definitive answer as to whether the difference in dose-response effects is due to species or to procedural differences.

Effects of THC on sensory capabilities. A number of studies on the sensory capabilities of several species of laboratory animals have been completed or are nearing termination. THC was given by mouth daily for periods of about two weeks to monkeys working on an auditory discrimination (click frequency) procedure. Doses of 2 to 4 mg/kg initially depressed both work rate and accuracy. Work rate recovered in the course of the chronic administration of the drug, but accuracy did not. In addition, urine samples were collected from these animals for use in preliminary attempts by the Division of Biochemistry, WRAIR, to detect metabolites of THC in the urine, with the long-term goal of development of a screening test for marihuana use. Pigeons run on intensity and frequency difference threshold paradigms were given oral doses of THC. Dose response curves were established for the drug. The purpose of the study was to investigate the possibility that the drug has deleterious effects on sensory thresholds as well as the more complex discriminations described above. To date, the data suggest that pigeons run on these auditory discrimination problems motivated by appetitive consequences (food) stop working under the influence of the drug. The length of the work stoppage is functionally related to the dose of THC administered. The auditory thresholds for all practical purposes are not affected.

THC and aggression. Two recently completed studies have dealt with the relationship between aggression and THC. In one of these, fighting was elicited in pairs of rats by applying pulses of electric shock to the grid floor of a small chamber. The probability of eliciting such fighting was unchanged by injections of THC. Manipulations of dose (64 mg/kg to 6400 mg/kg), previous drug history, vehicle (vegetable oil or propylene glycol), and time elapsing between injection and test (0.5 to 2 hours) were all ineffective in altering this finding. In contrast, chlordiazepoxide reliably decreased the probability of such fighting, with the magnitude of the decrease being directly related to dose level. The second study in this area assessed the effects of THC on a different type of aggressive behavior, i.e., spontaneous interspecific aggression. In this situation, doses of THC which failed to alter shock-elicited fighting reliably depressed the incidence of attack. Initially, it would appear then that the relationship between THC and aggression is a complex one, highly dependent upon the circumstances surrounding the type of aggression in question.

THC and frustration. The effects of THC were also studied using a very different type of stress from electric shock, but which is very common, i.e., frustration. Operationally, this involved training rats to lever press for food pellets delivered at intervals of one minute, contingent on a press. Prominent exteroceptive stimuli also accompanied the delivery of each pellet. After substantial training on this schedule, a probability factor was introduced, so that 25% of the time no pellets accompanied the exteroceptive stimuli usually associated with food. Rats reliably pressed twice as fast following this "frustration" than following receipt of a pellet. Injections of 4 mg/kg THC produced a slight enhancement of this effect, though a drastic reduction in work rate was by far the most prominent effect. Lower doses produced no consistent effects.

Effects of chronic administration of THC on food intake and body weight. One of the THC investigations completed was a study of body weight, food and water intake during and after an extended period of THC administration. Very little is known about the long-term effects of the drug, so rats were compared receiving daily i.p. (4 mg/kg) or oral (8 mg/kg) doses with appropriate placebo controls. While the latter gained weight at a steady rate throughout the experiment, the THC animals failed to gain at all during the thirty days of drug administration. Upon cessation of drug administration they began to gain weight again, but had not reached the weight of the control animals after thirty days without THC. This failure to gain weight appeared to be primarily due to a decrease in food intake rather than post-ingestional changes. Subsequent work has shown this finding to be dose-related, at least in the range of 0.5 to 32 mg/kg. In addition, all rats given intraperitoneal injections of THC, and only these, showed evidence at necropsy of having suffered moderate to severe irritative peritonitis. Both of these findings (reduced food intake, and peritonitis) have major methodological implications for all THC investigators.

BIOLOGICAL EFFECTS OF MICROWAVE EXPOSURES.

During the reporting period an entire laboratory has been completed and staff selected to investigate the biological effects of low-level microwave exposures. The bulk of the last year has been devoted to completing the physical alterations so research can begin. Several studies have been completed. These clearly indicate that exposure to levels at or below 20 mw/cm^2 can influence cell growth in tissue systems with high cellular turnover. Consistent with this observation is the finding that proliferative tissues of the fetus are also sensitive to exposures of less than 20 mw/cm^2. Behavioral, neurophysiological, and neurochemical studies are underway to determine, across a wide spectrum, the mediating mechanisms and their potential hazards, if any. Emphasis is being placed upon wavelength and other physical characteristics of exposure parameters which are of concern to operational Army radar systems.

Project 3A061102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 025, Analysis and management of behavior and stress

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PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01
Biochemistry

655

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

	DA 0A 6430	71 07 01					
70 07 01	D. C. Angel	E	H	BA	NL	Yes	No
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	CAOC 14122(2)						

(U) Biochemical Variations during Disease and Treatment (39)

012900 Physiology 002300 Biochemistry

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XA					
			71	13	195
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(U) Membranes

(U) Protein Synthesis; (U) Enzymes; (U) Genetic Interrelationships; (U) Gene Isolation

23. (U)The technical objective of this work unit is to provide a program of differentiating the response between the host and infecting organism of those diseases of military medical importance. Particular emphasis is paid to the nucleic acids, lipids, enzyme response and their interaction at the molecular and organ level.

24. (U)Macromolecular separations and characterizations, sequential analysis of nucleotide components and lipid layer separations. Isotopic precursor incorporation and degradation will be used to study host infecting organism interrelationships. Membrane studies employing spin labels are being conducted. The mechanism of hydrogen equivalent transfer across mitochondrial membranes is being elucidated.

25. (U) 70 07 - 71 06 All the major Enterobacteriaceae groups have been studied for their nucleic acid relatedness to strains of Escherichia coli. Pathogenic and non-pathogenic strains of an individual genera may be grouped on the basis of their nucleic acid sequence relatedness as the pathogens exhibit less divergence than the nonpathogens. A series of technologies for the separation of single stranded and double stranded DNA as well as DNA/RNA hybrids from hydroxyapatite has been completed. These technologies have led to improvement and standardization of either small or large batch preparation of either RNA or DNA. Studies with purified tRNA genes have failed to show homology between Escherichia coli, 5s or ribosomal RNA or yeast and rat liver tRNA. Using labelled tRNA and hybridization with pure genes, it has been shown that there are 3-4 cistrons per DNA fragment and further that tRNA genes are mostly contiguous. The mechanism of hydrogen equivalent transfer across liver mitochondrial membranes is being elucidated. Spin labeling of erythrocyte ghosts has been accomplished using various spin labels. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE AND WILL NOT BE USED AFTER JUNE 1968

PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical variations during disease and treatment

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DESCRIPTION.

The fundamental objective of this work unit is to investigate diseases at the molecular level. At the same time consultation on technical and investigative levels is also provided to other units. Specifically, this involves the study of variation in cellular processes caused by disease in terms of molecular biology, biochemical genetics, physico-chemical and structural studies of macromolecules using disease causing agents such as bacteria, viruses and mammalian cells. Detailed studies have been pursued to understand:

1. The mechanism of cellular protein synthesis.

2. The role of nucleic acids in this process and the mechanism of replication, transcription, translation and transfer of the genetic information.

3. The homology and divergence of nucleotide sequences in various species of bacterial DNA and its correlation to the pathogenesis of these species. This in turn will provide basic knowledge about the host-parasite relationship at the genetic level.

4. Interaction of antimetabolites with the macromolecules such as proteins, nucleic acids and the enzymes.

5. Isolation and *in vitro* characterization of structural and functional genes.

6. The effects of nutritional variations on the macromolecular functions in health and diseases.

7. Interaction of small molecules and ions with biological membranes.

8. The effect of alcohol on the mechanism of transfer of hydrogen equivalents across liver mitochondrial membranes.

9. Studies on the mechanism of radioprotection.

10. Studies on phototoxicity.

PROGRESS.

Since the first successful attempts last year in isolating the tRNA genes from E. coli, major efforts were concentrated in improving this technique and extending it for the isolation of other structural genes. Both these efforts were successful and the 5s RNA gene has been isolated and the method improved for the isolation of tRNA genes. A wealth of information was obtained regarding the character of tRNA genes in E. coli and its relatedness with other species. This advancement has also made it possible to characterize the gene products. Nucleic acid relationship studies among the Enterobacteriaceae have been extended with particular emphasis on the pathogenic E. coli strains and the differentiation of pathogenic Klebsiella species from the non-pathogenic Aerobacter species.

1. Nucleic Acid Relationship Studies.

Relationships of all main groups of enterobacteria to three strains of E. coli, two species of phytopathogenic Erwinia, Proteus mirabilis, Klebsiella pneumoniae and Enterobacter hafniae. Further detailed relationships have been determined among several dozen E. coli strains, Alkalescens-Dispar strains and Shigella strains; between Erwinia species, and between members of the Klebsiella-Aerobacter-Enterobacter genera. Both closely and distantly related nucleotide base sequences are assayed by varying the stringency of reassociation conditions so that only highly complementary or both highly and loosely complementary nucleotide sequences can form stable duplexes. By determining the stability of duplexes formed between species relative to the stability of duplexes formed from DNA of the same species one can assay the amount of divergence in related nucleotide sequences.

As reported previously, E. coli strains have diverged to a point where as much as 25-30% of their DNA is no longer capable of reassociating at stringent incubation conditions. All Alkalescens-Dispar strains tested and all Shigella species fall within this range. These closely related organisms, all of which are pathogenic or potentially pathogenic could well be placed within a single species. Frits Orskov, at the Staaten Serum Institute in Copenhagen, Denmar, has been able to place E. coli strains into three groups with respect to migration of "O" antigens in immunoelectrophoresis. He finds, with few exceptions, that there are three patterns of antigen mobility and that each pattern correlated well with the type of infection that the strain causes. His groups differentiate between E. coli strains

responsible for infant diarrhea, dysentery-like diseases and general parenteral infections. A collaboration has been started with Dr. Orskov in order to determine the gross DNA homology in groups of strains from each of the groups he has described. Thus far somewhat different patterns have been found than those observed with antigen mobility. It is clear that the E. coli strains within any group are significantly more related than are strains compared from different groups. We have verified and extended our results with strains from our own collection which fall into one of Dr. Orskov's groups. It is particularly important to be able to differentiate pathogenic from resident normal flora in an organism as ubiquitous as E. coli. It is hoped that the data obtained in these studies will aid the clinician in this regard.

In the course of carrying out reactions with E. coli strains of diverse origin, it was noted that several of the strains appeared to have significantly different genome sizes. This fact was subsequently confirmed by Dr. DeLey and his associates in Belgium. It is now known that E. coli strains differ in genome size by at least 25% and that at least a 40% range in genome size exists among the Enterobacteriaceae. All relatedness values obtained in the past, both in this laboratory and other laboratories, assumed that all organisms tested have the same genome size. This assumption is not valid and corrections for molecular weight must be made, especially if closely related organisms are tested or if the genome sizes are significantly different.

Results obtained from interspecies reassociation reactions with labeled DNA from Er. caratovora and Er. dissolvens indicates that the Erwineae are a diverse group of organisms; in some cases sharing less than 15% of their DNA under optimal conditions and less than 3% under stringent conditions. The Erwineae share as much as 1/3 of their genome with "true"enteropathogens. The Erwineae share as much as 1/3 of their genome with "true" enteropathogens such as Sh. flexneri and E. coli. These phytopathogens may be divided into two groups based on the type of disease they produce. The data indicate that considerable diversity exists in both groups. A detailed examination of these organisms will probably point up at least four groups and may further our knowledge of phytopathogenicity.

Differentiation of Klebsiella pneumoniae strains from the nonpathogenic Aerobacter aerogenes or Enterobacter aerogenes strains is a difficult task for the clinical bacteriology laboratory. Studies show that these organisms are only 50% related and that only about 15% of their DNA reassociates at stringent incubation temperatures. All Klebsiella species formed extensive and stable interspecies duplexes, whereas the Enterobacter species showed several levels of divergence. Ent. aerogenes and Ent. cloacae strains are closely related, while Ent. hafniae, and Ent. liquifaciens are only distantly related to Ent. aerobacter and to each other. These findings again emphasize the disparity in relatedness observed in pathogenic organisms as opposed to nonpathogens. The pathogens always show less divergence than the nonpathogenic organisms.

2. Methodological Advances in Separation of Single- and Double-Stranded DNA by Hydroxyapatite Chromatography.

During the past year, studies were continued to improve the techniques of separating single- and double-stranded DNA and DNA/RNA hybrid preparations from hydroxyapatite and with alternative methods including nitrocellulose powder and filter methods.

The addition of sodium lauryl sulfate completely precludes the batch to batch variation in nonspecific binding of DNA to hydroxyapatite. As a result of continued consultation with the supplier and with Dr. Dave Kohne, of the Carnegie Institute of Washington, this laboratory was instrumental in the development of a "DNA grade" of hydroxyapatite. This product is standardized for DNA work and offers large capacity, reasonable flow rate and excellent specificity. Batches of hydroxyapatite were tested in order to develop an ideal preparation of ribonucleic acids.

Gene isolation techniques have been standardized to a point where small or large scale preparations can be processed reproducibly and with minimal loss. In the past, a significant proportion of the genes to be isolated were lost during concentration steps and through leaching from hydroxyapatite. The first problem has been virtually eliminated by a combination of dialysis and concentration by rotoevaporation. The leaching problem has been minimized by lowering the salt concentration of the hydroxyapatite columns which results in RNA binding with greater stability.

3. Transfer-RNA Gene Studies.

Method for isolation of tRNA genes is standardized. This improvement eliminated the mechanical loss of material during isolation thus improving the yield. At the same time this technique has made it possible to increase the capacity to isolate the genes on a larger scale.

The purified genes which are ^{32}P labelled were hybridized with ^{32}S labelled tRNA. Upon gel chromatography on Sephadex-G-100 and $CsSO_4$ density gradient centrifugation, a tRNA:DNA ratio of 0.6 to 0.7 is obtained. This indicates that the DNA fragments (M.W. 125,000) contain 3 to 4 tRNA/DNA fragments. This further shows that tRNA genes are grouped in clusters of at least 3 or 4 genes.

Hybridization of pure tRNA genes with 5s RNA (E. coli), ribosomal RNA (E. coli), yeast and rat liver tRNA show that there is no cross reaction. This indicates the lack of homology between the RNA and E. coli tRNA. There is however, slight homology between E. coli tRNA and Neurospora tRNA.

Hybridization of pure tRNA genes with two isoaccepting tRNA's (pure) from E. coli namely formyl-methionyl-tRNA and methionyl-tRNA, show that they hybridize additively. This shows for the first time that isoaccepting tRNA are the products of the separate genes rather than modification after transcription. Attempts are being made at present to extend this finding to other isoaccepting tRNA's from E. coli.

DNA-DNA reassociation using pure tRNA genes and DNA from a series of enterobacteria indicate that tRNA genes are preferentially conserved. Under optimal reassociation conditions, tRNA gene relatedness ranged from 85% to approximately 66% in organisms showing 85% to 7% bulk DNA relatedness to E. coli. The nucleotide sequences held in common between tRNA cistrons from these organisms are largely stable as judged by experiments carried out at higher incubation temperatures, and by determinations of the thermal stabilities of individual duplexes. The degree of conservation evident in tRNA sequences may further be exploited to determine divergence in very distantly related groups of bacteria where the overall DNA relatedness is too small to increase.

Using pure tRNA genes as template and highly enriched DNA dependent RNA polymerase, it has become possible to obtain tRNA gene transcription products. This product migrates in tRNA regions when subjected to acrylamide gel electrophoresis. E. coli tRNA competes with this transcription product for hybridization with bulk E. coli DNA. This indicates that in vitro sequences of tRNA have been synthesized. The biological functionality and the structural features of this transcription product are being investigated at the present time.

The tRNA genes isolated by this procedure contain 3 to 4 tRNA genes per DNA fragment. In order to answer some of the questions regarding full understanding of the gene function in general, it became apparent that one would want to obtain a DNA fragment of the same size as tRNA, and of complementary sequences. To this end an endonuclease from Neurospora crassa mycelia which degrades single-stranded DNA at a much faster rate than double-stranded DNA was purified. This preparation had an initial ratio of degradation rates (dDNA/nDNA) of greater than 150.

E. coli transfer RNA and dDNA were hybridized by modification of the described procedure. The modification was of such a nature that it afforded maximum hybridization under this condition. The apparent maximal binding of tRNA to dDNA is 0.046% of total genome which agrees with the value reported by this laboratory and other investigators. However, this value is twice that of Marks and Spencer.

The hybrid (tRNA:dDNA) was separated from tRNA by agarose chromatography. The recovered hybrid was subjected to Neurospora endonuclease action under specific conditions. The digest was subjected

to the fractionation procedure to obtain the hybrid in pure form; this includes column chromatography on MAK column, $CsSO_4$ density gradient centrifugation followed by Sephadex-G-100 gel filtration. The density of this preparation was 1.500 which is within the range of DNA/RNA hybrid. The molecular weight of the hybrid is less than 100,000 and greater than 40,000, which is also the expected value for tRNA:dDNA hybrid. The tRNA:DNA ratio is approximately 0.5. It is planned to use this preparation to obtain specific questions regarding the nature of transcription of isolated genes.

4. 5s RNA Gene Studies.

Since the efforts to obtain tRNA genes were successful the 5s RNA gene was isolated. Unlike tRNA, 5s RNA is a single species of RNA. 5s RNA is a minor constituent of ribosomal RNA whose function is yet unknown. However, complete nucleotide sequences of this RNA have been determined. Other investigators showed that there are as many copies of 5s RNA genes as ribosomal RNA genes (5-6). Whether there is a specific initiation and termination site before or after each copy of 5s RNA genes is not known. Furthermore, in this case it will be relatively less difficult to identify the transcription product thereby facilitating understanding of the mechanism of transcription of isolated genes.

The isolation of 5s RNA genes was accomplished by concomitant hybridization of 5s RNA and tRNA with DNA and separation of unhybridized DNA from hybrid by hydroxyapatite column chromatography. After four such cycles each RNA was separately hybridized and further purified by repeated column chromatography.

Characterization of 5s RNA genes shows the homology and conservation similar to tRNA among various species of bacteria. There is essentially no homology with E. coli tRNA whereas there is considerable homology with ribosomal RNA.

Using highly enriched DNA dependent RNA polymerase and purified tRNA and 5s RNA genes, the transcription products have been obtained. This product has been analyzed at the present time for its size, sequence, conformation and biological functionality.

5. Structural Studies of Nucleic Acids.

In order to correlate the structures of nucleic acids to their function and further acquire better knowledge of the nature of interaction of protein and nucleic acid, a systematic study has been undertaken. An atlas of nucleotide sequences of all known RNA was compiled in order to facilitate these studies. The construction of this atlas was based upon the knowledge of nucleotide sequences, their conformation, the physical properties, their chemical activity and biological functions of these RNA's.

These studies are further extended to construct three dimensional atomic models of some of the nucleic acids.

Another project was initiated to differentiate the hydrogen bonded regions of RNA from non-hydrogen bonded regions. The main idea behind this project is to employ a nuclease which is relatively specific for single-stranded RNA. An endonuclease isolated and purified from Neurospora crassa has such activity. In the preliminary experiments purified tRNA was used as a substrate. The initial experiments were very encouraging.

6. The Effect of High Doses of Vitamin C on Male Reproductive Function.

It has recently been suggested that Vitamin C be ingested in large amounts to prevent sickness and improve general health. A simple procedure such as the above is potentially very useful in the Army. It has also been shown that large quantities of Vitamin C can cause sterility in rats when injected intramuscularly. Ten rats and 6 guinea pigs are now on a pilot diet study where they ingest large amounts of Vitamin C. The testicles, urinary bladder, and kidneys will be histologically examined at the termination of the program to determine what, if any, damage has been done to the above organs.

7. Interaction of Small Molecules and Ions with Biological Membranes.

Previous investigation by many workers has shown that membranes do not merely act as passive filters. Cation pumps, permeases, and in certain instances, enzymes of metabolic pathways have been associated with intact membranes and membrane fractions.

Small ion interactions have been studied and the influence of anesthetics and ATP on Ca^{+2} binding and configuration, respectively, have similarly been undertaken. Manery has further delinated the binding sites of Ca^{+2} using ^{45}Ca.

The erythrocyte membrane was chosen as a model system since preparation is relatively simple and almost pure intact ghosts are obtained. This membrane exhibits Na^{+} - K^{+} stimulated ATPase activity, Mg^{+2}-induced ATPase activity as well as enzymatic activities of the EMP-pathway. The presence of these enzyme centers coupled with work published on soluble enzymes and proteins using fluorescent probe molecules and spin label probes as an index of configurational changes was the basis for the use of such probes to examine membrane interactions under controlled conditions. Intrinsic tryptophase fluorescence was also followed.

Ghost preparations were either used intact or perturbed by using phospholipases A, C, D and neuraminidase, and Ca^{+2} was added as an added perturbation. With the fluorescent probe, 2-p-toluidinyl-6-naphthalene-sulfonate (TNS), which binds to lipophilic regions of the membrane, a statistical binding constant for the intact ghosts of 10^{-6}M was found. Neither digestion nor neuraminidase action significantly changed the results. Phospholipase A, however, seems to shift the emission maximum of membrane tryptophane. Added calcium increases the quantum yield of TNS, but also shows little difference in binding. Calcium has no effect on either the quantum yield or emission and absorption maxima of membrane tryptophane.

The spin-label probe, 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl, was attached to membranes intact and enzymatically treated. An increase of "free" spin-label is evident in phospholipase A treated ghosts. Other phospholipases and neuramindase exhibit patterns similar to the intact membranes.

The fluorescent probe, 5-dimethylaminonaphthalene-1-sulfonic acid (DNS), as the acid chloride, was covalently attached to free basic amines in the membrane. Lipid extraction and thin-layer chromatography shows this probe attached to the membrane phosphatidyl ethanolamine fraction. Also, DNS attached to membrane protein.

A concentrated effort has just begun using the latter probe in conjunction with ATP configurational influences. Drug blocking agents for the EMP pathway influence ATP levels in erythrocytes in vivo, and this study could possibly elucidate more fully the roles of such blocking agents in pathological circumstances.

8. The Effect of Alcohol on the Mechanism of Transfer of Hydrogen Equivalents across Liver Mitochondrial Membranes.

Knowledge on the mechanism of transfer of hydrogen equivalents in the form of NADH across mitochondrial membranes is fundamental to the understanding of the pathogenesis of alcohol-induced hypoglycemia, alcoholic fatty liver, and perhaps alcoholic cirrhosis. Moreover, it is significant for the understanding of the mechanism of production of hyperlacticemia in various disease processes.

Other investigators observed that intact liver mitochondria do not oxidize externally added NADH. It has therefore become generally recognized that hydrogen equivalents in the form of NADH do not directly traverse the mitochondrial inner membrane. A number of indirect substrate "shuttle" systems have been proposed to explain the known physiological transfer of hydrogen equivalents to and from the intramitochondrial space. Borst has suggested a substrate shuttle system which involves oxido-reduction catalyzed by malate dehydrogenase, coupled with transamination catalyzed by aspartate aminotransferase

on both sides of the mitochondrial membranes; i.e., equations 1-10, where o and i denote outside and inside the mitochondrial membrane respectively.

$$\text{Oxaloacetate}_o + \text{NADH}_o + \text{H}_o^+ \rightleftharpoons \text{malate}_o + \text{NAD}_o^+ \quad (1)$$

$$\text{Malate}_o \rightleftharpoons \text{malate}_i \quad (2)$$

$$\text{Malate}_i + \text{NAD}_i^+ \rightleftharpoons \text{oxaloacetate}_i + \text{NADH}_i + \text{H}_i^+ \quad (3)$$

$$\text{Oxaloacetate}_i + \text{glutamate}_i \rightleftharpoons \alpha\text{-oxoglutarate}_i + \text{aspartate}_i \quad (4)$$

$$\text{Aspartate}_i \rightleftharpoons \text{aspartate}_o \quad (5)$$

$$\text{Aspartate}_o + \alpha\text{-oxoglutarate}_o \rightleftharpoons \text{oxaloacetate}_o + \text{glutamate}_o \quad (6)$$

$$\text{Glutamate}_o \rightleftharpoons \text{glutamate}_i \quad (7)$$

$$\alpha\text{-Oxoglutarate}_i \rightleftharpoons \alpha\text{-oxoglutarate}_o \quad (8)$$

$$\text{NADH}_i + \text{H}_i^+ + 1/2\ \text{O}_2 \longrightarrow \text{NAD}_i^+ + \text{H}_2\text{O} \quad (9)$$

$$\text{Sum: } \text{NADH}_o + \text{H}_o^+ + 1/2\ \text{O}_2 \longrightarrow \text{NAD}_o^+ + \text{H}_2\text{O} \quad (10)$$

Previous studies have shown that the above scheme as suggested by Borst can be very active in isolated rabbit liver mitochondria and that the oxidation of externally added NADH can compete effectively with the synthesis of phosphoenolypyruvate from glutamate. Extramitochondrial NADH was not oxidized by liver mitochondria when added alone or when glutamate and aspartate were also present. However, when aspartate aminotransferase and malate dehydrogenase were added, extramitochondrial NADH was rapidly oxidized, and the oxidation of glutamate was markedly reduced. External NADH was not oxidized in the absence of added aspartate aminotransferase. However, the requirement for added malate dehydrogenase was only partial. Furthermore, increasing amounts of added aspartate stimulated the oxidation of extramitochondrial NADH and increased the inhibition of glutamate oxidation. These experiments strongly support the hypothesis that the antiglucogenic effect of ethanol in fasted animals is due to a competition between cytosolic NADH generated in the oxidation of ethanol and intramitochondrial NADH produced during the oxidation of glucogenic amino acids in the citric acid cycle, with the result that the net flow of precursors to oxaloacetate and phosphoenolpyruvate is diminished.

The fact that tne substrate "shuttle" system suggested by Borst has been made to work in vitro encourages the belief that it operates in vivo. Since the in vivo NAD/NADH ratio in the cytosol of liver of well-fed rats has been estimated to be 100-fold more oxidized than the ratio inside the mitochondrion, recently experiments were conducted to test whether such a redox gradient can be achieved in the reconstructed system which was studied. Preliminary data indicate that NAD/NADH ratio in the extramitochondrial compartment as high as 535 could be achieved when the intramitochondrial NAD/NADH ratio was kept at 4 by incubation of rabbit liver mitochondria at 20 μM 2,4-dinitrophenol. Presumably, this was accomplished by the asymmetrical distribution of one or more of the substrates across the mitochondrial inner membrane in the following relationship:

$$\frac{(NAD_o)/(NADH_o)}{(NAD_i)/(NADH_i)} = \frac{(\alpha KG_o)(Asp_o)(Glu_i)(Mal_i)(H_o^+)}{(\alpha KG_i)(Asp_i)(Glu_o)(Mal_o)(H_i^+)}$$

Continued investigation on this substrate "shuttle" system indicates that rat liver mitochondria behave similarly as rabbit liver mitochondria. Recently, the effect of butylmalonate on this "shuttle" mechanism in rat liver mitochondria was studied. Butylmalonate, being a specific inhibitor of malate-phosphate exchange translocator in the inner membrane of rat liver mitochondria, was found to be effective in inhibiting the transfer of hydrogen equivalents across this membrane. Other known inhibitors of malate translocation such as mersalyl and formaldehyde are being tested. Sutaldehyde, a product of ethanol oxidation, will also be tested.

9. Studies on the Mechanism of Radioprotection.

a. Studies of the Correlation between Structure and Function of the Aminothiol Radioprotectants. The effect of varying chain length in β-mercaptoethylamine (MEA) was studied in bacterial and mammalian systems. In contrast to mammalian systems, no abrupt reduction in radioprotection was observed when the carbon chain separating amino and thiol groups was lengthened from three to four or more units. From both bacteriological and free radical studies, the data indicate a gradual reduction in efficacy with no change in mechanism of action either in oxygen or nitrogen as might be expected for a homologous series.

These findings suggest that the aminothiols protect by specific interactions with cell components in addition to acting via a more general, non-specific radical scavenging and/or repair mechanism. In mammalian systems (mice) at the radiation and drug doses employed, only MEA showed a radioprotective effect. This result is in marked contrast to those of previous studies in bacteria where pharmacological effects were avoided and only physical-chemical effects were studied.

b. Effects of Sulfhydryl Dissociation on Free Radicals Stabilized by γ-Irradiated Frozen Aqueous Solutions of Cysteamine. Whereas the last stable radical formed in γ-irradiated frozen aqueous pH = 2 cysteamine solutions is the well-known organosulfur radical, the heretofore unidentified radical formed in pH = 9 solutions gives a triplet electron spin resonance (ESR) spectrum. Several distinct radical species appear to cause the pH = 9 spectrum. It is primarily a 1:2:1 triplet together with an underlying broad singlet, and a small contribution from the organosulfur radical. The yield and stability of the triplet vary with the thiolate anion concentration prior to freezing. This fact, together with other ESR characteristics, leads one to tentatively identify the triplet as a pH stabilized precursor to the organosulfur radical, an excited state neutral sulfur radical RS'.

c. The Microwave Power Saturation of γ-Irradiation Induced Glycylglycine Radicals. A technique has been developed whereby the shape of the first derivative electron spin resonance (ESR) spectra observed for γ-irradiated glycylglycine can be used to estimate incident power in ESR experiments in the 0.1 to 10 mW range. The technique is proposed as one means to determine that power saturation is being avoided when power measuring devices are not available for obtaining a classical power saturation curve.

d. Radiation Energy Migration in Bone and Bone Simulations. Preliminary investigations have been initiated in cooperation with LTC Brady of USAIDR to use ESR to study radiation energy transfer in bone and synthetic bone simulations. It has been shown that ESR centers in irradiated bone persist for extended time periods and moreover that energy initially deposited in the hydroxyapatite matrix can migrate to the protein component resulting in additional radiation damage to this protein.

The initial ESR study has characterized the radicals stabilized by amorphous calcium phosphate irradiated *in vacuo* at 77°K and subsequently annealed. It is planned to determine the nature of free radical interactions when the proteins, bovine or trypsin, are adsorbed to the inorganic matrix and to study the effect of tetracycline on this energy transfer. It has been shown that tetracycline administration can markedly reduce radiation damage in bone when it is administered immediately following irradiation. These ESR studies were designed to test the hypothesis that tetracycline intercepts migrating long-lived radicals initially formed in the inorganic matrix which would otherwise cause radiation damage in bone protein.

10. Phototoxicity Studies.

a. Reduction of WR-7930 Phototoxicity by Administration of MEA Prior to Exposure to UV Light - A Study to Test the Free Radical Intermediate Hypothesis (in collaboration with M. Grenan, Div of Med Chem).

The first product during the UV photolysis of the quinoline methanol, WR-7930, is a hydroxyl free radical from the methanol function. If such a reaction were to proceed in vivo, the observed phototoxicity might be the result of an indiscriminate reaction of such hydroxyl radicals with critical cellular macromolecules. Thus the situation would be quite similar to the "indirect effect" of ionizing radiation in cellular systems. Consequently, if a radioprotective dose of the aminothiol, MEA were administered immediately before UV irradiation of mice which had received a phototoxic dose of WR-7930, the phototoxic effect of the quinoline methanol should be markedly inhibited.

When the mice were examined immediately after irradiation and during the next two days no difference was noted in the phototoxic response of mice which had received WR-7930 whether or not MEA was administered prior to irradiation.

Several conclusions can be drawn from this study. Since MEA injected intraperitoneally has its maximal radioprotective effect during the first 15 minutes after injection, it is possible that any phototoxic protection obtained during this time was masked by subsequent damage during the following 1-3/4 hours. However, since no phototoxic protection at all was noted in mice receiving MEA, this is unlikely. More probably the results indicate that any OH' radicals released by WR-7930 are formed in such proximity to their biological target molecule that the scavenging effect of MEA cannot intervene. Such a mechanism appears likely in view of the proposed antimalarial mechanism of action which involves intercalation of WR-7930 within DNA. In this regard, it is perhaps pertinent that methoxypsoralen, a potent phototoxic drug, has been shown to form interstrand cross links in the DNA α-helix. Such an interaction between each end of the molecule and the DNA strands could proceed by a free radical mechanism. A similar phenomenon occurring between WR-7930 and DNA would account for the ineffectiveness of MEA in reducing phototoxicity.

b. A Study of the Free Radicals Induced at 77°K with WR-7930 in Methanol UV Irradiated with 3250 Å Light. As yet, only free radicals at the g = 2.004 region have been detected. It is likely that these radicals are related to the phototoxic effect described above. Although a definite fluorescence has been observed during UV irradiation at 77°K thus demonstrating the existence of a triplet state, attempts at demonstrating the triplet by ESR have not been successful.

SUMMARY.

All the major groups of Enterobacteriaceae have been investigated in their nucleic acid relatedness to several strains of Escherichia coli, with Shigella and Alkalescens-Dispar strains, in addition to other E. coli strains, showing the highest degree of relatedness, 80% or more, whereas the other enterobacteria tested have a relatedness of

less than 50% to that of E. coli. In addition it has been shown that pathogenic and non-pathogenic strains of an individual genera may be grouped by their nucleic acid sequence relatedness, the pathogenic strains exhibiting less divergence than the non-pathogens. The technology of separating single-stranded and double-stranded DNA and DNA/RNA hybrid preparations from hydroxyapatite has been improved considerably leading to the preparation of a "DNA-grade" hydroxyapatite by the manufacturer, thus leading to more standard methods with either small or large DNA and RNA preparations. Characterization of purified tRNA genes show that these genomes are very highly conserved in several microorganisms, however, there is no homology with E. coli, 5s or ribosomal RNA or yeast and rat liver tRNA. Using labelled tRNA and hybridization with pure genes it has been possible to show that there are 3-4 cistrons per DNA fragment and further that tRNA genes are mostly contiguous. The transcription product of isolated tRNA genes competed out with tRNA for hybridization with E. coli DNA, thus showing that in vitro synthesis of tRNA sequences is accomplished. A novel procedure for isolation of DNA:tRNA hybrid by digestion of single-stranded DNA with Neurospora endonuclease was developed. 5s RNA genes from E. coli have also been isolated and characterized. There are 4-5 copies of this gene in E. coli. The nucleotide sequence analysis of the transcription product of pure 5s RNA genes is in progress.

An atlas of known nucleotide sequences of RNA's was constructed. Based on these data and other physico-chemical information regarding these RNA's a three dimensional model of RNA is being constructed.

The effect of high doses of Vitamin C on male reproductive function in animals is being pursued.

Using spin-labelled densyl compounds and fluorescent probes, and other DNS derivatives, the interaction with biological membranes was pursued. Their site of attachement, involvement in enzymatic reactions and the metabolic effects was studied.

In order to better understand the mechanism of alcohol addiction, the effect of alcohol on the mechanism of transfer of hydrogen equivalents across liver mitochondrial membranes was studied. The metabolic involvement of ethanol in the electron transport system and the energy yielding mechanism of liver mitochondrial membranes was investigated.

When the effect of varying chain lengths in MEA as radioprotectants were studied in bacterial and mammalian systems, it was observed that aminothiols protect by specific interactions with cell components in addition to general non-specific radical scavenging and/or repair mechanism. In addition, the effect of sulfhydryl dissociation on free radicals stabilized by gamma-irradiated frozen aqueous solutions of cystamine, the microwave power saturation of gamma-irradiation induced

glycylglycine radicals and the radiation energy migration in bone and bone simulation studies were pursued.

The reduction of WR-7930 (an antimalarial) phototoxicity by administration of MEA prior to exposure to UV light was pursued in order to test the free radical intermediate hypothesis. The preliminary results show that any OH· radicals released by WR-7930 may be in such proximity to their biological target molecule that the scavenging effect of MEA cannot intervene.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical variations during disease and treatment

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	3. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6452	71 07 01	DD-DR&E(AR)636

2. 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
70 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	61102A	3A061102B71P	01	073
b. CONTRIBUTING				
c. XXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Molecular Pharmacology of Chemotherapeutic Drugs (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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17. CONTRACT/GRANT

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18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 71	7	175
FISCAL YEAR CURRENT 72	7	175

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Molecular Pharmacology; (U) Antimalarials; (U) Nitroacridine 3582; (U) Naphthoquinones; (U) Mechanisms of Drug Action

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

23. (U) This work is directly related to safeguarding the health of the Army against communicable diseases. Its objective is explanation of mechanisms of action of clinical or investigational drugs for the cure or prevention of communicable diseases of military importance, for example, malaria. From this: explanation of relationships between chemical structure and medicinal activity of drugs, formulation of principles for the design of new or improved drugs for which there is a military need, and ways to overcome natural insensitivity or acquired resistance to drugs in disease-producing microorganisms.

24. (U) Experimental studies at 3 levels of biological organization: 1. Biophysical studies on the interaction of drug molecules with their biopolymer sites of action. 2. Biochemical studies of the functional consequences resulting from the above interactions. 3. In vivo studies of the manifestations of the above events on the physiology and population dynamics of intact microbial cells.

25. (U) 70 07 - 71 06 DNA-complexing substances (antimalarials, antimicrobials, dyes) displace methyl green from its complex with DNA. Rates and endpoints of this reaction afford measures of affinities of such chemotherapeutic substances to their site(s) of action; they are also proportional to biochemical and genetic effects of such compounds. Nitroacridine 3582 kills susceptible microorganisms by blockade of DNA biosynthesis. It has lesser effect on RNA and protein synthesis. - A typical antimalarial naphthoquinone inhibits active transport of nutrients into or out of susceptible microbial cells. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

*Available to contractors upon originator's approval

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Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 073, Molecular pharmacology of chemotherapeutic drugs

Investigators.
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Description.

Experimental research studies in depth on the molecular pharmacology, biochemistry, biophysics, microbial physiology and biochemical genetics of the actions of antimicrobial drugs, especially antimalarials, with a view to elucidating principles of modes and mechanisms of drug action at the primary level, explaining phenomena of acquired drug resistance and offering conceptual guidance to both improved methods of chemotherapy of infections with existing drugs as well as rational development of novel chemotherapeutic substances.

Progress and Results.

1. Mode of Action of Chloroquine. Complex formation with native double-helical DNA through intercalation has been recognized as the mechanism of antimicrobial action of chloroquine; structure-activity rules for antimalarial action developed in this laboratory have stood the test of quantitative regression analysis and molecular orbital calculation by others. But, the detailed point-counter-point determination of the binding of the drug to DNA still remains to be worked out. For this purpose, the structure of chloroquine in solution has been further investigated by nuclear magnetic resonance spectroscopy (NMR). Initial studies to determine the importance of ionic charge in stabilizing an interaction between chloroquine and DNA in aqueous solutions utilizing NMR were hampered by the fact that the monoprotonated species of chloroquine is not soluble. Since any of the ionic species may be involved in an interaction with DNA, the effect of each ionic form on the conformation of chloroquine must be determined. These studies are feasible in nonaqueous solvent systems. When trifluoroacetic acid (TFA) is added to methylene chloride solutions of chloroquine (CQ) until the TFA/CQ molar ratio reaches 1:1, the spectral parameters indicate that protonation occurs at the diethylamino nitrogen atom; continued addition of TFA causes a second protonation to occur at the heterocyclic nitrogen atom. The results also indicate that the aliphatic chain of the monoprotonated species has formed a 7-membered ring as a result of a hydrogen bond between the diethylamino proton and the 4-amino nitrogen atom. Further

protonation of CQ restores the open-chain structure.

The proton exchange rates of the protonation equilibria indicate that the aliphatic chain of the free base chloroquine must also exist as a ring-like structure. To confirm this hypothesis and define exactly the conformation of the monoprotonated species would require a conformational study giving atomic dimensions similar to x-ray spectroscopic results. Until recently such structure determinations in solution were not possible; however, current development in NMR now makes such detailed analyses possible by the use of contact shift reagents.

The effect such reagents have on NMR signals comes as a result of binding of these reagents to N, S and O atoms in a molecule and subsequent effects on spectral parameters is related to structure by the following equation

$$\Delta\delta = \beta\left(\frac{3\cos^2\theta - 1}{r^3}\right)$$

where $\Delta\delta$ is a shift in a spectral parameter, r is the vector distance between the bound shift reagent and various proton nuclei in a molecule such as chloroquine, and θ is the angle between a molecular axis and the line defined by the vector distance r. If the parameters β and θ are known, then r is determined and the structure of a molecule in solution is known.

A study of the binding of Tris (dipivalomethanato)-praseodymium to chloroquine was undertaken in order to determine the precise structure of the free base, monoprotonated and diprotonated forms of the drug. Spectrophotometric studies of the binding interaction of CQ with praseodymium up to 10:1 molar ratios of $Pr(dpm)_3$ concentration to CQ concentration indicated a single binding site. NMR studies indicate that binding site to be at the heterocyclic nitrogen atom. Since the distances between praseodymium and the protons on the heterocyclic and carbocyclic ring are fixed and known from their changes in chemical shift, the exact position of the praseodymium attached to the heterocyclic nitrogen atom can be calculated determining the β and θ.

Using computer techniques the atomic coordinates of prasecdymium bound to CQ have been determined. From the relative changes in the shifts of the aliphatic proton signals the structure of the aliphatic chain in the free base is being calculated. This procedure is being used to determine the conformation of the other ionic species of chloroquine. With this information, studies to elucidate the conformation of CQ-nucleic acid complexes and the forces stabilizing them will be possible.

2. Mode of Action of One Antimalarial Naphthoquinone. It has previously been concluded that the abrupt, total and simultaneous

inhibition of *in vivo* synthesis of RNA, DNA, protein and cell wall polymer by 2-hydroxy-3-cyclohexylpropyl-1,4-naphthoquinone in *Bacillus megaterium* points to an effect of the drug on membrane-centered energy supplying reactions and/or transport of essential nutrients. Experiments measuring the uptake of uracil-^{14}C into the intracellular pool, as well as its incorporation into RNA, revealed that naphthoquinone blocked the entry of radioactive uracil into cells of *B. megaterium*. The accumulation of alpha-methyl-D-glucoside-^{14}C, a nonmetabolizable analog of glucose, was only slightly inhibited, an observation attributed to this compound being transported by a phosphoenolpyruvate-dependent phosphotransferase system rather than by an adenosine triphosphate-linked accumulation mechanism. Cellular ATP pool levels were not lowered by exposure of cells to naphthoquinone, however, naphthoquinone drastically uncoupled phosphorylation from oxidation in cell-free extracts. Apparently the drastic shutdown of energy-requiring biosynthetic reactions has a sparing action on the ATP pool. In addition, glucose may continue to be utilized fermentatively, thus maintaining a supply of ATP. The inhibition of active transport of nutrients by naphthoquinone in spite of the presence of a large pool of intracellular ATP, presumably accessible as an energy source for permease action, suggests that transport may be mediated by a high-energy respiratory chain precursor and not by ATP. With respect to the action of naphthoquinone, it is significant that the entire content of vitamin K, a respiratory naphthoquinone thought to participate in oxidative phosphorylation by acting as an intermediate phosphate carrier between electron transport and the final phosphorylation of ADP, is exclusively localized in the membrane of *B. megaterium*. An uncoupling or inhibition of respiration-linked phosphorylations by naphthoquinone might alter the supply of energy in such a manner as to preclude macromolecular biosyntheses, as well as the transport of essential metabolites. The work on this project has been completed and a final report in the form of a research article is in press.

3. Mode of Action of Primaquine. Although the 8-aminoquinolines are the oldest synthetic antimalarial drugs, they have seen the least amount of study of their mode of action. Incorporation of ^{32}P-orthophosphate into the nucleic acids of two plasmodia was reported in 1961 to be only slightly affected by 8-aminoquinoline antimalarials and more recent studies in tetrahymena, temperature-shocked into synchronous growth, have not demonstrated a specific biochemical mode of action of primaquine. The major obstacle in the study of primaquine's mode and mechanism of action had remained the unavailability of a convenient drug-sensitive test micro-organisms; bacteria had been considered to be insensitive to primaquine. We have found that the growth of a strain of *Bacillus megaterium* is prevented by a minimal inhibitory concentration of 52 micrograms per ml (2×10^{-4} M) of primaquine. When exponentially growing cultures received primaquine to a concentration of 6×10^{-4} M, the rate of growth was drastically reduced and no further growth occurred after 15 min of drug exposure.

Approximately 40 per cent of the cells were killed during one doubling time (45 min). Supplying primaquine to cultures 30 min after the addition of radioactive-labeled leucine or phenylalanine, diaminopimelic acid, uracil, or thymidine, caused an immediate and complete inhibition of protein biosynthesis. The formation of cell wall polymer and RNA proceeded at the rate established prior to the addition of drug, and no inhibition of DNA biosynthesis occurred for at least 15 min. This pattern of inhibition of macromolecular biosyntheses suggests that the major in vivo action of primaquine in B. megaterium is to block protein synthesis. It is typically encountered with broad-spectrum antibiotic inhibitors of protein biosynthesis but primaquine is the first major synthetic drug discovered to act in this manner. Studies are under way to pinpoint the reaction step in protein synthesis which is affected by primaquine.

4. Displacement of Methyl Green from DNA by DNA-Complexing Drugs. Antimalarial (chloroquine, quinacrine, quinine, quinoline methanols), antischistosomal (miracil D), antileishmanial (berberine) and antitrypanosomal drugs (ethidium bromide, berenil) as well as topical or systemic antibacterial acridines (acriflavine, Entozon) all act by forming complexes with DNA and inhibiting microbiological key processes in which DNA must participate. Some of these drugs act as mutagens, antimutagens or eliminators of episomal R-factors. The investigation of the various actions of such chemotherapeutic substances requires, therefore, the determination of their affinity for DNA as well as of the extent (stoichiometry) of their binding to DNA. We report that such determinations were made conveniently and simply by measuring, spectrophotometrically, the rates and extents to which chemotherapeutic drugs displace the histological dye, methyl green, from its stable complex with DNA. The colored form of methyl green is stable in its complex with native, double-helical DNA but unbound methyl green undergoes a spontaneous molecular rearrangement into a colorless isomer. Displacement of methyl green from its complex with DNA results, therefore, in a progressive decrease in absorbancy at 642 nm, the absorption maximum of the dye, in DNA-methyl green drug mixtures. In decreasing order of rate constants and endpoints, the following chemotherapeutic drugs cited in this Annual Report, displaced methyl green from DNA: quinacrine, ethidium bromide, Nitroakridin 3582, distamycin A, miracil D, chloroquine and berberine. Displacements by primaquine and quinine were negligible. Some displacements, particularly by compounds which displaced methyl green rapidly and almost completely, changed from an initial first-order to a subsequent second-order kinetic course while for others a reaction order could not be established unambiguously. Substances which bind to DNA by intercalation displaced methyl green at more rapid rates and to larger extents than did nonintercalators. Methyl green itself is not considered to bind to DNA by intercalation. We suggest that the release of methyl green from DNA is caused by local unwinding of the double helix.

5. Mode of Action of Nitroakridin 3582. Results reported in last year's

Annual Progress Report suggested that the inhibition of DNA biosynthesis was not completely responsible for the bactericidal effect of 3582. Specifically: (1) Exponential loss of viability was produced by the drug in bacteria suspended in buffered saline. (2) Accelerated loss of viability in metabolizing cultures was produced by concentrations of 3582 in excess of those which were sufficient to block DNA biosynthesis entirely. (3) Bactericidal concentrations of 3582 inhibited not only DNA biosynthesis but also RNA and protein biosynthesis. Among various alternative explanations, one is the assumption that intercalation of 3582 (and perhaps of other drugs) causes, by itself, irreversible damage to DNA. Consequently, the growth-inhibitory effect of 3582 has been studied in strain E. coli 3110 T^- and in a mutant of this strain (POL A^-) which lacks DNA polymerase I (Kornberg's repair enzyme). The repair-deficient mutant was much more sensitive to the bactericidal action of 3582 than was the parent strain. This suggests that failure or the ability to repair DNA which has been exposed to intercalator chemotherapeutic drugs may be a prime determinant of greater or lesser sensitivity. The nature of the intercalation damage and of its repair is now under study.

6. Mode of Action of Distamycin A. We reported last year the formation of a molecular complex of DNA with the antibiotic distamycin A. This subject has since come under intensive study in other laboratories. We report that the DNA-distamycin complex can not be dissociated by 1.0 M NaCl, 0.1 M Mg acetate, 6 M urea, 12 M formamide or 0.001 M silver ion. Likewise, dialysis against Tris buffer or even against 1 per cent sodium lauryl sulfate fails to displace distamycin from DNA. Finally, enzymatic hydrolysis of the DNA-distamycin complex with deoxyribonuclease I failed to restore absorption spectrum of free distamycin. These findings suggest that distamycin becomes bound covalently to DNA. Although the change in distamycin's spectrum upon contact with DNA is instantaneous, others report that it requires 10 min preincubation of distamycin with DNA before the antibiotic exhibits its full activity as a DNA template poison. Since distamycin preferentially stabilizes AT-rich DNA to heat denaturation, we hypothesize that the antibiotic binds either to deoxyguanylic acid but more probably to thymidylic acid in DNA. This is now under test.

7. Mode of Action of L-cycloserine. L-cycloserine is the synthetic enantiomer of the natural antibiotic D-cycloserine. The D-compound inhibits the biosynthesis of the bacterial cell-wall polymer by virtue of being a structural analog and antimetabolite of D-alanine. We found that L-cycloserine inhibits the growth of E. coli in mineral glucose medium but only marginally in brain-heart-infusion broth (BHI). Graded quantities of BHI added to mineral medium reverse proportionally the bacteriostatic action of L-cycloserine. The drug does not inhibit cell-wall biosynthesis, as found earlier in our laboratory. Based on the structural analogy between L-cycloserine and the natural amino acid L-alanine, we hypothesized that the drug was an L-alanine antagonist, probably in protein synthesis. In fact, the bacterial

growth rate which was reduced by subinhibitory concentrations of L-cycloserine could be restored to that of a drug-free control culture by supplying an excess of L-alanine to the inhibited culture. D-alanine, likewise, restores the growth rate. This is probably explained by the fact that E. coli possesses an active alanine racemase which interconverts the two enantiomers of alanine. The hypothesis that L-cycloserine is a specific inhibitor of protein biosynthesis in competition with L-alanine is under test.

8. Effects of Quinacrine on Reaction Steps in Protein Synthesis. While the principal antimicrobial effect of quinacrine is due to the drug's binding to DNA and inhibition of DNA biosynthesis, it has a definitive but smaller side effect on protein biosynthesis. Quinacrine inhibits the polycondensation of phenylalanine in an E. coli ribosome-polyuridylic acid cell-free model system of protein synthesis. The synthesis of phenylalanyl-transfer RNA is only slightly affected by quinacrine and the poly U-directed binding of phenylalanly-tRNA to ribosomes is unaffected. It is concluded that quinacrine acts directly on ribosomes and on some ribosome-mediated step in protein synthesis. Indeed, ribosomes alter the absorption spectrum of quinacrine, quinacrine labilizes ribosomes to heat, and an excess of ribosomes reverses the drug's action on polyphenylalanine formation.

Conclusions.

Structures of the different ionic species of chloroquine have been determined by nuclear magnetic resonance spectroscopy using praseodymium as a contact shift reagent. One antimalarial naphthoquinone inhibits active transport of metabolic precursors. Primaquine is a specific inhibitor of protein biosynthesis in sensitive microorganisms. DNA-complexing chemotherapeutic drugs displace methyl green from its complex with DNA, offering a convenient method to determine affinity and stoichiometry to drug binding to DNA. Nitroakridin 3582 causes a lesion in DNA by complexing with the polymer; ability or inability to repair this lesion determines lesser or greater sensitivity to the drug. Distamycin A forms a covalent complex with DNA, possibly by reaction with thymidylic acid. L-cycloserine inhibits growth of E. coli in synthetic media in competition with its structural analog L-alanine. Quinacrine, as a side effect, inhibits protein synthesis at the ribosomal level of the reaction sequence.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 073, Molecular pharmacology of chemotherapeutic drugs

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6453	71 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
70 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	61102A	3A061102B71P	01	074
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

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(U) Molecular Basis of Biological Regulation (60)

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69 07	CONT	DA	C. In-House

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a. DATES/EFFECTIVE: NA EXPIRATION:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	50
	CURRENT 72	2	50

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Episomes and R-factors; (U) Antimutagens; (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) Selected studies with the common objective of rendering the drug treatment of communicable diseases of military importance either (1) safer, or (2) effective in those instances in which multiple drug resistance is encountered or emergence of resistance to individual drugs is predictable.

24. (U) Experimental studies on (1) elimination of circular DNA episomes from bacteria by DNA-complexing compounds and determination of the rate of episomal DNA, (2) nature of complexes of biologically active compounds, for example, antimalarials, with circular DNAs, (3) molecular genetics of the action of quinacrine and other antimutagens and of their apparent ability to block microbial mutations to drug resistance in vitro.

25. (U) 70 07 - 71 06 Quinacrine forms an intercalation complex with superhelical DNA from bacteriophage PM2 infecting Pseudomonas sp. BAL-31. Graded concentrations of the drug produce typical transitions in the sedimentation coefficient of this DNA which are indicative of gradual unwinding of the superhelix, attainment of the open circular configuration of this DNA and formation of another superhelix owing to an excess of Watson-Crick helical turns. - The recently discovered gentamicin R-factor, harbored by a strain of Klebsiella, shows a strong tendency of spontaneous genetic segregation; studies are in progress to enhance this effect by episome-eliminating chemicals in order to "cure" these bacteria of gentamicin resistance. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

*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 67 (FOR ARMY USE) ARE OBSOLETE

PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 074, Molecular basis of biological regulation

Investigators.
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Associate: CPT Richard G. Allison, MSC; Jennie Ciak, M.S.; Anne K. Krey, M.S.; Alan D. Wolfe, Ph.D.

Description.

A work unit comprised of experimental research studies in molecular biology concerned with the physiology, biochemistry, biophysics and molecular genetics of the title processes with a view to elucidating mechanisms underlying biological events of special importance to military medicine, for example, currently, the biological role of supercoiled DNA in micro-organisms, the influence of chemicals on DNA's function as a carrier of heredity and the perturbation of ribosomes by planar heterocyclic compounds.

Progress and Results.

1. Conformational Changes in Superhelical DNA Produced by Quinacrine and Quinine. Alteration of the superhelical density of supercoiled DNA by drug molecules is known to be largely a property of drugs which bind to DNA and produce changes in the biophysical properties of DNA usually interpreted by an intercalation model. The greater sensitivity of the sedimentation and viscosity properties of superhelical DNA, as compared to linear DNA, to intercalation drugs, suggests that it be used as a system for investigating the effect and the intercalating properties of antimalarials and other drugs that might bind by this mechanism. The effect of ethidium bromide (EB) on polyoma virus DNA has been previously reported. However, miligram quantities of superhelical DNA needed to routinely study drugs by this method are not realistically available from polyoma virus. The DNA needed is now prepared in sufficient amounts from bacteriophage PM2. We have found that ethidium bromide demonstrates, quantitatively, identical effects on the sedimentation of PM2 DNA. The sedimentation properties of PM2 DNA in the presence of quinacrine and quinine were examined by conventional ultracentrifugation techniques. Quinacrine produces alterations of the sedimentation coefficient of PM2 DNA equivalent to those produced by ethidium bromide. Conversion to an open circular form occurs at a drug-(added)-to-DNA-Phosphate ratio (D/P ratio) of 0.034. Maximum inversion of the superhelical coils, estimated by increased sedimentation rate, is obtained at the D/P ratio of 0.069. These values do not differ significantly from those obtained for EB by others and confirmed by us on PM2 DNA. Quinine also alters the

sedimentation properties of PM2 DNA. These changes were observed up to as experimentally limiting D/P ratio of 0.2 where difficulties were encountered due to the high concentration of quinine. Extrapolation to the D/P ratio which would correspond to the open circular form can be used to predict that this conversion, if indeed possible, will not occur at a D/P ratio less than 0.5.

2. Elimination of Episomes by Drugs Intercalating into DNA. Episomal elements of bacteria such as the F factor or resistance determinants can be eliminated, e.g. in E. coli, by treatment with aminoacridine drugs. Since these drugs form complexes with DNA by intercalation, we assumed that other intercalative drugs would also eliminate episomes by conformational changes in circular episomal DNA. We reported earlier that the F' lac episome from E. coli was eliminated in a decreasing order of potency by a series of antimalarial DNA-complexing drugs. We hypothesized that the ability to eliminate or "cure" certain episomal determinants is a group property of DNA intercalators. E. coli K-12, RS-2 containing R-factors for chloramphenicol, kanamycin, streptomycin, sulfadiazine and ampicillin was grown in the presence of acridine orange, ethidium bromide, quinacrine, chloroquine, quinine, berberine, methylene blue and p-rosaniline at concentrations of 10^{-4} M. The cultures were then plated on drug free-agar and agar containing, individually, the above antibacterial drugs. Individual R-factors showed marked differences in their susceptibility to elimination by DNA-complexing compounds. Chloramphenicol and kanamycin resistance determinants were eliminated by the strong intercalators, ethidium bromide, quinacrine and acridine orange. The R-factor for ampicillin was partially cured by ethidium bromide and quinacrine. Berberine, for which intercalation has recently been shown, exhibited borderline curing activity for kanamycin and chloramphenicol. The quinolines, chloroquine and quinine, were less active and eliminated the kanamycin factor only. Methylene blue which binds to DNA in a manner suggestive of the possibility of intercalation showed no curing activity at the standard concentrations. However, at a much higher concentration there was marginal curing for the chloramphenicol factor. p-Rosaniline which does not intercalate was devoid of activity. Resistance to streptomycin and sulfadiazine could not be eliminated under our standard test conditions. In the past year, during a hospital outbreak of serious infections, a strain of Klebsiella was discovered to harbor a transferable R-factor for gentamicin. Studies are now in progress to determine whether intercalative drugs are capable of "curing" Klebsiella of the gentamicin R-factor.

3. Enhancement of Thermal Disassemblage of Ribosomes by Intercalator Drugs. The selected DNA intercalants, quinacrine, chloroquine, Nitroakridin 3582 and ethidium bromide, in direct proportion to their planar heterocyclic area, reduce the stability of E. coli ribosomes to heat. Since results suggested that, in general, DNA intercalants will reduce ribosome stability, a total of fourteen assumed intercalants

were tested for their ability to increase the hyperchromic rates of ribosomes melting at a constant temperature (52°C). The two intercalants with the largest total planar area, propidium iodide and ethidium bromide, were most active, while those intercalants with the smallest total planar area, the quinolines, chloroquine and quinine, were the least effective. Only one compound, quinine, at the low concentration of 2×10^{-5} M, failed to labilize ribosomes. Additionally, intercalants enhanced thermal disassembly of ribosomes in direct relation to the fraction of drug which bound to supercoiled DNA unwinds it to open circles (Waring, M., J. Mol. Biol. 54:247, 1970). Finally, isopycnic centrifugation of ribosomes in the presence of ethidium bromide or propidium iodide induced ribosome disassembly with separation of proteins from RNA and formation of "core particles" with sedimentation coefficients of 25s and 41s. Isopycnic centrifugation of ribosomes was carried out in approximately 5 M CsCl which restricts binding of ethidium or propidium to nucleic acids to intercalation. Our results are consistent with the hypothesis that heterocyclic drugs, including the antimalarials quinacrine and chloroquine, bind by intercalation to ribosomal RNA and reduce the stability of ribosomes.

Conclusions.

Quinacrine and quinine produce configurational transitions in superhelical DNA indicative of intercalation and suggestive of conversion of DNA into nonfunctional supercoils. Intercalative drugs, foremost quinacrine and ethidium bromide, eliminate certain drug resistance determinants from bacterial episomes. Flat heterocyclic intercalator substances enhance thermal disassembly of ribosomes, probably by an intercalation-type binding to ribosomal RNA.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 074, Molecular basis of biological regulation

Literature Cited.

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PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03
Entomology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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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
70 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	61102A	3A061102B71P	03	035
b. CONTRIBUTING				
c. [redacted]	CDOG1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Ecology and Control of Disease Vectors and Reservoirs (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 005900 Environmental Bio 010100 Microbiology

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18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	3	60
	CURRENT 72	3	60

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Arbovirus; (U) Trypanosomes; (U) Ecology; (U) Mosquitoes; (U) Disease Vectors; (U) Tsetse flies; (U) Control;

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

23. (U) Studies emphasize control of vectors of arbovirus and parasitic diseases of military significance. Objectives are incrimination of vectors and understanding of host-parasite relationships initially, understanding of vector biology and disease transmission mechanisms ultimately in order to develop more effective control procedures

24. (U) Invertebrate vectors and vertebrate reservoirs and hosts are collected in areas of known disease activity. Infection rates are determined, as are flight ranges, blood meal sources, breeding habits, and other biological characteristics. Other biological processes, such as pathogen transmission, flight physiology, and diapause are studied in the laboratory.

25. (U) 70 07 - 71 06. Over 100,000 mosquitoes collected in an area in Maryland endemic for arbovirus disease. All collection data obtained since January 1969 analyzed to establish patterns of distribution and movement. Blood meal sources determined for over 2000 mosquitoes collected. Vector potential ratings assigned to species studied. Nearly 200 mammals examined for antibodies to California encephalitis virus, percent positives ranged from 0 to 40%, varying with species and area collected. Three unidentified virus isolates obtained from Aedes canadensis, one from A. vexans. Relationship of temperature and photoperiod to diapause in two potential U.S. arbovirus vectors determined. Trypanosoma congolense successfully transmitted by tsetse flies under laboratory conditions. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon originator's approval

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03, Entomology

Work Unit 035, Ecology and control of disease vectors and reservoirs

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Description

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

Progress

1. Eastern Maryland Arbovirus Study

a. Introduction. A program to investigate the ecology of arthropod-borne virus diseases in swamp habitats of eastern Maryland has been underway for several years by investigators at WRAIR. Until last year, the emphasis had been on isolation of virus agents from invertebrate and vertebrate animals. Last year, intensive entomological studies were begun, with an emphasis on population dynamics and feeding, oviposition, and flight habits of the most abundant species occurring in the swamp, and especially those species yielding virus isolates. This report covers field data accumulated since last year's annual progress report and also results of data analyses made since that time. For sake of convenience results of field collections made in Delaware and Afghanistan are also reported here. Strictly virological aspects of this program are reported under Project 3A061102B71Q, Communicable Disease and Immunology, Work Unit 166, Viral Infections of Man. For background of the overall study, and a detailed description of the study site, see WRAIR annual reports for previous years.

b. Objectives. The entomological aspects of the study sought to study the possible role of species other than Culiseta melanura Coquillett as vectors of mosquito-borne virus diseases in the Pocomoke Cypress Swamp. In addition to evidence of present involvement as vectors, evidence was sought pertaining to factors which relate to potential involvement. These factors include: flight range, seasonal

occurrence, susceptibility to virus infection, host preference, daily activity patterns, and larval breeding habitats. Until these factors can be fully evaluated for the most abundant species occurring in endemic areas, the likelyhood of involvement of other vertebrates, including man, in virus transmission under altered ecological or climatic conditions cannot be assessed.

c. Methods. The same 5 major habitat types (macrohabitats) present in the Pocomoke Cypress Swamp, Maryland were sampled in 1970 as in the previous year: Closed swamp, open swamp, upland forest, marsh, and farmland areas transitional between these types were also sampled. Collection sites were numbered arbitrarily as shown in Fig. 1. Macrohabitats were again sampled weekly for mosquito larval breeding and presence of adult mosquitoes. Collection techniques included light traps augmented with solid CO_2, human biting collections and sweeping collections. Engorged females were identified and frozen for virus isolation after the abdomens had been cut off and saved for precipitin test identification of the blood meal. Unengorged females were identified and frozen without dissection. Collection records were coded on a standard machine data punching format (WRAMC Form 1200) and all data were stored on magnetic tape for machine data processing.

d. Results

(1) Mosquito population studies

(a) Larval surveys

During the period January-December 1970, collections of mosquito larvae were made. Table 1 lists the species collected by month. Collections consisted of 14,196 mosquito larvae comprising 21 species. Collections were made from established sites in 5 ecologically diverse areas. The sites sampled were the same as those sampled in 1969 (see WRAIR Annual Progress Report for FY 70). The 5 most abundant species collected, in order of decreasing abundance, were: Aedes canadensis, Culex territans, Culiseta melanura, Culex restuans, and Aedes vexans. The results of the larval surveys demonstrated again that the distribution and abundance of larval mosquitoes among habitats within and around the swamp are not necessarily correlated with the distribution and abundance of adult mosquitoes. The univoltinism of Aedes cantator in this area was again confirmed, as was the phenological relationship between Culex salinarius and C. restuans - the former appeared in the swamp approximately one month later than the latter species.

(b) Adult Collections

Included in last years' annual progress report were results of collections made during the period March-September 1969. Collections have been processed and identified now for the period September 1969 - May 1970. During 1969, 346,691 adult mosquitoes were eventually captured and during 1970, through May, 93,389 adults were

captured and identified. No testing of 1970 pools for virus has been accomplished to date. Aedes canadensis continues to be the mosquito most frequently captured by far, accounting for 92% of the mosquitoes captured in 1970. As collections from later in the year are identified, however, this percentage should drop considerably.

(c) Analysis of mosquito collection data from 1969

In order to assess quantitatively the validity of the 5 habitat types selected in 1969 for sampling mosquito populations, the 1969 light trapping data were transformed to natural logarithms of individuals captured per trap night. Analyses of variance were then performed on the data for the 4 dominant species. Two sites from each habitat were selected for analysis which were considered to be representative of the 5 habitat types sampled (Tables 2 and 3). Data from sites located in areas transitional between habitats were not analyzed. In all but one instance differences between habitats and between weeks were found to be highly significant. There were no significant differences between habitats and between weeks were found to be highly significant. There were no significant difference found between trapping sites within the same habitat. The one exception was Aedes cantator, for which differences among habitats were found not significant.

It can be seen by inspection of Table 2 that there are obvious differences in capture rates among the species. While found commonly throughout the swamp and surrounding areas, Aedes canadensis had a significantly lower capture rate ($p = .05$) at the two least shaded sites (15 and 17). Larvae were found in standing water at virtually all of the sites, especially in the spring, and could be found year-around. (Fig. 2).

Culiseta melanura had significantly lower capture rates ($p = .05$ in upland forest sites (10 and 11), cultivated area sites (14 and 15) and in swamp sites closest to the river (1 and 2). The pattern of larval distribution conformed roughly to the pattern of adult captures. C. melanura larvae were present in the swamp all year, but they were never collected outside the swamp. Adults of Culex salinarius were most abundant on the southwest periphery of the swamp (sites 14, 15, 16, 17) and scarcest in the interior of the swamp. There was little correlation between adult and larval abundance, larvae being found most numerous in the northern part of the swamp after the heavy mid-summer rains, and in sites outside the swamp.

Aedes cantator adults were more evenly distributed than those of the other dominant species. In general, the largest collections occurred on the southwest periphery of the swamp, the smallest in the northern part of the swamp. Too few larvae were collected to establish a definite distribution pattern, but the largest numbers were collected near site 1, the poorest adult site.

Most of the remaining species could be placed into one of four basic distributional patterns: (1) Swamp species; larvae and adults found virtually only within the swamp --Aedes atlanticus, A. infirmatus, A. aurifer, A. thibaulti, Orthopodomyia signifera, Toxorhynchites rutilus septentrionalis and Uranotaenia sapphirina. (2) Peripheral species; adults occur in largest numbers in the periphery of the swamp, larvae found more frequently outside of the swamp than within -- Anopheles crucians-bradleyi, An. punctipennis, Culex erraticus, C. pipiens, Psorophora ciliata and P. confinnis. (3) "Invader" species; those for which no evidence of larval breeding could be found in the swamp, but were present as adults -- A. sollicitans, A. taeniorhynchus, and An. quadrimaculatus. (4) Undetermined; species that either showed no preference or were seldom collected -- most others with the exception of Mansonia perturbans. Circumstantial evidence pointed to it being a swamp or peripheral species, but larvae were not collected in or near the swamp.

Among the factors which determine the vector potential of various mosquito species is their distribution in time and space. The larval stages of mosquitoes, being relatively sedentary, are confined to generally well defined aquatic habitats which can be roughly correlated with patterns of vegetation, especially those patterns which are produced as a result of edaphic factors. Adult mosquitoes, on the other hand, are more motile, and their distribution in space may or may not conform to the distribution of the larval stages. If suitable larval habitats are present in one type of area, but suitable blood feeding hosts are scarce or absent, adult females must migrate from their breeding areas to other areas in order to obtain blood necessary for reporduction. We sought to examine the relation of both larval and adult mosquito stages to the major ecological habitats present in the vicinity of the swamp, in an attempt to discover the potential of the various mosquito species to introduce arbovirus into the swamp as well as to export virus from the swamp.

The analyses of variance confirmed that inter-habitat differences in adult collecting rates differed significantly for the four species analyzed, whereas intrahabitat differences were not significant. That these differences were not, in each instance, merely reflections of differences in larval distribution, can be seen by examining species such as Culex salinarius where adult and larval distributions varied considerably. Such differences in distribution may be attributable in part to differences in collecting methods used, but in many cases actual migration from breeding areas apparently took place, a conclusion which was reinforced by the results of the blood meal identifications (see following section). Adult and larval distribution of Culex territans, for example, corresponded closely. Adults tended to remain close to their primary breeding area probably because frogs, their preferred host, were present in abundance there. Culex salinarius adults, on the other hand, apparently dispersed from areas of heavy breeding within the swamp to other areas, especially those adjacent areas to the south where their preferred hosts (domestic mammals) were more abundant. Adult females of Culiseta melanura were present in greatest numbers within the swamp habitats.

This is consistent with the availability of birds in the swamp where the greatest abundance of mosquito larvae are present. This is probably a major factor in the confinement of EEE and WEE to swamp habitats in the eastern US during non-epizootic periods. An extreme situation is demonstrated by the collection of adult Aedes sollicitans, A. taeniorhynchus, and Anopheles quadrimaculatus in the swamp where they were never found in the larval stage. It is difficult to say, however, whether they are attracted into the swamp by the adult collecting technique (light + CO_2) or by hosts which occur in the swamp. The results of the blood feeding study show that these species, although primarily mammal feeders, will feed on birds and reptiles, both of which are common in the swamp. A mechanism for "spillover" of virus from the swamp to areas inhabitated by man and domestic animals is thus present, probably dependent upon unusually large populations of "invader" species entering and leaving the swamp.

From the standpoint of spatial distribution, the tendency in and around the swamp is toward aggregation of adult mosquitoes in large measures as a function of differences in host preferences and availability. This aggregation is exaggerated somewhat by the use of CO_2-augmented light traps which are biased toward blood-seeking females. The effect of this aggregation on arbovirus transmission is toward high transmission rates within the swamp where Culiseta melanura and birds reach peak densities, and low transmission rates in surrounding areas.

The seasonal population peaks of many mosquito species in the swamp are roughly synchronous and occurred in 1969 during the period of peak virus isolation in mosquitoes. All recoveries of EEE and WEE from C. melanura were from mosquitoes collected 15 July - 8 September, although serological evidence from sentinel quail indicate that transmission was occurring at least from mid-June through late October (reported elsewhere in this report).

The 1969 midsummer peaks of Aedes canadensis, A. atlanticus, Culex salinarius and Psorophora ferox seem clearly related to the heavy July and August rains. Some common mosquitoes have seasonal distributions which did not conform to this pattern, however. Aedes cantator adults reached peak density in the spring and were present in very low numbers during the summer. This observation is in contrast to reports of its being a multivoltine species in other areas. Aedes vexans and Mansonia perturbans captures peaked in early summer, and although they could be involved in early season virus transmission, they would be of little importance as vectors during peak periods of virus transmission.

(2) Mosquito blood meal determinations

Blood meal determinations were made on engorged mosquitoes from Afghanistan, Delaware and the Pocomoke Cypress Swamp, Maryland. Specimens were collected using a variety of techniques, including CDC miniature light traps with and without dry ice, D-vac® vacuum sweeps

around caged animals, resting box collections, Malaise traps, net sweeps and human biting collections. The trituration and testing of engorged mosquitoes followed the techniques of Tempelis and Lofy (1). Only antisera with a minimum homologous titer of 1:10,000 and no crossing with heterologous antigens at 1:1,000 were used. All screening antisera were prepared in rabbits and specific antisera in roosters. Precipitin tests were conducted in capillary tubes with the readings being recorded at 1 and 2 hours, and then overnight. Controls were set up daily for all antisera used. Where crossing occurred with anti-deer, anti-goat and anti-bovine with heterologous antigens, the cross reactions were virtually eliminated by serial 2 fold dilutions of the antisera up to 1:16 using phosphate buffered saline, the same diluent as used to grind mosquito abdomens. Where crossing could not be eliminated at this dilution, test results were recorded as deer-goat, deer-bovine or deer-goat-bovine. All multiple feedings were retested.

(a) Pocomoke Cypress Swamp

About 2000 blood-engorged mosquitoes specimens were collected from May through November, 1969. Mosquitoes were first screened for bird, reptile or mammal feeding. If negative, a benzidine test was done to verify the absence of blood. Results of these screenings are shown in Table 4. Specific host determinations were then done on all positive reactors from the screening tests. Results of these specific determinations are shown in Tables 5 and 6. Five species of mosquitoes were considered in depth in light of their strong vector potential:

Aedes canadensis: This species fed readily on mammals and reptiles, and to a limited extent on birds, and was found to be avidly attracted to humans throughout the year. Especially noteworthy was the extremely high rate of deer feeding, comprising about 47% of the total feeds. The omnivorous feeding habits and abundance of A. canadensis indicate its potential as a vector of arboviruses. By feeding avidly on mammals and occasionally on reptiles and birds, it is exposed to all potential virus reservoirs. This same feeding pattern then becomes an excellent means of dissemination. The degree of feeding on deer and the reported deer involvement with CEV, WEE and Cache Valley agents again point to its vector potential from the standpoint of range of host feeding. Isolation of undetermined arboviruses from A. canadensis pools from our study area as well as CEV from pools collected elsewhere furnishes further evidence of its involvement, while its attraction to man makes it of possible medical importance. The percent of A. canadensis found positive for reptile feeding was constant throughout the year, except in late spring. At that time there appeared to be increase in reptilian feeding. We find this observation to be consistent with previously reported findings. We believe that the increased feeding by A. canadensis on reptiles, especially turtles, is dependent on host availability and that the apparent increase in reptilian feeding reflects the oviposition behavior of the turtles. In our study area, turtles leave the swamp habitat in spring and travel to higher ground where the female deposit their eggs. This movement takes them from the protection of the swamp

and increases their exposure to mosquito feeding. Should a poikilothermic virus reservoir exist in our area as has been reported from other areas of the U.S., this feeding by A. canadensis could be a vehicle by which virus is reintroduced into a warm-blooded host cycle.

Aedes atlanticus: This species was frequently attracted to small mammals, especially sentinel rabbits, and occasionally to caged rats and raccoons. It was not recorded from domestic animals outside the swamp proper. Reptiles appeared to play a role as hosts of this species whereas birds did not. It was frequently attracted to humans.

Psorophora ferox: The feeding patterns of A. atlanticus and P. ferox were almost identical. Both species were attracted to small mammals and humans and to a lesser extent to birds. Neither species was found in large numbers on the swamp periphery close to domestic animals. Both species fed with equal frequency on sentinel rabbits, but only P. ferox fed avidly on caged rats. Although attracted to them, it did not feed on reptiles. Though representing a small portion of the mosquito population, the feeding activity of A. atlanticus and P. ferox becomes significantly important considering their intensive feeding on rabbits. Substantial involvement of rabbits with CEV has been well established. This suggests that these two mosquito species could be involved in the natural transmission of this arbovirus. Feeding on humans by these species implies a possible vector potential for CEV to man as well.

Culex salinarius: When collected, this species was most often found engorged near domestic animals on the periphery of the swamp. Birds and reptiles did not appear to play a substantial role as hosts for this species. This species is of potential medical importance through its feeding habits as well. It is exposed to arbovirus activity by its feeding on sylvatic animals, while its apparent preference to leave the sylvatic habitat and feed upon the surrounding domestic livestock suggests a possible mechanism for virus movement from the swamp to domestic animals. Credence is lent to this possibility by the isolation of EEE from pooled C. salinarius specimens.

Culiseta melanura: This species showed the least variation in its host selection of any of the mosquitoes studied. Over 90% of the C. melanura tested were found to contain avian blood, the majority of these being from passerine birds. Occasional quail-fed specimens were found, but no chicken positives were, even though chickens were present on a farm near the swamp. All mammal positives were found in late summer or fall, after the main peak of abundance of the C. melanura population was past. No reptile or human positives were found. Although C. melanura is probably primarily responsible for disseminating EEE and WEE throughout the avian population, we feel, based on our findings showing minimal non-avian feeding, that under the conditions prevailing in the swamp at the time of our study, it is unlikely that this species is responsible for the spread of these viruses into the mammalian and reptilian vertebrates. It is possible, however, that this feeding pattern may vary under conditions other than those examined. A

temporal analysis of C. melanura feeding reveals an interesting observation. Although few in number, all mammal feedings were found to occur during the latter part of the year, after the mosquito population peak had subsided. Earlier studies have reported similar findings. The number of birds found in the swamp during this period increases due to fall migration, suggesting that bird availability alone is not the reason for this apparent shift. However, it is possible that C. melanura feeds most readily on a particular summer resident. The departure of the summer residents in fall could account for increased feeding on secondary mammalian hosts. By preferential feeding, each mosquito species is exposed primarily to that group of arboviruses utilizing a particular vertebrate host as its reservoir or amplifying mechanism. Thus, Culiseta melanura by feeding primarily on avian hosts, is most likely to contract EEE or WEE, both associated with birds. Figure 3 graphically compares group feeding preferences of the 5 mosquitoes we have looked at in depth in an effort to demonstrate this type of preferential feeding.

California encephalitis virus group agents have most often been associated with rabbits as their vertebrate reservoir or amplifying mechanism. Thus, comparing the attractiveness of rabbits against other small mammals could indicate a preference by certain mosquito species for this host, thereby increasing or decreasing their exposure to this group of arboviruses. Figure 4 presents comparative results on vacuum sweep collections around caged sentinel rabbits, rats and raccoons, showing the frequency of collection for each mosquito species. Figure 5 shows the frequency of collection for each species in human biting collections. This demonstrates each mosquito species' attractiveness to humans, thus implying their potential to transmit acquired arboviruses to man.

(b) Delaware

A limited number of engorged mosquitoes were analyzed for the University of Delaware. No information was available as to exact trapping method, locality or date of capture. Results of these tests are shown in Table 7.

(c) Afghanistan

Fifty Anopheles pulcherrimus and one undetermined culicine mosquito were collected from the village of Angourbugh, Afghanistan on 21 and 24 July, 1970. Results of these blood meal determinations are shown on Table 8.

(3) Isolation of viral agents from field collected mosquitoes and bird blood clots.

During the current year virus isolations have been attempted from a variety of field collected material by primary passage in suckling mice. Attempts to isolate virus from 120 pools of deer flies

(family Tabanidae) containing 7 deer flies per pool were negative. Aliquots of triturated mosquito pools passed by the Department of Virus Diseases for virus isolation in a vertebrate cell culture system were also inoculated intracerebrally in suckling mice. From 2,048 mosquito pools collected in 1969 in the Pocomoke Cypress Swamp, Maryland, 4 presumptive virus isolates have been recovered. One isolate has been recovered from a pool of 4 female Aedes vexans collected 19-20 May and 3 isolates have been recovered from A. canadensis containing 20-25 mosquitoes per pool collected during August. Characterization and identification of these agents is being done by the Department of Virus Diseases. Five virus isolates were recovered by primary mouse passage from mosquito pools from which isolates had previously been recovered by vertebrate cell culture, thus validating the original isolations. Details of virus isolations from mosquitoes are shown in Table 9.

Six virus isolates have been recovered by primary mouse passage from 9 wild bird blood clots and 4 isolates from 4 sentinel bird blood clots collected during 1969 from the Pocomoke Cypress Swamp. Identifications of these isolates has not yet been made.

(c) Serological studies of CEV in mammals

(a) Serological survey

Mammal sera collected from the Pocomoke Cypress Swamp and adjacent areas between 1969 and 1971 were examined for evidence of past infection of California encephalitis virus (CEV) agents. Plaque reduction neutralization tests were employed using 3 strains of CEV. Preliminary work included producing stock seed virus for each strain to be used, producing mouse hyperimmune ascitic fluid against each strain and analysis of various cell culture lines to determine the most sensitive virus/cell culture system available. Results of virus/cell culture analysis are shown in Table 10.

Sera to be examined were first heat inactivated and diluted 1:10, then screened against Keystone strain CEV. This strain is the only CEV agent that has been isolated from Maryland to date. Results of these preliminary efforts are shown on Table 11. Future studies will include determination of the specific strain or strains of CEV causing the reduction shown in screening tests against Keystone strain CEV, and experimental infection of laboratory rabbits to determine the extent of viremia as well as the titer, duration and crossreactivity of the resulting antibody produced.

(b) Sentinel rabbits

Paired sentinel rabbits were caged in the Pocomoke Cypress Swamp from 17 May to 16 November, 1970, at collection sites 3, 7, and 16 (Fig. 1). Pre-swamp exposure bleeds as well as bi-weekly bleeds were taken from each sentinel rabbit. Three groups of 6 rabbits each were used throughout the year. Only 1 rabbit showed positive antibody formation to CEV agents as a result of the swamp exposure. That

rabbit was caged at Site 7 from 28 July to 7 September. Bleeds of that rabbit on and subsequent to 25 August showed 100% reduction against Keystone strain CEV, while the prebleed and all bleeds previous to 25 August showed no antibody present. It is estimated that the rabbit was infected about 11 August. Tests to determine the strain of CEV infection showed greatest reduction against Keystone strain CEV.

2. Basic Biology Studies

a. Mosquito flight studies

During the year, basic techniques of tethered mosquito flight were refined and standardized using 4 mechanical flight mills modified from the design of Rowley and Graham (2). The objectives of this study are:

(1) to determine interspecific differences in flight capacity among various vector mosquito species.

(2) to use flight mill performance as a measure of quality of insectary-reared mosquitoes.

(3) to determine the effect of pathogen infection on flight mill performance.

(4) to use flight mill performance as a measure of physiological state of individual mosquitoes.

The principal problem area in tethered flight studies is in identifying, measuring, and eliminating or otherwise controlling variation in speed, duration, and distance of flights so that causal effects above can be evaluated. In one series of experiments, freshly emerged female Culex salinarius and C. restuans were flown 1 and 2 days after emergence, without having been furnished any food as adults -- only water. The results of 31 females so flown are shown in Table 12. Under these circumstances all energy for flight must have come from glycogen accumulated during the larval stages. In spite of the elimination of the variation resulting from adult intake of food, total variation was very large, and the difference in distance flown in a 2-hour test period between 1 and 2 day old females was not significant by "t" test. Future work will include studies on Nosema-infected and non-infected Anopheles stephensi flight and on the effect of light and temperature conditioning of females on flight performance.

b. Overwintering of culicine mosquitoes

A preliminary study has been completed on the overwintering mechanisms and phenology of 2 common eastern U.S. mosquitoes: Culex restuans Theobald and Culex salinarius Coquillett. The objective of this study was to determine the response of C. restuans and C. salinarius to simulated late fall conditions of temperature and photoperiod and to determine if the observed responses were consistent with that observed

in species such as C. pipiens known to undergo adult diapause. Additionally, we hoped that their respective physiological responses would furnish clues to the difference in reported geographical distribution of the two species.

The seasonal and geographic distribution records which form the basis for Figs. 6-9 were obtained primarily from 170 published references. These were supplemented by published and unpublished records of mosquito survey programs of US Army Area Laboratories. The distributional maps were constructed by first making a map for each month of the year for each species, using dots to indicate sites of collections where dates of collection of adults were recorded. Records were discarded where specific identification was questionable. Lines were then fitted by eye to encompass the northermost dots. Isolated dots hundreds of miles from any other dot were not included, but were marked on the composite maps.

Various simulated environments were produced in modified BOD incubators. The light source used was a 40 W incandescent appliance lamp. A wire-wound power resistor calibrated to furnish the same heating effect as the lamp was wired to the light programmer so that when the lamp was off, it was on, and vice versa. Females of both species were subjected during all life cycle stages to 4 combinations of temperature and photoperiod: 20°C, LD 16:8; 20°C, LD 8:16; 27°C, LD 16:8; and 27°C, LD 8:16. Standard larval rearing procedures were used and were identical for both species.

Blood feeding trials were conducted for each species and treatment by randomly placing 10 females in each of 6 glass jars and providing a baby chick as a blood meal source. The lamps in all incubators were programmed to come on at varying times, but to go off simultaneously. The feeding trials were conducted in the incubators during the 2 hour period after the lamps went off. Only females which had emerged during the same 24-hour period were used in the feeding trial, and in each instance the trials took place 4 days after emergence.

(1) Geographic distribution.--The approximate northermost limits of activity of C. restuans and C. salinarius, based on published collection records, are shown in Figs. 6-9. For any given month, the range of C. restuans adult activity extends farther north than does C. salinarius. Both species reach their maximum geographical limits during the month of August. At this time, the northermost limit of C. restuans extends well into southern Canada, where it has been collected above the 50th parallel, whereas the northernmost limit of the latter appears to be roughly 46°N. The range of C. restuans also extends much farther west than does that of C. salinarius.

(2) Response to temperature and photoperiod.--The number of female C. restuans and C. salinarius taking blood after being subject to 4 combinations of temperature and photoperiod is shown in Table 13. The proportion of C. salinarius taking blood was higher at all treatments

than that of C. restuans. Blood feeding frequency among C. salinarius females varied inversely with length of photoperiod and temperature--the proportion taking blood at 27°C, LD 16:8 was significantly less than at 20°C, LD 8:16. With C. restuans, the pattern was strikingly different. There was no significant difference in blood feeding between short and long photoperiods at 27°C, nor between the 2 temperatures under long photoperiod. Under conditions of low temperature and short photoperiod, however, blood feeding frequency was significantly less (about 1/10 the frequency observed under other treatments). Fed females of both species developed ovaries fully under all conditions tested. No gonotrophic dissociation was observed.

In mosquitoes, an altered physiological state under endocrine control in response to an environmental trigger can be considered diapause, whereas a lowering of general activity due to chilling is not. The responses of C. restuans and C. salinarius to a simulated environment of short photoperiod and low temperature is consistent with the hypothesis that the former species undergoes diapause prior to overwintering, whereas the latter species does not. The more northerly distribution of C. restuans also supports this hypothesis. Unfortunately, very few field studies have been done on the overwintering of these 2 species, but the few documented discoveries of overwintering habitats would indicate that their overwintering behavior differs. The only fully documented recovery of C. salinarius females which survived a winter (where the climate does not permit year-around breeding) known to us is from woodchuck burrows in Delaware. Culex restuans, on the other hand, has been recovered from a variety of overwintering habitats, in Connecticut and New Jersey.

The recognition of 2 different overwintering mechanisms way have bearing on the question of overwinter survival of arboviruses. Considerable evidence indicates that hibernating Culex mosquitoes are predominently nulliparous. It has been shown experimentally that in C. pipiens, females showing gonotrophic dissociation and females fed a straight carbohydrate diet developed fat bodies equal to those found in naturally occurring prehibernating females. Gonoactive mosquitoes of the same age produced significantly less fat. Recently however, reports of collections of overwintering mosquitoes in New Jersey have shown that at least under certain conditions parous C. pipiens can accumulate sufficient fat reserves to overwinter. It would seem likely that these would be relatively old females which had oviposited relatively early in the season, early enough to have had time to build up a reserve of fat. They would also have had to suspend blood feeding activity in response to fall conditions of temperature and photoperiod or to have undergone gonogrophic dissociation after subsequent blood meals. Interspecific differences in cold hardiness and temperature differences of larvae development also play important roles in determining geographical ranges and phenology. Culex restuans is widely known as a "cool season" mosquito, a fact which is evidenced by its earlier appearance wherever it and C. salinarius have common ranges. This could be due to the ability of C. restuans to function at lower

temperature than C. salinarius, and also because at the northern limits of the range of C. salinarius, hibernation is not possible, and adults which are active in the warmest months have migrated north from warmer regions.

c. Colonization attempts

(1) Culex restuans. Our colony continues at a low level maintained by forced mating. Attempts, so far unsuccessful, to establish a self-mating colony are continuing.

(2) Little difficulty was encountered in rearing field collected larvae and pupae through to adults. The adults feed readily on chicken or human blood, and the engorged females are easily mated using the forced mating technique. Our attempts were directed toward methods of handling eggs which would result in higher hatch rates. Two batches of eggs were conditioned, yielding the following results:

Lot Number 1. On 3 July 1970, 15 female Aedes canadensis engorged on human blood. The engorged females were force-mated and placed separately in Styrofoam cups containing dampened cotton and lined with paper towels. Between 3 July and 10 July 313 eggs were removed from the cups and placed in a petri dish lined with a dampened filter pad. The petri dish was sealed with tape to maintain conditions of high humidity and was then stored at a temperature of 40°F. On 20 August the eggs were flooded with a mixture of tap water and water collected from a freshwater swamp. The eggs remained flooded for 3 days but failed to hatch. The eggs were then recovered and exposed to sub-freezing temperatures for 24 hours, then returned to a temperature of 40°F.

On 10 September the eggs were again flooded but did not hatch. They were then stored at 78°F. in the insectary (70% r.h.). On 14 September the eggs were placed in a dessicator jar and an attempt was made to hatch the eggs by means of lowering atmospheric pressure with a vacuum pump. Between 14-15 September 6 larvae hatched. Eight days later adults emerged. Beginning 3 days after adult emergence, 4 females were offered human blood meals. On 5 October, 1 female fed on a human host and was force mated. It died without ovipositing. The other 3 females refused to take blood and died.

Lot Number 2. This material was processed 15 January to 23 March 1971 and consisted of 96 eggs obtained from 3 females which had fed on chicken blood before being forced mated. The females were obtained by laboratory rearing of field - collected larvae. Eggs were collected and sorted in the same manner as for Lot Number 1. The conditioning procedure and the results are shown in Table 14. The overall hatch rate obtained, 20.8%, indicates that diapause has not been completely terminated by the laboratory procedures followed. These procedures could result in low level colony production, but the efficiency would be very low.

3. Glossina studies

Small colonies of Glossina austeni and G. morsitans are being maintained for studying the dynamics of transmission of African trypanosomes under laboratory conditions.

Transmission studies have commenced with another trypanosomes, T. congolense. The parasite is maintained in white mice by blood transfer or cyclical transmission. Unlike T. brucei, there is no indication that the age of the tsetse fly affects the susceptibility of the fly to infection with trypanosomes.

During the current year, studies have been made to determine whether tsetse flies could be infected with a parasite strain which had a long history of blood passage, what would be the most appropriate donor host for infecting flies and whether it would be possible to use laboratory infected flies to infect a calf with trypanosomiasis.

Between 12 February - 19 March 1971 G. austeni and G. morsitans were fed on infected calves at Fort George Meade which served as controls for T. congolense immunization experiments conducted by the Department Medical Zoology, DCD&I. Similar feeds were made at WRAIR on mice infected with the same strain of T. congolense. Dissection of samples of tsetse flies indicated that 4/39 or 10% of flies which fed on parasitemic mice developed trypanosome infections while 6/25 or 24% of flies exposed to infected calves were infected.

Conclusions and recommendations

1. Capture rates of adult and larval mosquitoes vary among ecological habitat types in a freshwater swamp in eastern Maryland. Different species differ in their capture rates and within the same species, capture rates of adults among habitats differ from those of larvae. This reflects differences in density and points out that factors responsible for adult density in a given area can differ markedly from those responsible to larval density. The main determinant of larval density is the relative abundance of suitable aquatic breeding sites whereas, the determinant of adult density as measured by light trapping is the relative abundance of suitable vertebrate hosts for blood feeding.

2. Culiseta melanura feeds predominantly (>90%) on avian blood. Mammal feeds which are detected occur in mosquitoes captured in the fall of the year. On this basis, C. melanura is unlikely to transmit EEE and WEE from infected birds to domestic mammals or man. Mosquito species which are abundant in the swamp and which commonly feed on mammals and occasionally on birds include: Psorophora ferox, Culex salinarius, and Aedes canadensis. Laboratory experiments should be performed to determine susceptibility to infection and ability to transmit EEE and WEE.

3. California encephalitis virus is active in the area of the Pocomoke Cypress Swamp, based on antibody surveys of vertebrate animals. Attempts to isolate viruses of this group from mosquitoes should be done.

4. Culex restuans and Culex salinarius differ in their ability to overwinter in Maryland. Both species should be tested for their ability to survive simulated hibernation conditions after virus infection.

5. Tsetse flies can be readily infected with Trypanosoma congolense either from infected mice or calves. The calf appears to be a more suitable donor since a greater proportion of exposed flies develop trypanosome infections. Additional transmission experiments are required to establish the minimal number of infected flies required to establish an infection in the calf and the maximal period that a fly can transmit T. congolense.

TABLE 1

Larval mosquito collections, 1970, Pocomoke Cypress Swamp, Maryland (Number of larvae)

Species	Month												Total
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
Aedes canadensis	113	512	746	1731	354	300	396	162	-	1	83	18	4416
Culex territans	-	-	-	-	290	787	647	292	344	156	71	-	2587
Culiseta melanura	62	58	39	150	79	273	365	191	73	134	537	74	2035
Culex restuans	-	-	-	-	337	580	178	1	241	190	150	-	1677
Aedes vexans	-	-	-	-	3	286	355	18	48	158	90	-	958
A. triseriatus	-	-	50	351	147	25	15	8	-	-	-	-	595
O. signifera	2	-	4	9	1	9	23	101	142	164	131	2	588
Culex salinarius	3	-	-	-	-	58	4	15	96	17	31	-	224
C. pipiens	-	-	-	-	-	-	49	-	44	38	69	-	200
Culex spp.	-	-	-	100	-	-	-	42	21	37	-	-	200
P. confinnis	-	-	-	-	-	-	21	-	43	70	2	-	136
Aedes cantator	-	5	12	64	15	36	-	-	-	-	2	-	134
Anopheles spp.	-	-	-	1	1	5	21	5	75	12	3	-	123
P. ferox	-	-	-	-	4	29	18	54	3	3	-	-	111
Aedes atlanticus	-	-	-	-	1	8	35	4	2	-	-	-	50
Other species*	-	-	9	6	-	8	75	28	13	16	6	-	161
Totals	180	575	860	2312	1332	2404	2202	921	1145	996	1175	94	14196

*In order of decreasing abundance: Uranotaenia sapphirina, Toxorhynchites rutilus septentrionalis, Aedes spp., Aedes aurifer, Anopheles quadrimaculatus, A. infirmatus, Anopheles bradleyi, Aedes sollicitans, Psorophora howardi.

TABLE 2

Light trap collection rates of 4 species of mosquitoes from sites in 5 types of habitats in Pocomoke Cypress Swamp, Maryland, 1969*

Habitat	Mosquito Species			
	Aedes canadensis	Culiseta melanura	Culex salinarius	Aedes cantator
Closed Swamp	3.07	3.81	1.75	1.18
Open Swamp	2.47	3.79	1.92	0.88
Upland Forest	2.78	2.44	1.21	1.00
Marsh	1.45	3.67	3.56	1.17
Cultivated Area	1.62	2.89	3.27	1.07

*Mean of Log_e mosquitoes per trap night of 2 sites considered representative of each habitat type.

TABLE 3

Analyses of variation in light trap capture rates of adults of 4 species of mosquitoes, Pocomoke Cypress Swamp, Maryland, 1969 (F values)

Variation Source	Mosquito Species			
	Aedes canadensis	Culiseta melanura	Culex salinarius	Aedes cantator
Habitats	19.89***	13.55***	11.34***	1.59
Weeks	12.69***	8.06***	11.19***	9.40***
Habitats x weeks	1.42	1.20	1.05	0.96
Traps (error)	-	-	-	-

p*** = <.001

TABLE 4

Results of Pocomoke Cypress Swamp mosquito blood meal screening tests

Mosquito Species	Number Mosquitoes	Mammal	Bird	Reptile	Negative
Aedes atlanticus	67	48		6	13
A. aurifer	1	1			
A. canadensis	1151	664	42	246	223
A. cantator	23	16	2	5	
A. infirmatus	8	7	1		
A. sollicitans	132	105	6	23	2
A. taeniorhynchus	17	10	3	4	
A. triseriatus	7	6			1
A. vexans	16	13		3	
Anopheles bradleyi-crucians	20	19			1
An. punctipennis	3	3			
An. quadrimaculatus	8	8			
Culex restuans	5		5		
C. salinarius	96	91	3		2
C. territans	7			5	2
Culiseta melanura	358	10	304	11	48
Mansonia perturbans	5	5			
Psorophora confinnis	2	2			
P. ferox	46	40	1		5

TABLE 5

Specific host determinations, mammal positives

Mosquito Species	Bovine	Deer	Dog	Goat	Human	Opossum	Pig	Rabbit	Raccoon	Rat	Squirrel	Deer/Bov.	Deer/Goat	D/G/B	Rat/Squir.	Undet. Mam.
Aedes atlanticus*	1	15						6	1							
A. aurifer																1
A. canadensis*	13	315	5	11	4	7	7	55	12	11	2	9	4	1	1	58
A. cantator	7	5		1	1		1					1				
A. infirmatus		3						2	1							2
A. sollicitans		3	87		14			1								
A. taeniorhynchus		43	6													
A. triseriatus		1			1				5							
A. vexans		12							1	2						
Anopheles bradleyi-crucians	7	3		5	1		6	7	1							
An. punctipennis		4						1								
An. quadrimaculatus	2	2		3			1									
Culex salinarius*	2	11	1	9		1		3		1						2
Culiseta melanura*		3						1	1							
Mansonia perturbans	1	1							2	1						
Psorophora confinnis							2									
P. ferox*		2							2							1

* Engorged material from light traps and resting box collections only.

TABLE 6

Specific host determinations, bird and reptile positives

Mosquito Species	Passeri-formes	Columbi-formes	Galli-formes	Other Orders	Undet. Bird	Reptile Positives
Aedes atlanticus*						1
A. canadensis*	22	2	1	1	15	113
A. cantator			2			5
A. infirmatus			1			
A. sollicitans			4			26
A. taeniorhynchus			3			4
A. vexans						3
Culex restuans	5					
C. salinarius*				2		
C. territans						5
Culiseta melanura*	272		10	25	23	
Psorophora ferox*					1	

* Engorged material from light trap and resting box collections only.

TABLE 7

Engorged mosquitoes from Delaware

Mosquito Species	Total Tested	Host	Negative
Aedes canadensis	2	Human (1) Bird (1)	0
A. atlanticus	4	Human (3)	1
A. vexans	6		6
Psorophora ferox	2	Rat (2)	0

TABLE 8

Engorged mosquitoes from Afghanistan

Screening Host	Number	Specific Host	Number
Mammal	51	Bovine	31*
Bird	0	Dog	0
Reptile	0	Goat	1
		Horse	6
		Human	4
		Rabbit	0
		Other	9**

* Including culicine specimen.
** Neither anti-camel nor anti-buffalo serum was available, even though these hosts were present in the study area.

TABLE 9

Isolation of viruses from female mosquitoes collected at Pocomoke Cypress Swamp, Maryland, 1969

Mosquito species	Isolation No. mosquitoes	(No. pools)
Aedes atlanticus	0/3,302	(151)
" aurifer	0/7	(3)
" canadensis	3/27,104	(1,116)
" cantator	0/4,089	(177)
" grossbecki	0/2	(2)
" infirmatus	0/723	(39)
" sollicitans	0/279	(19)
" taeniorhynchus	0/525	(24)
" triseriatus	0/129	(9)
" vexans	1/224	(18)
Aedes sp.	0/146	(19)
Anopheles bradleyi	0/60	(13)
Anopheles bradleyi crucians	0/355	(20)
Anopheles punctipennis	0/4	(2)
Anopheles sp.	0/1	(1)
Culex restuans	0/21	(12)
Culex salinarius	0/7,016	(303)
Culex territans	0/1	(1)
Culex sp.	0/2	(2)
Culiseta melanura	5/1,246	(75)
Orthopodomyia signifera	0/3	(2)
Psorophora ferox	0/876	(40)

TABLE 10

Tissue culture susceptibility to California encephalitis virus agents

Cell Line	LLC			Vero			Chick Emb.			ATCC, MK-2		
CEV Strain	BFS-283	Key	Triv	BFS-283	Key	Triv	BFS-283	Key	Triv	BFS-283	Key	Triv
Dilution of stock resulting in 100 PFU per 0.1 ml	1:2 10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-4}	10^{-5}	10^{-4}	10^{-4}		10^{-5}		10^{-5}
Plaque Size*	VS	L	VS	S	S	M	L	M	NO PLAQUES FORMED	S	NOT TESTED	S
Plaque Defination**	F	E	F	F	F	E	E	P		E		F
Plaque Production Rate ***	4	4	4	4	4	4	4	4		5+		5+

* VS=very small; S= small; M=medium; L=large.
<1 mm dia. 1 mm dia. >1 mm dia. 2-3 mm dia.

** P=poor; F=fair; E=excellent.

*** Time in days

TABLE 11

Serological evidence of Keystone strain CEV infection by plaque reduction neutralization test

Locality	Host*	Number Tested	% Positive**	% Equivocal***
Pocomoke Cypress Swamp & surroundings, Md.	Raccoon	19	10%	10%
	Opossum	15	0	0
	Squirrel	18	27%	27%
	Pig	32	0	0
	Deer	16	25%	31%
Assateague Island, Va.	Raccoon	46	4%	8%
	Opossum	6	0	0
Snowhill, Md.	Deer	45	26%	40%

* Serum diluted 1:10
** Positive = 80--100% reduction
*** Equovocal = 50--79% reduction

TABLE 12

Distance (M.) flown in 2 hour test period by one and two-day old *Culex salinarius* and *C. restuans* (unfed as adults)

Culex salinarius:

	1 day old	2 days old
N	18	11
$\bar{X}$	518.17	421.36
$S_{\bar{x}}$	80.13	134.22

Culex restuans:

N	-	2
$\bar{X}$	-	548.00
$S_{\bar{x}}$	-	168.50

TABLE 13

Number of female mosquitoes taking blood meal after being held under different conditions of temperature and photoperiod*

Photoperiod (Hours)	*Culex restuans* 20°C	*Culex restuans* 28°C	*Culex salinarius* 20°C	*Culex salinarius* 27°C
8	0.33 ± 0.33 (a)	3.20 ± 0.58 (b)	8.83 ± 0.31 (c)	6.83 ± 0.40 (c)
16	3.60 ± 0.51 (b)	4.00 ± 0.77 (b)	7.83 ± 0.48 (c,c)	5.83 ± 0.48 (c)

* Mean ± s.e. of 6 replications, 10 females per replication. Means not sharing a common letter differ significantly at 1% level by student's "t" test.

TABLE 14

Results of conditioning of a batch of 96 Aedes canadensis eggs obtained from laboratory reared females

Blood meal	Number Fed	Number Mated	Number Eggs	Collected
20 Jan 71	3	3	96	26 Jan 71

Storage Temperature	Dates	Days Exposed
78°F.	26 Jan-5 Feb 71	10
40	6 Feb-15 Feb 71	9
58	16 Feb-17 Mar 71	29
32	18 Mar-19 Mar 71	1
78	19 Mar-21 Mar 71	2

Flooded	Hatched	Percent*
22 Mar 71	6	8.3
23 Mar 71	9	12.5
Total	15	20.8

*Although the original batch consisted of 96 eggs, some were removed periodically to check for embryonation. Percentages are based on 72 eggs remaining at the time of flooding.

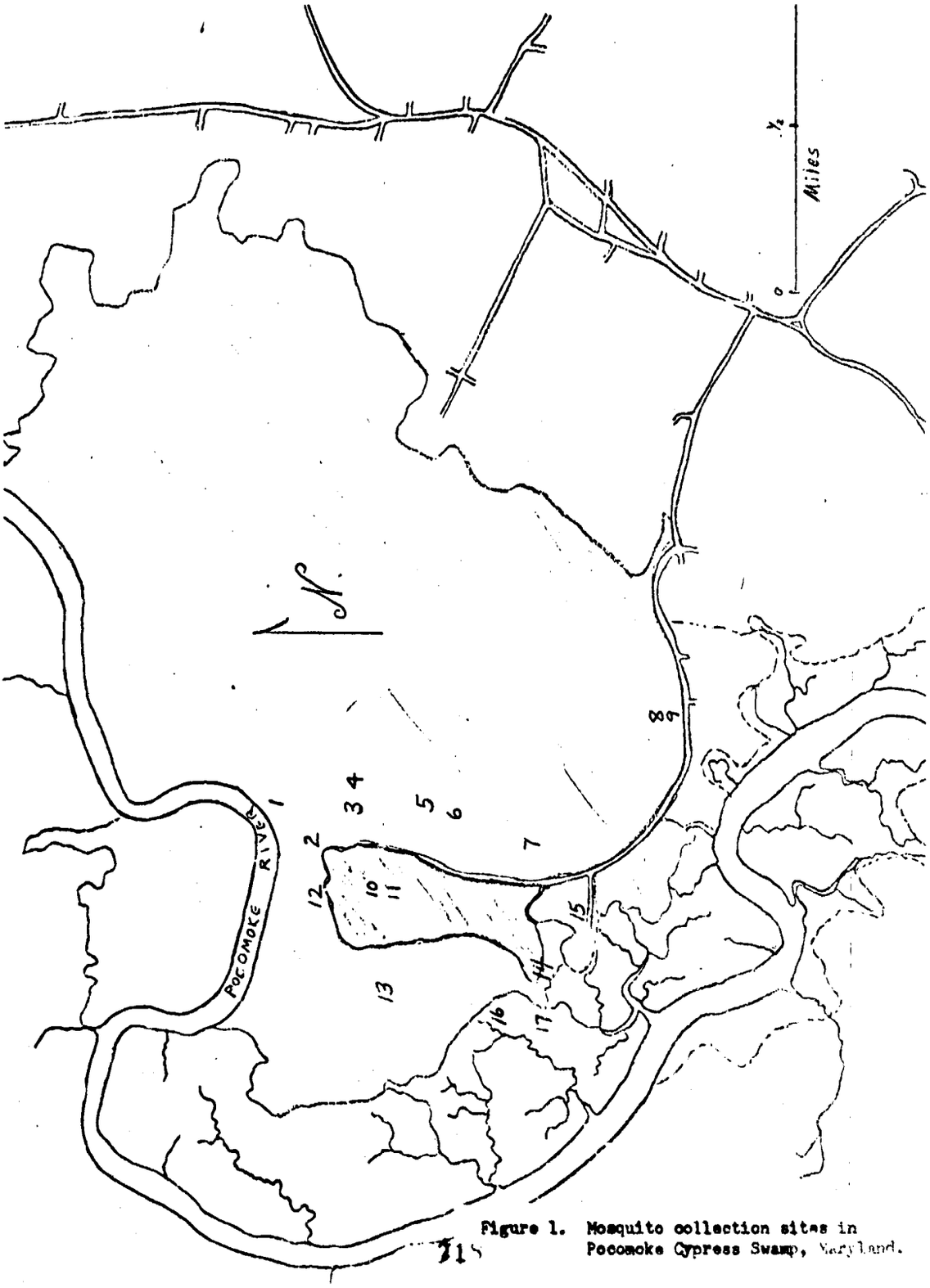
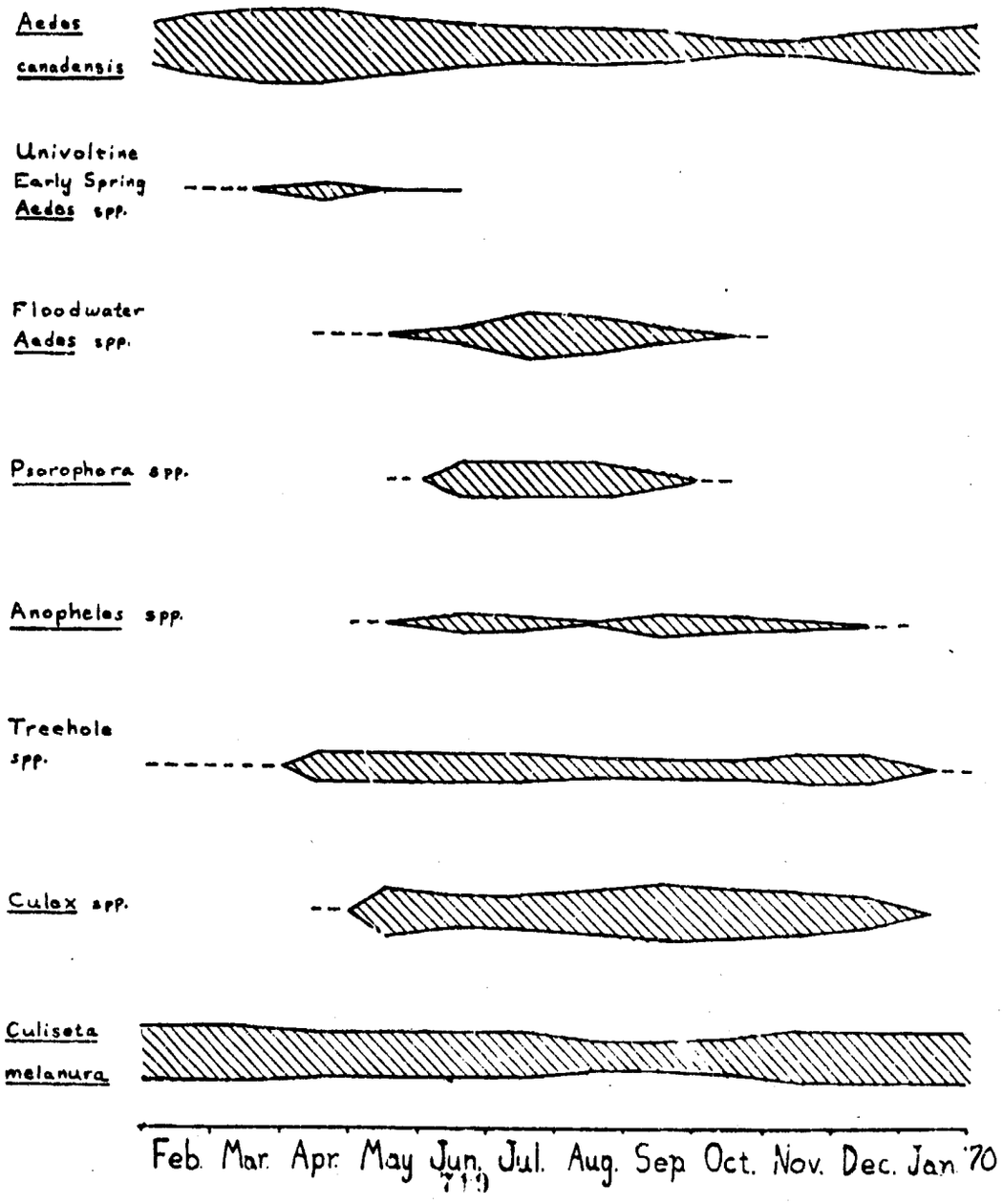


Figure 1. Mosquito collection sites in Pocomoke Cypress Swamp, Maryland.

Figure 2. Relative larval abundance, Pocomoke Cypress Swamp, Maryland, 1969.



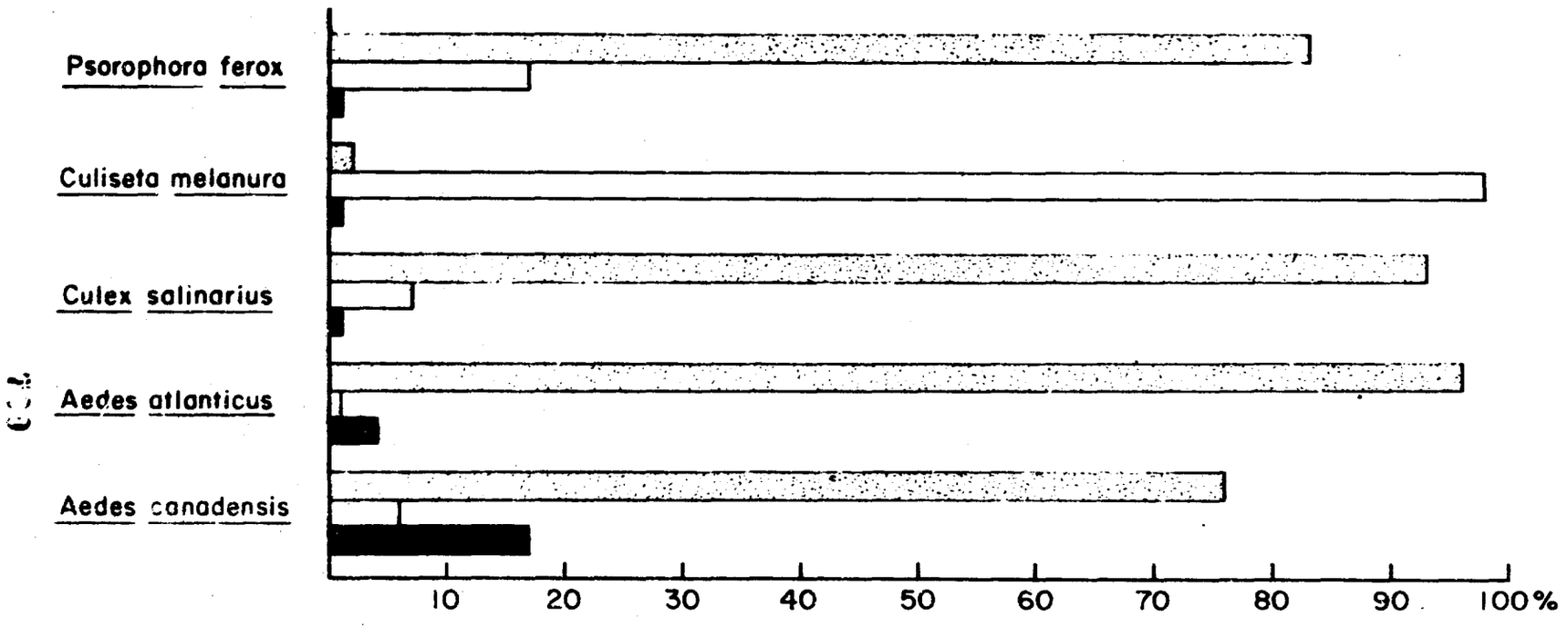


FIGURE 3. Result of Precipitin Tests on Engorged Mosquitoes Collected Without Bias

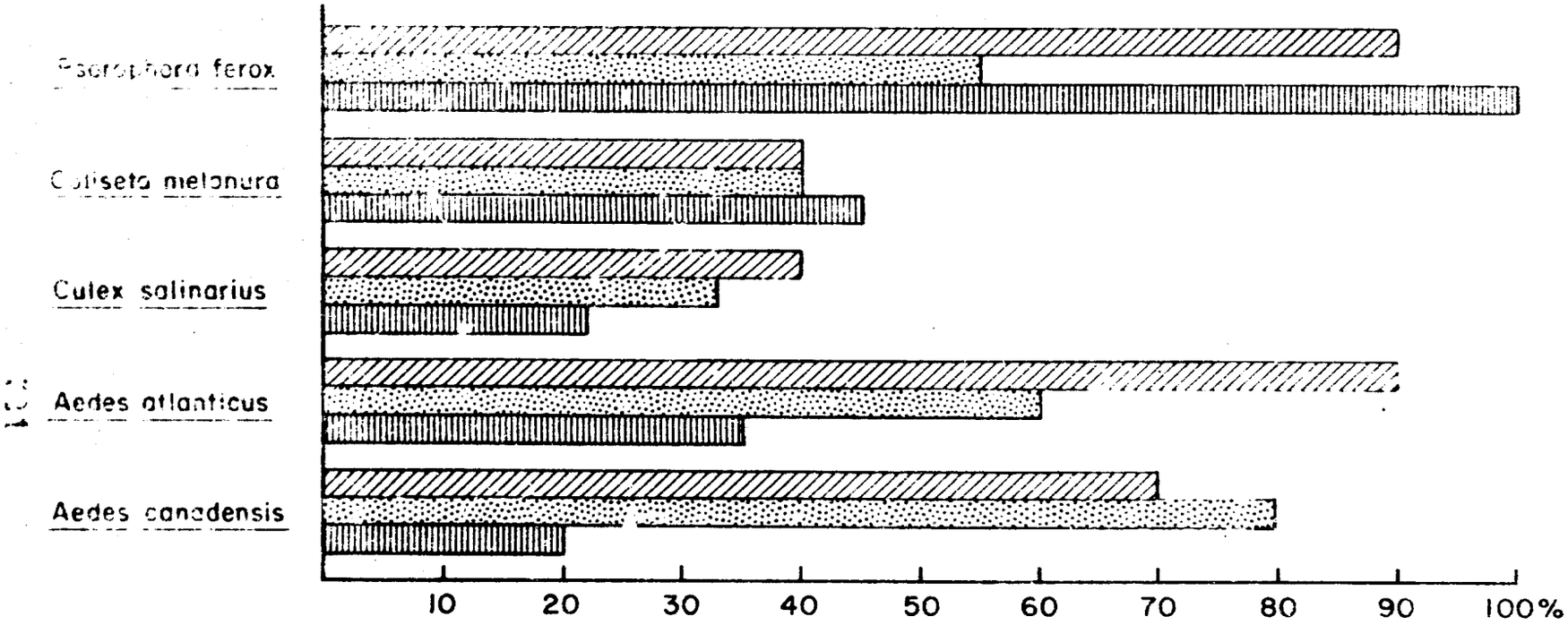


FIGURE 4. Frequency Present in Vacuum Sweeps from Small Mammals

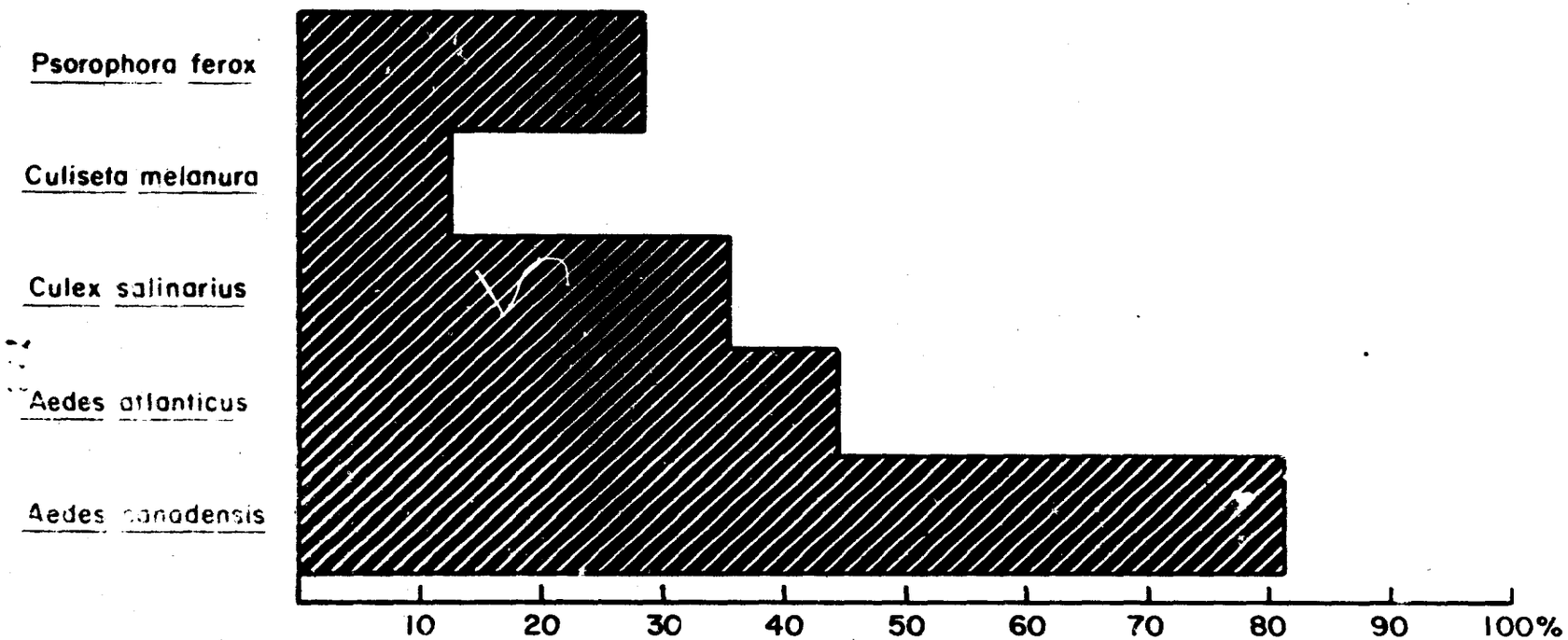


FIGURE 5. Frequency Present in Human Bait Collections

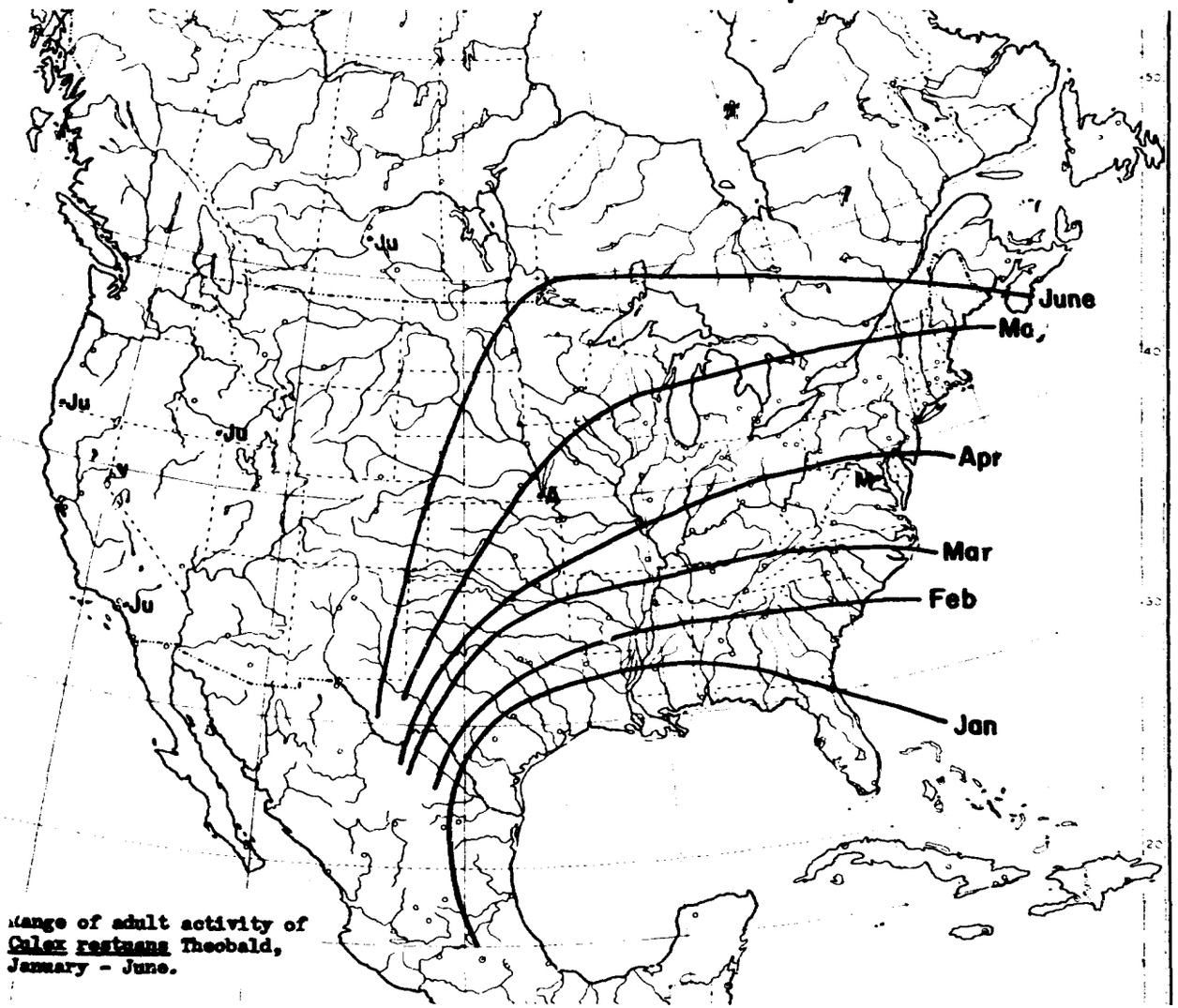


Figure 6. Range of adult activity of Culex restuans Theobald, January - June.

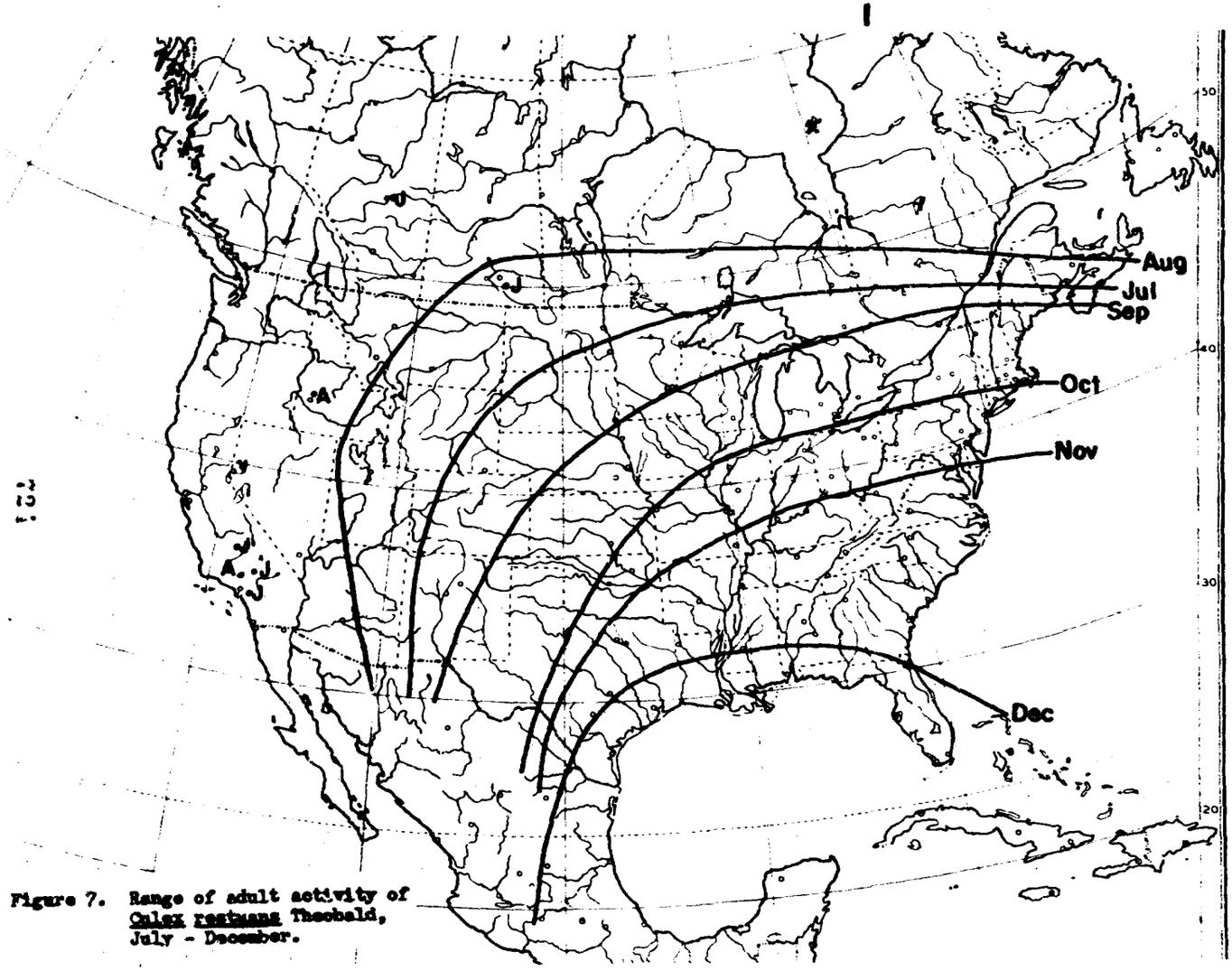


Figure 7. Range of adult activity of Culex restuans Theobald, July - December.

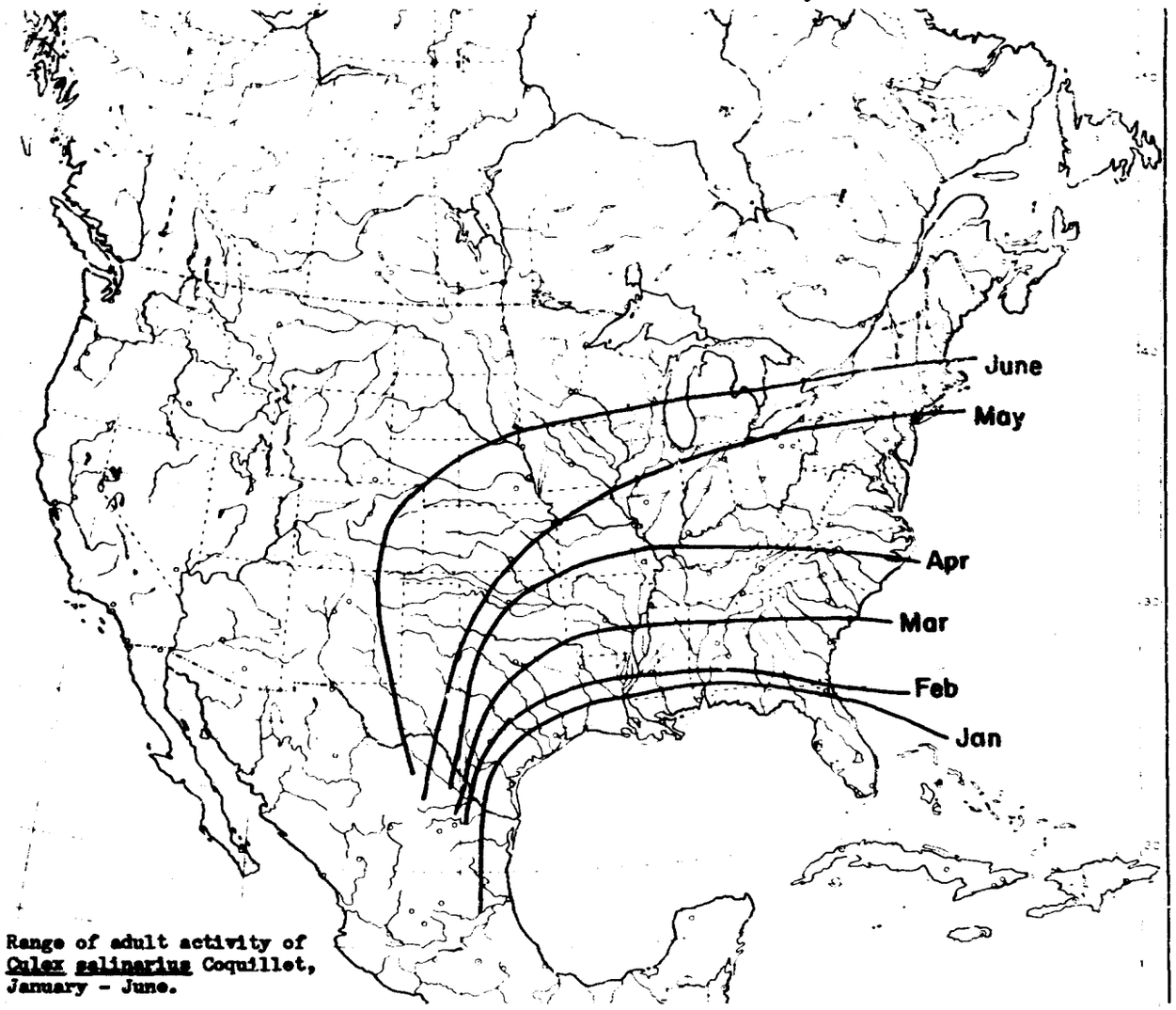
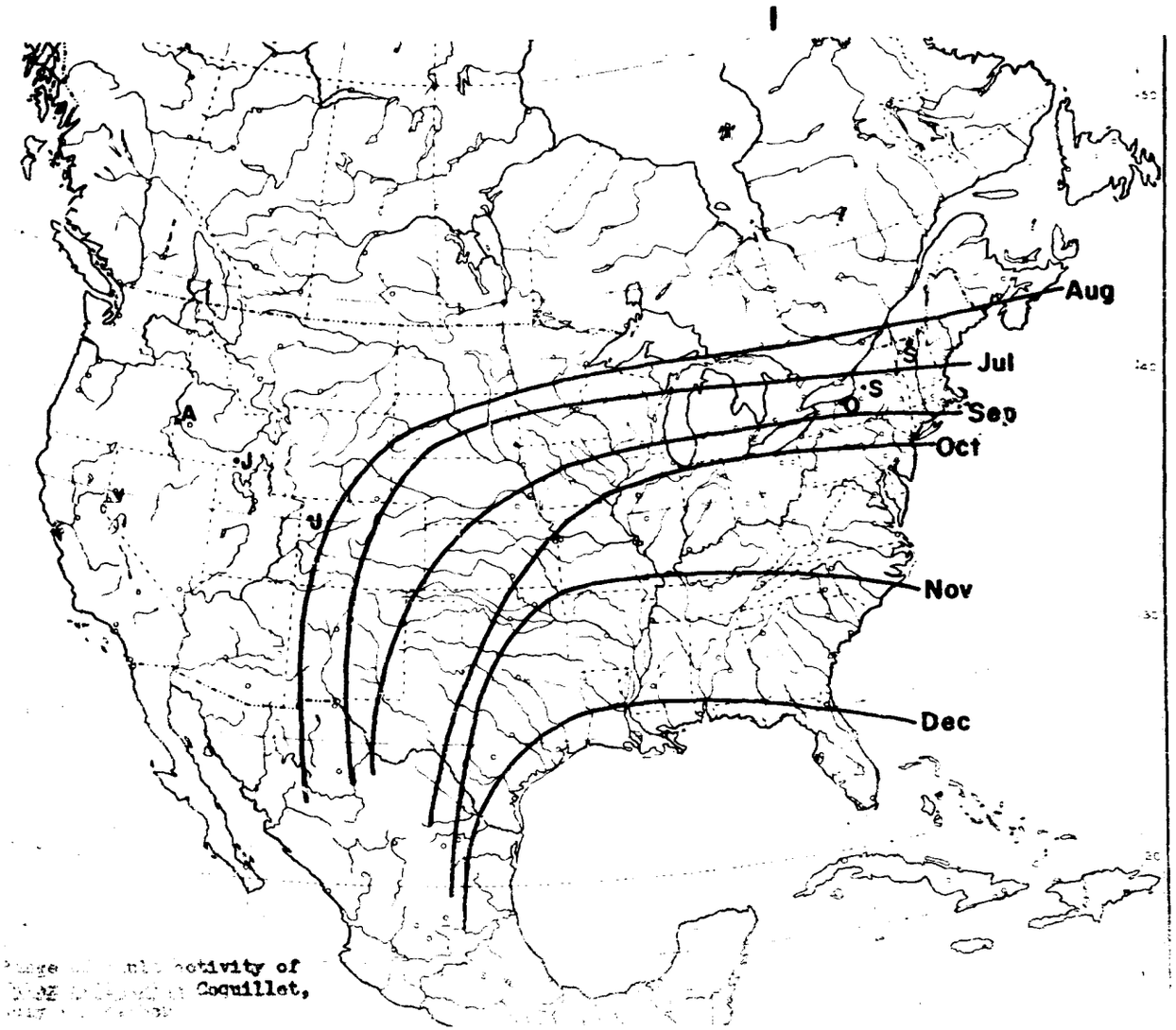


Figure 8. Range of adult activity of Culex salinarius Coquillet, January - June.



...ctivity of
Coquillet,

Project 3A061102B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03, Entomology

Work Unit 035, Ecology and control of disease vectors and reservoirs

Literature Cited.

References:

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2. Rowley, W.A., Graham, C.L. and Williams, R.E. A Flight Mill System for the Laboratory Study of Mosquito Flight. Ann. Entomol. Soc. Amer. 61:1507, 1968.

Publications:

1. Ward, R.A., and Bell, L.H. Transmission of *Trypanosoma brucei* by colonized *Glossina austeni* and *G. morsitans*. Trans. Roy. Soc. Trop. Med. Hyg. 65:236, 1971.

PROJECT 3A061102B7P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04
Immunology

728

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6432	71 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMAR	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
70 07 01	C. 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	61102A	3A061102B71P	04	015
b. CONTRIBUTING				
c. XXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Antigen-Antibody In Vivo and In Vitro (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 71	7	160
	CURRENT 72	7	160

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 202-576-3665

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ADDRESS: Washington, D. C. 20012

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

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Allergy; (U) Enzymes; (U) Immunology; (U) Antigen; (U) Antibody; (U) Hypersensitivity

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

23 (U) This work unit is concerned with the study of the basic mechanisms of the immediate type allergies, the development of methods for the isolation and characterization of the enzymes involved in hypersensitivity reactions. This looks to the ultimate control of such allergies by a specific inhibition of these enzymes.

24 (U) The antibodies and mechanism of the leukocyte-dependent histamine release from platelets of the immediate hypersensitivity reaction to various parasitic diseases are being studied. The distribution of blood group antibody between fluid phase and human erythrocyte is being investigated under various conditions of temperature and concentration of cells and antibody, towards the recognition of the dangerous universal donor.

25 (U) 70 07-71 06 The results obtained strongly suggest that the class of antibody involved in the leukocyte-dependent histamine is of the gamma E class. Studies are presently underway to establish what cell type is involved in this reaction, as well as an in vitro sensitization with purified antibody. Evidence has been obtained with selected sera that specific binding of the naturally occurring anti-B isohemagglutinin are characteristic of the ABO genotype. However, discrepancies were obtained when random anti-B sera were tested; the reason for this is being studied. For technical report see Walter Reed Army Institute Annual Progress Report, 1 Jul 70 to 30 Jun 71.

*Available to contractors upon originator's approval

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

PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 015, Antigen-antibody reactions in vivo and in vitro

Investigators.

Principal: John F. Barbaro, Ph.D.

Associate: D.T.O. Wong, Ph.D; M.B. Gibbs, Ph.D.; LTC R. Wistar, MC; MAJ C. Alving, MC; L. Hafner, M.S.; J. Ortaldo, M.S.; R. Jenks, M.S.; E. Seno, M.S.; MAJ O. J. Lawless, MC; LTC J. Miller, MC; J.P. Bingham, M. J. Schoenbechler

Description.

The purpose of this task is to study the enzymatic and other mechanisms of allergic reactions and the agglutination reactions of the human groups.

Progress.

1. In vivo passive sensitization of rabbit leukocytes.

(a) The previous annual report described the in vivo sensitization of normal rabbit leukocytes with antiserum demonstrating homocytotric (reagin) antibody. It was established that only those antisera possessing homocytotropic antibody were capable of in vivo sensitization. Further, the degree of sensitization was directly related to the homocytotropic antibody titer of the serum and sensitization was maximal at 3 days.

(b) This work was continued to demonstrate that the antibody responsible for the leukocyte sensitization was the same antibody causing the skin sensitization. Antiserum demonstrating homocytotropic antibody was treated in various ways and these treated sera were tested for skin and leukocyte sensitization. It was found that heating antiserum at 56°C for 4 hours resulted in concomitant loss or decrease of both activities.

(c) The results obtained with 2-mercaptoethanol reduction and alkylation were the only parameter studied that showed a poor correlation between skin sensitization and leukocyte sensitizing activity. Both of the 2-ME treated sera tested demonstrated a complete loss of skin sensitizing capacity, whereas only one showed a decreased leukocyte sensitization. There are several explanations for the poor correlation between the leukocyte and skin sensitizing ability of mercaptoethanol treated antiserum. First, the reduced-alkylated antiserum maintains its affinity for skin tissue, however, this affinity is considerably less than that of untreated IgE. Second, the mercaptoethanol treated antisera does not persist in the skin for long periods. Both of these have been demonstrated with reduced-alkylated human IgE antibody. If the rabbit homocytotropic antibody is similar to the IgE, then the equivocal re-

sults with 2-ME could be explained by either the greater sensitivity or longer persistence of antibody in the leukocyte mediated release in comparison to the passive cutaneous anaphylaxis activity. Third, it is possible that this discrepancy might indicate more than one homocytotropic antibody with differing mercaptoethanol sensitivities and differing capacities to give skin and leukocyte sensitization.

(d) Adsorption of homocytotropic antibody with a specific anti-rabbit IgE was done and the adsorbed antiserum was tested for leukocyte and skin sensitizing ability. This anti-rabbit anti-IgE was supplied by Dr. Zvaifler and demonstrated no reactivity to any of the known rabbit immunoglobulins. The adsorbed serum completely lost both skin and leukocyte sensitizing capacity.

(e) Although the results obtained utilizing the in vivo sensitization strongly suggest the involvement of the homocytotropic antibody in leukocyte mediated histamine release from rabbit platelets and its correlation with skin sensitizing capacities, it is difficult to establish conclusively unless an in vitro method of leukocyte sensitization is developed. Presently, a collaborative study with Dr. Zvaifler is planned, where he will supply us with purified IgE antibody so we can develop an in vitro method for leukocyte sensitization.

2. Electron microscopic study of leukocyte-dependent histamine release.

(a) An electron microscopic study of the leukocyte dependent histamine release reaction was undertaken in collaboration with Dr. Aikawa from the Institute of Pathology of the Case-Western Reserve University in an attempt to elucidate the mode of histamine release from platelets.

(b) In suspensions of platelets and sensitized leukocytes not activated with antigen, there was no apparent interaction between the leukocytes and platelets with no morphological changes observed in these cells.

(c) There were demonstrable changes in their physical relationship as well as their morphology when platelets were mixed with either antigen activated leukocytes or in the presence of leukocytes and antigen. There were many aggregates of platelets intermingled with leukocytes, usually composed of a few small lymphocytes and monocytes. Many of these platelets were irregular in shape and possessed pseudopods extending towards the centrally located leukocytes. Often the platelet pseudopods were in close contact with these leukocytes and were interdigitated with the microvilli of the leukocytes. The tips of the pseudopods of the platelets were frequently inserted into the cytoplasm of these leukocytes, forming a cytoplasmic anastomosis with no limiting membrane observable in these areas.

(d) The cytoplasmic matrix of platelets located near the leukocyte became more electron opaque than in the control group. The number of α granules decreased and the number of vesicles increased. The remaining α granules occasionally underwent changes in which their matrix became partially electron translucent. Although the platelets appeared to be vacuolated and to have lost some of their granular inclusions there was no lysed platelets observed in these preparations.

(e) Although the results of this study demonstrates that both the small lymphocytes and monocytes are closely associated with platelets, they do not conclusively establish that these cells are the effector cells of this reaction. Recent published results indicate that the basophil may be the effector cell in the leukocyte-dependent histamine release reaction. Further work is needed to establish precisely what leukocyte and mechanism is involved in this reaction.

3. Studies on blood group antigens and antibodies.

(a) Investigation directed towards the recognition of the "dangerous" universal group O donor by characterization of the binding properties of natural and immune anti-A and anti-B isoagglutinins were continued. As summarized in the previous annual report, evidence has been obtained with selected sera from young adults of known genotypes in support of the Wurmsers' conclusion that naturally occurring anti-B agglutinins differ in their binding affinities according to the ABO genotype of the individual. However, when random anti-B sera from group A and O individuals were tested for their binding affinities, the results did not agree with those obtained with the selected sera. Consultation with the Wurmser group disclosed the possibility that the log-probit assay procedure may not be capable of providing the same information on the binding of anti-B antibodies to B sites of the red blood cells as the Wurmsers' assay procedure. To resolve this issue, a collaborative study was initiated with Dr. Salmon, of the Central Blood Transfusion Service of Paris. Antisera have been exchanged and the results obtained in two laboratories with the Wurmsers' assay and the log-probit assay procedure will be compared.

(b) Numerous technical problems were encountered with the Wurmser assay procedure. The method proved to be far more complicated, tedious and time consuming than indicated by their publications. Most of the major problems have been resolved and the reproducibility of the procedure may be more sensitive in discerning small differences in the binding properties of anti-B agglutinin than the log-probit procedure. Simplification of the Wurmser's procedure will be attempted.

(c) In the previous annual report a method for the identification of A, B and H antigen using sonicated saline extracts of blood stained material to inhibit hemagglutinations of specific antisera was described. Extensive effort to extend the usefulness of this procedure to the identification of the M, N, S, s and P antigens of human red blood cells have been unsuccessful. Sonicated saline extracts of blood stains from

fabrics, paper and wood did not react with the specific antisera to cause inhibition of hemagglutination with these antigens. It was concluded that sonication destroyed the ability of M, N, S, s and P antigens to combine with their respective antibodies.

4. Studies of the blood group A and B substance activities of vaccines.

(a) The following commercial vaccines were tested for the presence of blood group A and B activity: 20 plague, 24 cholera and 28 typhoid. No detectable A or B blood group substances were found in any of the plague, cholera or in 26 of the typhoid vaccines. Two typhoid vaccines contained 0.0005 mg of blood group A substance but no group B substance.

5. Studies of microaggregates of human blood.

(a) Swank (N. Eng. J. Med. 265: 723, 1961) and recently Mosely and Doty (Annals of Surg. 171: 329, 1970) presented evidence that multiple microaggregates are routinely infused into patients during the administration of stored blood. In collaboration with Dr. Solis, Div. of Surgery, WRAIR, a study of the development and removal of microaggregates of leukocytes, platelets and other amophorous debris in human blood stored for transfusion was undertaken. The model T Coulter particle counter was adapted to obtain quantitative date on the frequency and size distribution of microaggregates in stored human blood. Whole blood stored 23 days in plastic bags had 121.7±12.7 small (10-29μ), 17.8±8.0 medium (30-99 μ) and 1.8 ± 0.8 large (100-164μ), 17.8 ± 8.0 medium (30-99μ) and 1.8 ± 0.8 large (100-164μ) particles per mm^3.

(b) A comparison was made of the relative effectiveness of a Dacron wool filter, a 40μ and the 170μ screen filter currently used in removing microaggregates present in out-dated ACD units of whole blood (i.e. blood stored in excess of 21 days). The Dacron wool filtration effectively removed all small, medium and large particles of out-dated blood, whereas the 40μ pore filter removed only the large particles and the standard 170μ screen filter did not remove any of the microaggregates. These results clearly indicate that the Dacron wool filter should be used, particularly when large quantities of blood are administered.

(c) The time of occurrence and size distribution of the aggregates that develop in ACD whole blood stored in plastic bags at 4°C was investigated. It was found that microaggregates do not occur in stored blood until the 5th day of storage. A progressive increase in frequency and size of the particles was noted over the 21- day period of storage. It was demonstrated that the increase of microaggregates were associated with a decrease of both platelet and leukocyte counts.

6. The synthesis of novel phosphonates.

(a) Three new phosphonates were synthesized. Their elementary and physical properties are given in Table 1.

7. Production and characterization of guinea pig homocytotropic antibodies.

(a) The previous annual report described the characterization of mouse heat labile homocytotropic antibody (reagin-like) with regard to its production following a single antigenic stimulus and its separation from 7S γ, heat stable homocytotropic antibody. Attention has now been directed towards conditions for the production of heat-labile homocytotropic antibodies by guinea pigs.

(b) Preliminary evidence has been obtained demonstrating the production of reaginic-like antibody using p-amino benzoic acid diazotized to purified guinea pig albumin as an antigen. The antibody has the necessary characteristics of heat lability and persistence in the skin for long periods. It appears in the serum of guinea pigs following a single foot pad injection of antigen in complete Freund's adjuvant between 10 and 13 days. The titer obtained thus far has been low and the antibody has been demonstrated in only a small number of immunized animals. Experiments are planned to determine (1) if repeated antigenic challenge will evoke an increased production of this antibody (2) what adjuvants are best for its production and (3) if possible genetic factors are involved in its production.

(c) The unique aspects of this reaginic antibody is its very narrow specificity. The specificity is directed neither towards the carrier protein nor the hapten but appears to be link-specific; i.e. a positive reaction is obtained only when the homologous antigen used to elicit the formation of the antibody is used for the challenge, while the carrier protein alone or the hapten conjugated to a heterologous carrier do not give a positive reaction. However, generalized blueing results when animals are challenged with the hapten conjugated to a heterologous protein. If it can be established that the generalized blueing is not causing inhibition of the reaginic antibody by the heat stable 7S γ antibody, then under these conditions this reaginic antibody is different from other gamma E antibodies that are hapten specific. This type of specificity is usually associated with the delayed hypersensitivity reaction.

8. Immunologic study of pigeon breeders disease.

(a) Allergic alveolitis in pigeon breeders disease, (as well as other diseases dependent on inhalation of antigen which provoke intrapulmonary reactions) is considered to be due to an Arthus reaction in the lungs. In collaboration with Dr. Lawless of WRAMC Allergy Clinic, a study involving patients afflicted with pigeon breeders disease was initiated to investigate the cellular and humoral mechanisms of this disease.

(b) Presently, two patients are being studied in detail. Blood samples from each demonstrate precipitin, hemagglutinins and complement-fixing antibodies in high titers although the patients represent the clinical extremes that can be found in this disease. These results may indicate that mechanisms other than numoral antibodies are involved.

(c) The antigens to which these patients are responding can be found in pigeon serum as well as pigeon fecal extracts and appears to be predominantly the IgG immunoglobulins. Fragmentation of purified pigeon IgG immunoglobulin with pepsin to yield $F(abT)_2$ fragments as well as reduction and alkylation to obtain purified light chains demonstrate that both these preparations are reactive with each patient. The fecal antigen has a fast gamma mobility in electrophoresis, and is approximately 7S in size on gel chromatography. In double diffusion studies comparing pigeon fecal extract and serum antigens the results suggest that the fecal antigen probably represents a more complete antigen. Work is presently proceeding to isolate the fecal extract antigen with the idea that it may represent a secretory immunoglobulin which has not been previously described in avian species.

(d) Although both patients exhibit negative delayed skin reactions, their lymphocytes are capable of transformation in response to pigeon serum antigens as determined by incorporation of thymidine. This response of patients serum can be suppressed by repeated washings of their lymphocytes at least 5 times before the addition of antigen. These results indicate a loose association of antibody on the lymphocytes which reacts with antigen to cause transformation. Further, the serum from a patient with acute disease contains a "factor" which stimulated the DNA turnover of normal lymphocytes. These results suggest that the "factor" may represent antigen-antibody complexes. Work is currently underway to determine if preformed antigen-antibody complexes of known size and antibody to antigen ratios are capable of causing lymphocyte DNA synthesis.

9. Immune reactions with liposomes.

(a) It has been demonstrated that a system composed entirely of artificial lipid dispersions (liposomes) and purified lipid antigen (Forssman antigen) was capable of mimicking intact cells exactly with regard to their immunologic behavior. This system has been used to study the molecular basis of immune cytolysis. Work has started with particular emphasis on three approaches to the use of liposomes; namely, (1) the incorporation of different antigens onto liposomes; (2) the use of liposome as an adjuvant for producing antibodies against "poor" antigens and for testing antibodies that are produced and (3) the use of liposomes for the study of immune phagocytosis.

(b) Cerebroside is biologically important because it may comprise as much as 40% of the chemical composition of myelin and is responsible for all brain-specific antigenic activity associated with lipids. Because of this, it may be very important in chronic degenerative nerve

activity such as that associated with traumatic injury to peripheral nerves, or with encephalitis or encephalomyelitis. Since cerebroside is a lipid and it is very difficult to assay for the presence of antibodies, it was felt that liposomes could be used as an assay procedure.

(c) Anti-cerebroside antibodies were produced by incorporating purified cerbroside into the structure of liposomes. The presence of antibodies was detected by the release of trapped glucose from cerebroside containing liposomes. The activity of anti-cerebroside antibody was found to reside only in the IgM fraction. The amount of antibody protein as well as the amount of C1 bound could be calculated. In these experiments it was established that protein binding is affected by the concentration of specific antibody and the amount of antigen (cerebroside) contained in the liposomes.

(d) Using cerebroside containing liposomes, it was decided to assay for anti-cerebroside activity in patients. Serum was obtained from a variety of patients at WRGH. Preliminary evidence indicate that out of four sera tested, one patient with degenerative nerve disease may have anti-cerebroside activity.

(e) A model system was developed for quantitative analysis of phagocytosis of liposomes. This consisted of a fixed monolayer of mouse peritoneal macrophages above which liposomes were floating. The degree of phagocytosis was assayed by quantitatively measuring the amount of liposomal phosphorus in the fluid above the cells. It was found that the phagocytosis of liposome, in contrast to all other model systems, was a slow process. Phagocytosis depended on both specific antibody and complement. After 24 hours, in the presence of antiserum alone, about 20% of the liposomes were taken up. When complement was present, however, phagocytosis increased to 70%. Experiments are in progress to determine which complement components are required and the effects of altering the antigen concentration on phagocytosis.

Summary and Conclusions.

1. Normal rabbit leukocytes can be passively sensitized in vivo with rabbit sera demonstrating homocytotropic antibody. All the parameters tested that affect skin sensitizing ability of homocytotropic antibody also affect leukocyte sensitization.

2. The antibody responsible for leukocyte sensitization appears to belong to the gamma E class, similar to the human reaginic antibody.

3. There was no physical interaction between platelets and sensitized leukocyte not activated with antigen. Combination of platelets and leukocytes activated with antigen demonstrated remarkable changes in their physical and morphological relationships.

4. Platelets are irregular in shape, vacuolated, and lose some of their granular inclusions. Platelet pseudopods are in close contact

with leukocytes and their tips are often inserted into the cytoplasm of these cells forming an anastomosis.

5. The hemagglutination-inhibition reaction for the detection of M, N, S, s and P antigens in dried blood sample was unsuccessful. It was concluded that sonication destroyed the ability of these antigens to combine with their respective antibodies.

6. The model T Coulter particle counter was adapted to obtain quantitative data on the frequency and size distribution of micro-aggregates in stored human blood.

7. The microaggregates do not occur in stored blood until the 5th day of storage and increased progressively both in size and frequency up until the 21st day.

8. The relative effectiveness of various filters in removing micro-aggregates in stored blood established that the standard 170u screen filter does not remove any of the microaggregates, whereas, the Dacron wool filter was very effective.

9. Three new phosphonates were synthesized.

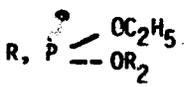
10. Preliminary evidence has been obtained demonstrating the production of reaginic-like antibody in guinea pigs. The unique aspects of this antibody, in comparison to other gamma E antibodies, is its very narrow specificity.

11. The antigen responsible for the allergic alveolitis appears to be gamma G immunoglobulin, which is also found in fecal extracts. The evidence obtained thus far suggest the involvement of antigen-antibody complexes as the mechanism of this disease.

12. Anti-cerebroside antibodies have been prepared using cerebroside containing liposomes. These cerebroside-containing liposomes indicate that they may be useful in detecting anti-cerebroside antibodies in patients with demyelinating diseases.

13. A model system for the quantitative analysis of phagocytosis using liposomes has been developed.

Table I



	R_1	R_2	n_D^{25}	B.P. ^oC	C		H		P		N	
					T	F	T	F	T	F	T	F
$C_{17}H_{21}O_3P$	$C_6H_5(CH_2)_3$	C_6H_5	1.5342	Xylene[a]	67.1	65.7	7.0	7.4	10.2	9.9		
$C_{16}H_{19}O_3P$	$C_6H_5(CH_2)_2$	C_6H_5	1.5396	Xylene[a]	66.2	66.0	6.6	7.0	10.7	10.7	-	-
$C_{15}H_{24}NO_5P$	C_7H_{15}	$C_6H_5NO_2$	1.5041	Xylene[a]	54.7	54.6	7.4	7.9	9.4	9.6	4.3	3.8

(a) Falling Film Molecular Still

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 015, Antigen-antibody reactions in vivo and in vitro

Investigators.

Principal: John F. Barbaro, Ph.D.

Associate: D.T.O. Wong, Ph.D.; M.B. Gibbs, Ph.D.; LTC R. Wistar, MC; MAJ C. Alving, MC; L. Hafner, M.S.; J. Ortaldo, M.S.; R. Jenks, M.S.; E. Seno, M.S.; MAJ O. J. Lawless, MC; LTC J. Miller, MC; J. P. Bingham, M.J. Schoenbechler

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Publications.

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PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6433	71 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. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 07 01	H. Termination	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71P	04	016
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Immunization Studies of Militarily Significant Viral Diseases (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	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	90
	CURRENT 71	3	120

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Eckels, K. H.

NAME: Hampton, C. M. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Chikungunya; (U) Dengue; (U) Vaccine; (U) Immunology; (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) Chikungunya and dengue are arthropod-borne viral infections of man having widespread geographic distribution throughout populated areas of the world. Although mortality is low the incapacitation effected by these viruses and their associated sequelae could have serious impact on military timetables and troop mobility. This investigation is concerned with the development, production and evaluation of vaccines suitable for use in man.

24.(U) Both, chikungunya and dengue are characterized by antigenically-related strains within their respective groups. However, the broad cross-protection elicited by strains of the chikungunya virus from diverse geographic areas (India, Southeast Asia and Africa), is not shared by the dengue virus prototypes 1, 2, 3 and 4, which show little if any protection between each other. Our primary objective is the isolation and purification of antigenic subunits from specific types of the dengue virus which will facilitate the production of a broad-spectrum protective vaccine against the major dengue prototypes.

25.(U) 70 07-71 06 A formalin-killed, freeze-dried chikungunya vaccine prepared in green monkey kidney tissue culture has been administered to more than 50 volunteers. Significant levels of neutralizing antibody have been demonstrated in these individuals 1 year after vaccination. Rhesus monkeys subjected to a live challenge with the chikungunya virus 6 months and 1 year after vaccination were solidly protected against viremia, whereas control monkeys were viremic for periods up to 5 days. Antigenic subunits exhibiting high serologic specificity are being produced for Group B arboviruses including Dengue, West Nile, Japanese B and St. Louis encephalitides. This work unit now incorporated into Division of Communicable Disease & Immunology 70 08 23. For technical reports see the Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04 Immunology

Work Unit 016, Immunization Studies of Militarily Significant Viral Diseases

Investigators.
Principal: V. R. Harrison
Associate: K. H. Eckels
C. M. Hampton

Little work was accomplished on this project number during the past fiscal year, since it was terminated on August 23, 1970. Progress is now reported under Project 3A061102B71Q, Communicable Diseases and Immunology, Task 00 Communicable Diseases and Immunology, Work Unit 166, Viral Infections of Man.

PROJECT 3A061102B71P

Task 07
Pharmacology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6448	71 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
70 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	61102A	3A061102B71P	07	036
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Pharmacological Studies (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	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 71	2	40
	CURRENT 72	2	40

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME: DEMAREE, LTC G. E. DA
NAME: EINHEBER, Dr. Albert

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pharmacology; (U) Medicinals; (U) Shock Therapy; (U) Drugs; (U) Stress

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

23. (U) Research is directed toward investigating the pharmacology of promising medicinal agents, drug interactions, developing and refining animal models for the study of hemorrhagic, septicemic, and traumatic injury shock as well as the exploitation of Army procured chemicals in the treatment and prevention of shock. Studies are directed toward determining the mechanisms of action of therapeutic agents as well as the nature and type of chemicals which would be useful in shock therapy. The goal of this research is to develop a highly effective, non-toxic drug which would be useful in the treatment or prevention of trauma associated with battlefield injury.

24. (U) Drugs are tested in animal models for effectiveness in preventing or treating experimental shock resulting from hemorrhage, endotoxin, traumatic injury, and anaphylactic stress.

25. (U) 70 07 - 71 06. IND was approved by AIDRB for Phase I testing of WR 2823AB. Preclinical pharmacology and toxicology studies are being pursued in preparation for submitting an IND for WR 149,024. In animal models, the latter agent appears to be about four times as effective as WR 2823. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

*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 68 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 07, Pharmacology

Work Unit 036, Pharmacological studies

Investigators.
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC Gale E. Demaree, MSC; MAJ James A. Vick, MSC;
CPT Robert W. Caldwell, MSC; Albert Einheber, Ph.D.

GENERAL PHARMACOLOGY OF WR 149,024

BACKGROUND: Preclinical investigations are continuing pursuant to proposing the clinical trials of WR 149,024 for the treatment of shock states in man. The effects of this agent appear to be nearly identical to those of WR 2823 but it is about four times more potent and much more prompt in onset.

METHODS: Cardiovascular effects were studied in anesthetized dogs. Effects on plasma and whole-blood volumes in dogs subjected to hemorrhagic hypotension were studied using Cr^{51}-labelled RBC. Efficacy of WR 149,024 to prevent mortality from hemorrhage was studied in anesthetized monkeys.

RESULTS: WR 149,024 exerts a prompt, reversible blockade of alpha-adrenergic receptors at doses of 5 to 15 mg/kg given intravenously to dogs. The chemical reversibly reduces cardiac output and cardiac contractility which appears not to be dose related. WR 149,024 did not prevent hemoconcentration in hemorrhagic dogs following reinfusion of blood. Three of three monkeys treated with WR 149,024 survived an hemorrhagic episode that was fatal to 8 of 12 untreated monkeys. Plasma and whole blood volumes of dogs treated with WR 149,024 (10 mg/kg) were higher than those values in untreated dogs following the reinfusion of shed blood to both groups. The red cell masses of the two groups were not different.

DISCUSSION: WR 149,024 has proven efficacious in the prevention of mortality in animal models of hemorrhagic, endotoxin, traumatic or anaphylactic shock. The minor adverse pharmacological effects of the chemical can be readily reversed if they should occur in humans. The mechanism of efficacy appears to be related to fluid volume shifts. This agent appears to hold considerable promise as a potential therapeutic agent for use in treatment of shock. The great degree of purity and stability, along with its high potency, clearly warrants its consideration for a potential clinical trial.

MECHANISM OF HYPOTENSIVE EFFECT OF WR 2823

BACKGROUND: Preclinical pharmacodynamics studies revealed that WR 2823 has a direct vasodepressor effect when administered intravenously in several species. One objective of preclinical studies is to learn how to prevent or reverse adverse effects of new drugs in man. These studies were conducted in order to determine the mechanism of the hypotension in the anesthetized dog.

METHODS. Anesthetized dogs were challenged repeatedly with hypotensive doses of WR 2823 to test for tachyphylaxis. Dogs were treated with atropine, pyrilamine, propranolol or phenoxybenzamine to test mediation of hypotension through release of acetylcholine, histamine or other biogenic amines and to test empirically for methods to prevent the hypotensive episode.

RESULTS: None of the procedures modified the hypotensive response to WR 2823.

DISCUSSION: The hypotension from WR 2823 is probably not due to the release of biogenic amines nor mediated through agonism of any such receptors. None of the classical blocking agents for these amines offers hope for preventing the hypotension from WR 2823. The most desirable method now available for treating this hypotension, should it present clinical difficulties, appears to be timely administration of a pressor substance such as norepinephrine or angiotenin.

EFFECT OF WR 149,024 ON URINARY OUTPUT IN THE MOUSE

BACKGROUND: WR 149,024 has been reported to prevent mortality and hemoconcentration from anaphylactic shock. This study was done to investigate the possible role of drug effects on renal function in this response.

METHODS: Half of 38 hydrated mice received WR 149,024 (50 mg/kg I.P.). The remainder received saline injections as controls. Urinary output was measured for 24 hours.

RESULTS: WR 149,024 had no effect on the urinary excretion by hydrated mice.

DISCUSSION: WR 149,024 probably plays no role through modification of renal functions in its prevention of mortality and hemoconcentration in anaphylaxis.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 07, Pharmacology

Work Unit 036, Pharmacological studies

Literature Cited.

Publications:

1. Caldwell, R.W. and Demaree, G.E.: Treatment of hemorrhagic shock with 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane tetrahydrochloride (WR 149,024). Fed. Proc. 30:508 ABS, 1971.

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10. Phillips, S.J. and Vick, J.A.: The pretreatment of E. coli endotoxin shock with a new adrenergic blocking agent. Pharmacologist 12:284, 1970.

PROJECT 3A061102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08
Physiology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6438	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'S INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	61102A	3A061102B71P	08	076
b. CONTRIBUTING				
c. [redacted]	CDOG 14124(2)			

11. TITLE: (U) Brain mechanisms maintaining vital functions during surgical and medical shock: Anatomical and Physiological Coorelates. (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 002400 Bioengineering 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	cont	DA	C. Inhouse

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 71 (PRECEDING)	6	
FISCAL YEAR 72 (CURRENT)		

19. RESPONSIBLE DOD ORGANIZATION
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20. PERFORMING ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
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NAME: Siegel, H., Cpt, MD DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS: (U) Neurophysiology; (U) Neuropsychiatry; (U) Neuroanatomy (U) Combat Shock (U) Autonomic Nervous System (U) Biomedical Engineering

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

23. (U) The principal objectives are combined and coordinated studies of the anatomic and physiologic basis of central nervous system control of blood pressure adjustments occurring in medical and surgical (blood loss) shock; the anatomical and physiological basis of pain of visceral origin; the intergrative functions of the autonomic nervous system during health, disease, and during enviromental and emotional stress.

24. (U) Determination of central nervous system pathways and terminations involved in control of blood pressure adjustments by histological and histochemical techniques. Recording and analysis utilizing computer techniques, of cardiovascular responses to controlled hemorrhagic shock in awake and chronically implanted monkeys. Recording and analysis of single and multicell activity in the central nervous system and its relationship to physiological adjustment mechanisms.

25. (U) 70 07 - 71 06 Autonomic cell groups in the spinal cord have been identified. New centers have been described for the first time. Sensory fiber connections from the internal organs to these spinal cord cell groups have been studied, described and their clinical implications discussed. A new pathway carrying information from spinal cord to the brain has been documented physiologically. Interactions between autonomic centers in the brain and spinal cord are being studied. The relationship of the vestibular system and other sensory informations are under study. Cardiovascular responses to controlled hemorrhagic shock are being quantitated. The above research efforts constitute the basis for favorably modifying life-threatening physiologic responses in stress and disease and provide new ideas to explain the structural and functional basis of normal ongoing autonomic functions. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 69 - 30 June 70 and 2 July 70 - 30 June 71.

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PII Redacted

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 076, Brain mechanisms maintaining vital functions during surgical and medical shock: Anatomical and physiological correlates.

Investigators.
Principal: J. M. Petras, Ph.D.
Associate: John F. Amann[1], Ph.D.; LTC Robert D. Cardiff, MC; CPT Jeffrey N. Crown, VC; John F. Cummings[2], D.V.M., Ph.D.; CPT John J. Dropp, MSC; MAJ Howard L. Fields, MC; CPT Richard C. Howe, MSC; CPT Howard W. Siegel, MC; MAJ C. Fred Tyner, MC; David L. Winter, M.D.; Richard M. Wylie, Ph.D.

DESCRIPTION

The research program of the Department of Neurophysiology attempts to: (1) provide fundamental neuroanatomical and physiological information, both scientific and applied, regarding behavioral functions of the limbic system and the physiological and behavioral functions of the autonomic nervous system acting to integrate and regulate, within normal limits, the body's temperature, heart rate, blood pressure, blood flow, water balance, exocrine glands, endocrine balance, gastrointestinal motility, digestion and absorption, and other vital functions, particularly as these functions may be related to the medical, surgical or psychiatric care of military patients; (2) define the enviromental or physiological circumstances contributing to or causing stress, and surgical or blood-loss shock; (3) suggest applied methods of corrective therapy for stress avoidance and recovery from surgical or medical shock. These studies have applied cardiovascular, physiological, neurological and neurosurgical implications.

The knowledge and research methods of neuroanatomy, neurophysiology, neuroendocrinology, cardiovascular physiology, and experimental psychology are utilized in the department's studies. In some cases the expertise of one discipline is applied to a particular research task, but whenever possible multidisciplinary approaches are utilized to study stress. The following problem areas are under study:

(1) the anatomy and physiology of sensory pathways to the autonomic nervous system, and the anatomical basis of reflex functions in the visceral nervous system;

1. Postdoctoral Fellow in Neuroanatomy
2. Consultant, Department of Anatomy, New York State Veterinary College, Cornell University, Ithaca, New York

(2) the pathways over which the autonomic nervous system can exert its motor effects;
(3) the physiologic mechanisms controlling heart rate, blood pressure, shifts in blood volume throughout the vascular bed during stress and slow blood-loss shock;
(4) bladder regulation;
(5) the physiology of the vestibular system; and
(6) the physiological behavior of cortical neurons and their relationship to somatic, visceral or multimodal afferent signals.

PROGRESS

A. Neuroanatomical Studies.

1. Sympathetic division of the spinal cord.

a. Identification and description of spinal cord sympathetic nuclei. Using the method of axonal reaction following sympathectomy, the location of preganglionic nuclei in the thoracolumbar region of the spinal cord was established. A description of the normal cell complement of these nuclear groups was also described in detail. The experimental results show that three separate nuclear groups send axons into the sympathetic chains. These studies were performed in rhesus monkeys. The findings are being extended by comparison with the occurrence of the same cell groups in other primates and in the cat and the dog.

The results in the rhesus monkey demonstrate a wider dispersal and greater number of preganglionic neurons in primates than was known before. Some of these cells are very large neurons. This finding opens the possibility of studying visceral motor neurons using single-unit neurophysiological techniques. Single-unit analysis has been attempted before in the cat, but all efforts ended in failure. The presence of such large visceral cells in the rhesus monkey was unknown at that time; this discovery should provide better opportunities for cell behavioral analysis.

b. Dorsal root afferent connections to autonomic nuclei of the spinal cord. Having established the location of preganglionic neurons in the cord, dorsal root lesions were made at appropriate spinal cord levels following sympathectomy. Utilizing the Nauta and Fink-Heimer stains for degenerating terminal endings, the distribution of dorsal root fibers on spinal neurons was documented. It was found that sensory fibers did not terminate directly on autonomic preganglionics, thus establishing, for the first time, that the autonomic spinal reflex system must be polysynaptic[14].

2. Parasympathetic division of the spinal cord.

a. Experimental identification and description of the parasympathetic nuclear groups. Studies are underway to determine the nuclei of origin for the outflow of the parasympathetic division of the nervous system. These cells innervate the gastrointestinal tract distal to the splenic flexure and supply efferent fibers which innervate the pelvic viscera and the external genitalia. Axons of the parasympathetic neurons are selectively cut to produce central chromatolysis of the nerve cell body. The axonal reaction displayed by the nerve cell bodies will permit identification of the nuclei of origin for parasympathetic fibers.

3. Afferent fiber connections of sympathetic and related cell groups of the spinal cord.

a. The relationship of reticulospinal fibers to sympathetic cell groups. Wide areas over the medullary and pontine reticular formation project to the spinal cord. Nerve cells of this region are known to participate in respiratory and cardiovascular events. The efferent fiber connections of this region of the tegmentum with sympathetic neurons has not been carefully studied. The possibility of a monosynaptic connection, or the presence of polysynatic pathways to sympathetic motor neurons is now being investigated.

b. The vestibulospinal projection and it relationship with sympathetic motor neurons. Labyrinthine stimulation occuring during air, land or sea travel is known to influence heart rate, blood pressure, exocrine glandular secretions and sometimes results in nausea and vomiting. The pathways over which vestibular sensory signals may reach and influence spinal autonomic centers are being investigated.

c. The corticospinal projection and its relationship to sympathetic nuclei. Abdominal sympathectomies were performed unilateraly and followed by cortical lesions in the same rhesus monkeys. As in the above studies, the brains and spinal cords will be serially cut. The tissues will be stained according to the several variants of the Nauta and the Fink-Heimer methods in order to study the resulting fibers of passage and terminal degeneration. A prominent direct projection to select somatic motor neurons has been previously described and its role in rapid, graded, limb movements and fine finger dexterity in rhesus monkeys has been implicated. The current investigation attempts to compare the cortical input to somatic motor neurons with that to visceral motor neurons with the view of describing the means by which cortical fields may exert their effects at segmental levels of the autonomic nervous system.

4. Ascending visceral afferent pathways to the brain stem. A fiber degeneration study is continuing in order to determine the course and termination of a newly discovered visceral-somatic afferent

pathway[5]. Lesions have been made using dorsal and ventral approaches to the ventral funiculus of the spinal cord. The available data is still being studied. However, preliminary findings point toward degenerated ascending components in the spinal ventral funiculus which may correspond to the ventral pathway of Fields and Winter.

5. The effects of behavioral stress on the neurons and neuroglia of autonomic ganglia. Cytological changes have been found in autonomic ganglia following behavioral stress. In the rat, evidence has been gathered to show that neuroglial proliferation, neuronal hypertrophy and neuronal nuclear and nucleolar hypertrophy takes place. These changes have been found in autonomic ganglia only. The increase in 3H-thymidine uptake is presumptive evidence of DNA synthesis leading to cell division. The glial response is perhaps a concomitant of increased metabolic demands upon the autonomic neurons.

6. Mast cells in the central nervous system. A survey of the distribution of mast cells in the central nervous system of rodents was continued. There is evidence that biogenic amines stored and released by mast cells have pharmacologic effects on neurons and neuroglia[11, 16]. Mast cell secretions have recently been implicated in the genesis of histological brain damage[1,8]. There are a few reports of the occurrence of mast cells in the mammalian brain and spinal cord, but a comprehensive review and study of the occurrence of these cells has not been undertaken. The present evidence demonstrates a wide distribution of mast cells in the brains of kangaroo rats, gerbils and albino rats. These cells are most abundant in diencephalic nuclear groups and tracts, are moderately abundant in the cerebral hemispheres and mesencephalon, least abundant in the pons and medulla oblongata, and were not found in the spinal cord.

7. Tectothalamic and tectomesencephalic connections in the rhesus monkey. Lesions of the superior colliculus were made utilizing stereotaxic surgical methods for the placement of electrodes, and by suction ablation performed by direct visualization of the midbrain roof. The efferent fiber connections are being studied and mapped. Tectothalamic connections have been found with the intralaminar nuclei limitans, parafascicularis, centralis lateralis and paracentralis, with nuclei medialis dorsalis pars parvocellularis, medialis dorsalis pars densocellularis and medialis dorsalis pars multiformis. Projections to the nucleus pulvinaris inferior and the nucleus pulvinaris lateralis were also seen. The tectal projection to the intralaminar nuclei may provide a pathway for visual impulses to reach the striatum and extra-pyramidal motor system via a transthalmic trajectory. A similar transthalamic route utilizing the nucleus medialis dorsalis to connect the superior colliculus with the frontal eye fields is under consideration.

Tectomesencephalic connections have been partially analyzed. Ipsilateral connections are established with the nuclei gresium pontis and the nucleus paralemniscalis of Olszewski and Baxter[12]. A large contralateral projection is seen to the nucleus reticularis pontis of Bechterew. Afferent connections to this tegemental nucleus appear not to have been identified before.

8. Efferent connections of the occipital lobe. The efferent connections of the peristriate (area 19) and parastriate (area 18) areas of the occipital lobe are under study in rhesus monkeys. Initial findings show that both areas project to the superior colliculus and pretectal areas and to the nuclei gresium pontis. A dense terminal field is present in the nucleus pulvinaris lateralis, nucleus pulvinaris inferior and the lateral geniculate body following area 18 ablations. A projection to the nucleus pulvinaris medialis, or the area of junction between the medial and lateral pulvinar, is seen following cortical ablation of area 19. This fiber degeneration also continues rostrally and appears to invade the most caudal cell population of the dorsolateral sector of the nucleus lateralis posterior. Distinctions between the rostral zones of the pulvinar and the adjoining lateralis posterior territory pose difficult anatomical questions.

9. Connections of the posterior parietal cortex with speculations regarding the relationship of the parietal lobes with aphasias, apraxias and agnosias[3, 6, 7, 13]. The efferent connections of the posterior parietal cortex were studied in rhesus monkeys subjected to selective lesions of the superior and inferior parietal lobules, which correspond approximately to Brodmann's areas 5 and 7, respectively[13].

Following ablations of either the superior or inferior parietal lobule, axon degeneration, stained with the Nauta and Fink-Heimer methods, was traced into the extreme, external, and internal capsules, and into the cerebral peduncle. This degeneration extended into the ipsilateral insular cortex, cingulate gyrus, prefrontal and premotor cortices, and the precentral and postcentral gyri. In addition to these connections, the superior lobule sends fibers to the ipsilateral inferior parietal lobule and superior temporal gyrus, and via the corpus callosum to the contralateral superior and inferior parietal lobules, whereas the inferior parietal lobule sends fibers to the ipsilateral superior parietal lobule and to the contralateral superior and inferior parietal lobules. A prominent fiber system to the ipsilateral temporal lobe degenerates following lesions in the inferior parietal lobule (area 7); in such cases fiber degeneration appears in the superior, middle and inferior temporal convolutions, and in the fusiform and parahippocampal gyri.

Both lobules evidently project to the claustrum and body of the caudate nucleus. Both, moreover, have massive efferent connections with the dorsal two-thirds of the putamen. By contrast, no

evidence of projections from the parietal cortex to the globus pallidus was found in any of the cases studied.

A further subcortical projection from the posterior parietal cortex involves the nucleus reticularis thalami and the nucleus lateralis posterior thalami. The inferior lobule projects directly to the nucleus lateralis dorsalis and to the mediodorsal region of the nucleus lateralis posterior that closely adjoins two thalamic cell groups: the nucleus lateralis dorsalis and the intralaminar nucleus centralis lateralis. The superior parietal lobule, by contrast, projects massively to a ventrolateral district of the nucleus lateralis posterior.

Parietosubthalamic connections could be traced from areas 5 and 7 to the zona incerta and fields H_2 and H of Forel, but evidence for terminal connections with the nucleus subthalamicus (Luys) could not be found.

Both areas 5 and 7 project massively to the pretectal area and the deeper layers of the superior colliculus. This parieto-mesencephalic connection is amplified by a fiber connection from the inferior parietal lobule (area 7) to the lateral densocellular region of the circumaqueductal gray matter. No evidence of parietal cortico-nigral fiber connections was found. Finally, both parietal lobules were found to project to the pontine nuclei.

10. Electron microscopy. The RCA4A electron microscope was modified in February 1970 in order to correct its persistent malfunctioning. These modifications brought the scope up to date and this instrument is essentially a RCA4B electron microscope. This microscope has functioned reliably and we have logged 227.4 hours of operating time. Tissues from 36 experiments have been prepared for study with the electron microscope. The remainder of our work in electron microscopy has largely been devoted to: (1) the training of professional and technical personnel in our department and in other Divisions of the WRAIR, and (2) expansion of our facilities for electron microscopy. We have also attempted to redefine the basic functions of these resourses.

a. Training in electron microscopy. Mr. Cyril Wingfield, electron microscopy technician, was appointed service manager for all electron microscopes in the Walter Reed Army Institute of Research, and now devotes approximately 50 per cent of his time to this job. In addition to his previous training in the use and maintenance of the electron microscope, he undertook an intensive two-month training program which included a formal electronics course, and an informal apprenticeship with the local RCA service manager, and a formal training course at the RCA plant in Cherry Hill, New Jersey.

Mr. Cyril Wingfield and LTC Robert D. Cardiff have conducted informal orientation and training programs to acquaint WRAIR personnel, both investigators and technicians, with the use and functions of the electron microscope. Within our division, Dr. J. M. Petras has participated in this program in preparation for electron microscopic research on the nervous system.

b. New facilities and responsibilities. Following the recommendation of LTC Charles Angel, Director, Division of Biochemistry and LTC Dana Slack, Director, Supply and Service Division, WRAIR, the RCA3M electron microscope in the Division of Medicine was transferred to this Department. A room was remodeled to accommodate this electron microscope. This microscope is now functional. We have also absorbed the personnel and equipment assigned to MAJ Andrew Saladino, Division of Medicine; this includes one civilian technician, two enlisted men and several ultra-microtomes. Additional space was assigned to this Department to help provide working areas more suitable for the increase in service functions of electron microscopy.

B. Physiological Studies.

1. Autonomic responses in hemorrhagic shock. The effects of slow hemorrhage in the development of circulatory shock are being studied in chronic, extensively instrumented monkeys. The basic aim of these studies is to detail the mechanisms and sequences of breakdown of the normal homeostatic autonomic reflexes involved in the maintenance of blood pressure and blood flow. Blood pressure, blood flows and derived values such as cardiac work and cardiac output have been documented for the slow hemorrhage model. The project is presently divided into two phases.

a. Phase I Cyclic variation in blood pressure and heart rates in the conscious primate. The purpose of this work is to study the cyclic variations in systolic and diastolic blood pressures and heart rates in the conscious animal during 24 hour periods, and to determine the degree of interaction between these two variables. Rhesus monkeys have been implanted with chronic indwelling femoral arterial catheters placed high in the abdominal aorta. The monkeys are restrained in Mason chairs during the study period. Techniques were developed to keep these catheters patent for indefinite time periods, usually from 1 to 4 months. Our electronic equipment is capable of recording on a beat by beat basis, systolic and diastolic pressures, heart rates, and derivative functions such as the dP/dt. These parameters are recorded in analog fashion and converted to digital data and recorded on tape for later computer analysis. A flexible editing and averaging program has been developed in conjunction with Miss Eileen Sussman of the George Washington University computer center. This program was first developed on an IBM 360 system and then modified to run on the WRAMC CDC 330 computer.

Program development and instrumentation interfacing required about three months. We have so far collected data from 3 pilot animals for 11 hour periods. This demonstrates a complex systolic waveform with multiple harmonic frequencies. We are currently analyzing this data with the Biomed 02T time series program now running on the WRAMC computer. Although the data analysis is still in a relatively early stage, our power spectral estimates indicate definite active frequency bands occurring at 3 to 5 minute periods, and 7 to 9 minute periods throughout the 11 hour observation periods. These phenomena are in addition to other well known rhythms such as the circadian fluctuations in blood pressure, respiratory variations, sleep/REM activity and other physiologic factors. We also have some suggestive data at present which points to coherence between heart rate and systolic blood pressure at certain frequencies but not at others. The usual acute response to stress is an elevation of heart rate and blood pressure. The hyperptensive state is frequently characterized by a high blood pressure at normal heart rates. Use of reinforcement techniques for the cyclic periods of high blood pressure without concomitant high heart rate may prove interesting in attempting to develop a good laboratory model of essential hypertension.

We are continuing to collect data on longer time series, and to refine the computer processing procedures. Future work in this area will involve the following studies: (1) documentation of the blood pressure and heart rate rhythms present in awake, and sleeping primates; (2) acquisition of flow data and integration of this into the current model; (3) use of psychoactive cannabinols (THC), detailing their effects, if any, in normal bio-rhythms; (4) use of time series techniques to analyze data obtained from the hemorrhagic shock model to gain some insight into how shock and the post-shock state may alter these bio-rhythms.

b. Phase II Autonomic responses and circulatory variations in hemorrhagic shock. This phase represents a continuation of the work previously carried out in this laboratory by MAJ John Adkins. We are currently analyzing these results before continuing Phase II.

Training Mr. Andrew Pryzbylik, electronics engineer, has received training in bioelectrical engineering, physiology, and endocrinology. CPT Howard W. Siegel has attended two computer courses during the year to learn the necessary programming skills for data analysis. These included a course in Fortran 3000 given by the Control Data Corporation in Rockville, Md., and a course in assembly language given at the Hewlett Packard Company at Forest Glen. The purpose of the latter course was to enable possible integration of the computer facilities at Forest Glen as an on line system in the processing of our data.

Progress A switching system was developed for calibration of electromagnetic flow probes. A computer program is being developed

which will process the calibration data and demonstrate its linearity. This portion of our computer capabilites will be made available to other investigators in the WRAIR and in the biomedical community of the Washington area. This computer capability will accomplish both the classical calibration curves as well as calibrations for flowmeters which use integrators to measure stroke volume directly.

Future work Following probe calibration, 8-12 mm electromagnetic probes will be chronically implanted in monkeys at the base of the aorta. Smaller flow probes, 1-3 mm, will also be placed around the superior mesenteric artery. Using our hemorrhagic shock model, we will attempt to gather data on mesenteric flow during slow blood loss. This is important, for if it can be demonstrated that mesenteric blood flow is severely compromised during the shock state, a physiological basis would be established for the release of either endotoxin or Lefer's myocardial depressant factor (MDF) which could lead to heart failure and consequent circulatory collapse. We will attempt to study the effects of the drug WR-2823 on shock. This drug is currently under study in the Department of Pharmacology, WRAIR. It is believed to be a "protective agent" in hemorrhagic shock. Lastly, we plan to use adrenalectomized animals, which are chemically supported, in order to determine the role of the adrenal glands in homeostasis and shock.

2. Somatic and visceral receptive fields in the ventral funiculus[4]. Neurons of the medullary and mesencephalic reticular formation receive converging inputs from widely separated receptive fields and from afferents subserving different modalities. It is not known whether this convergence occurs on brain stem neurons or on the spinal neurons which project to them. Although there is extensive knowledge of the receptive-field properties of neurons comprising the tracts of the dorsal half of the cord, the supraspinal course of these tracts largely bypasses the brain stem reticular formation. Thus, fibers of spinal origin that terminate in the medullary, pontine, and mesencephalic reticular formation must ascend primarily in the ventral quadrant. Convergence on fibers of the ventral quadrants has been demonstrated, but detailed information about receptive fields of these fibers is meager. To determine the degree of convergence that takes place at the spinal level, the receptive-field organization of ventral quadrant fibers was studied in spinal cats. Since visceral and somatic inputs converge onto single ventral quadrant fibers, the present study has included the testing of visceral inputs. Anatomical studies have demonstrated conclusively that the axons of cells in the spinal gray matter branch extensively in the segment of origin (often bilaterally) prior to entering the ventral quadrant. In addition, since most ventral quadrant fibers terminate within the spinal cord, the results of the present study have relevance to the organization of segmental polyneuronal reflexes, propriospinal reflexes, and ascending input to the so-called "nonspecific" systems of the brain stem.

In summary, single units located in the ventral quadrant white matter of the upper lumbar cord were studied in the spinal cat. Spatially widespread excitatory and inhibitory visceral and somatic inputs were found in most units. All units with visceral inputs also had somatic receptive fields. A regional relationship between visceral and somatic inputs was noted. On the basis of electrical stimulation at C1, the fibers were classified as either ascending or propriospinal. No significant differences were found in the properties of those fibers which ascended to supraspinal structures as compared to the propriospinal fibers. Using the criterion of somatic spatial convergence, units were divided into two types. One type had wide spatial convergence of input and a tendency for bilateral symmetry of receptive-field location. The other type had relatively small receptive fields, usually restricted to the ipsilateral hindlimb. Small amounts of barbiturate greatly reduced the size of excitatory somatic receptive fields but did not eliminate the response to visceral stimulation.

3. Cushing reflex[10]. An increase of intracranial pressure is followed by a rise of blood pressure (Cushing reflex). Cushing believed that this arterial hypertension was a protective reflex enhancing the chance for survival of central neurons. He also emphasized that there are several phases in the response of an organism to progressive intracranial hypertension involving fluctuating patterns of changes in vital signs. He thus implied that vital signs must be monitored closely over a prolonged period of time to enable one to make accurate inferences about an individual patient. Cushing emphasized Kocher's four stages of intracranial hypertension: (1) Stage of compensation The intracranial pressure rises without producing pressure signs or symptoms; (2) Stage of early manifestations Pressure symptoms such as headache and irritability develop; (3) Stage of maximum manifestations Pressure symptoms become severe, the systemic arterial blood pressue rises, and there is variable bradycardia and bradypnea; (4) Stage of paralysis The patient becomes deeply comatose, and a reversal of vital signs is seen, namely arterial hypotension and tachycardia, ending with apnea and death. This study was undertaken in the course of a series of experiments on autonomic control mechanisms, and serves to elaborate the role of various modifications of the Cushing reflex as mediated through spinal cord mechanisms.

The findings were as follows: (1) The efferent limb of the Cushing reflex is the spinal sympathetic nervous system. Pressure or hypoxia acting directly on these spinal neurons elicits a graded vasopressor response (Cushing reflex) whether or not the spinal centers have been neurally isolated from the brain stem; (2) Vagal activity inhibits the Cushing reflex as does anesthesia, hypovolemia, and adrenergic or ganglionic blocking agents; (3) Hypoventilation (hypoxia), hypoglycemia, and blood in the cerebrospinal fluid facilitates the Cushing reflex; (4) Spinal autonomic and somatic neurons

show facilitory and then inhibitory responses to abrupt changes in intradural pressure with markedly different time courses for the two neural systems; (5) The Cushing reflex is initiated by both ischemia (hypoxia) and direct pressure effects usually acting in concert. There is no evidence for a specialized intradural baroreceptor.

4. Blood pressure regulation[9]. In 1876, Mayer first described a periodic waxing and waning of blood pressure with a much longer time course than the respiratory cycle. Frequently confused with Traube-Hering waves which are synchronous with respiration, Mayer or third-order blood pressue waves occur at 1-5 cycles/min and are most commonly seen in deteriorating animal preparations. Although it has long been postulated that Mayer waves reflect rhythmic changes in medullary vasomotor function, there are diverse opinions about the factors causing this rhythmicity. Mayer waves were induced in the systemic circulation of spinalized (C_8), vagotomized dogs under barbituate anesthesia by the acute elevation of spinal subarachnoid pressure to between 100 and 150 mm Hg. Sixteen spontaneously discharging lumbar sympathetic units were isolated in which the rate of firing fluctuated in fixed phase relationship with blood pressure waves; the maximum neural discharge preceding the peak in the arterial wave by one-eighth cycle. The data suggest that the spinal centers alone are capable of mediating Mayer waves and that the response is probably the result of oscillation of a spinal vasopressor reflex induced by ischemia.

5. Studies of the vestibular system. The vestibular system has been implicated in autonomic changes occurring in sleep and in homeostatic adjustments of vascular responses to changes of body position. Vestibular-autonomic interactions are of importance in problems of motion sickness and in problems of medical evacuation flights. Current experiments have focused on the neurophysiology of vestibular afferent signals.

The electron microscopic studies of Sotelo and Palay[15] have demonstrated "gap" junctions in the lateral vestibular nucleus of the rat. "Gap" junctions have been associated with the presence of electrical transmission in many nervous systems, although never before in the mammalian central nervous system. The present investigation was undertaken to determine whether or not electrical transmission across synapses takes place between vestibular afferents and second order cells in the lateral vestibular nucleus of the rat.

Microelectrodes were used to record the extracellular action potentials evoked from cells in the vestibular region of the rat by electrical stimulation of the vestibular nerve. By using a marking technique, it was possible to determine the stereotaxic coordinates of the vestibular nuclei. Latencies of the electrical events accompanying transmission through the vestibular nuclei have also been determined.

The afferent volley arrives at 0.3 msec. Many cells identified as post-synaptic units discharged with in 0.5 msec of the arrival of the afferent volley, a delay shorter than the accepted synaptic delay of 0.5 to 1.0 msec. By appropriate adjustment of stimulus parameters, many of the cells can be shown to have a bimodal distribution in their latencies, with a discontinuity between what will be called early firing and late firing. Early firing occurs at an interval too short to allow a normal synaptic delay, and later firing occurs at an interval allowing a normal synaptic delay. Twenty-two cells have been found within the lateral vestibular nucleus which displayed early firing. Additional cells have been found in the superior and descending vestibular nuclei, although these nuclei have not been systematically explored.

Intracellular studies are now in progress to determine the events underlying early firing. Synaptic potentials have now been observed in 17 cells with latencies to onset of less than 0.2 msec following the arrival of the afferent volley. These potentials characteristically rise to a peak within 1.2 to 0.3 msec and often trigger a spike on their rising phase. In some cases, the underlying synaptic potential is revealed only after blocking spike generation by passing hyperpolarizing current through the cell. This early, rapidly rising potential is followed by a more slowly rising potential with a latency to onset greater than 0.5 msec following the afferent volley, corresponding to the typical synaptic delay observed in the mammalian central nervous system.

To determine whether the short latency excitatory postsynaptic potential (EPSP) might be electrically generated by current flowing in pre-synaptic axons, or generated through the mediation of a chemical transmitter agent acting upon the ionic conductances of the post-synaptic membrane, the effect of passing current through the cell on the EPSP amplitude have been studied. To date, these studies have been successfully completed in 7 cells. In all the early EPSP is not affected by either hyperpolarizing or depolarizing currents, whereas the amplitude of the late EPSP is voltage dependent. Such voltage dependence is typically observed at synapses where transmission is mediated by a chemical transmitter.

The results of the present study suggest that there is a dual mechanism of synaptic transmission in the vestibular nuclei of the rat. There is direct electrical coupling of the afferent fibers to post-synpatic cells such that current flowing during the invasion of the afferent terminals flows through the post-synaptic cells. In addition, the invasion of the afferent terminal releases a chemical transmitter agent which acts upon the ionic conductances of the post-synaptic membrane, generating a voltage dependent EPSP. These conclusions are compatible with the observations of Sotelo and Palay, namely, that single afferent terminals form both "gap" junctions and synpatic complexes associated with vesicles.

6. Physiologic properties of interhemispheric neurons in the somatosensory cortex. Sensorimotor cortex in the cat contains a variety of cell types. Three major types can be distinguished on the basis of peripheral receptive field size: those with small (s), intermediate (sb) and large (m) fields[18]. The last of these groups, the m cells, provide most of the axons which leave the tissue for the brain stem and spinal cord. The m cells are often responsive to auditory and visual stimuli as well as to somatic sensory input; there is also reason to believe they are sensitive to visceral input.

Somatosensory cortex of one hemisphere receives dense connections from the opposite somatosensory cortex via the corpus callosum[2]. Although the m cell fraction is know to provide most of the corticofugal axons to the spinal cord and brain stem, it is not known whether they also provide the axons which pass through the callosum to the opposite hemisphere[17]. The present study is designed to answer this question. Single unit records are being made in the cortex of cats. Electrical stimuli are delivered to the footpads, the pyramids and the corpus callosum. The goal is to identify, by antidromic activation, those cells which send their axons through the callosum. If successful, this will identify the source of one of the largest outputs of sensorimotor cortex.

C. Technical Developments.

Under the supervision of Mr. Maurice Swinnen, the staff of the Electronics Laboratory has supported the reseach program in all departments of the Division of Neuropsychiatry. Their efforts have produced the following designs or results in improvements by up-dating existing equipment:

(1) Blood pressure averager,
(2) Light stimulus unit,
(3) Dermoohmeter,
(4) Precision thermometer for use in radiation experiments,
(5) Control light for speakers platform for Division of Preventive Medicine,
(6) Electrode marker units,
(7) Data display device coupled to memory scope,
(8) Amplifiers of high impedance for microelectrode studies,
(9) Pressure strain gauge processor for the Division of Medicine,
(10) Temperature control for tissue oven,
(11) Triple strain gauge to measure muscle tension,
(12) Hydraulic blood pressure stimulator to calibrate strain-gauge pressure transducers,
(13) Cardiotachometer, plug-in type for use with Offner recorders,
(14) Restoration of polygraph recorders,
(15) Eight-channel integrated circuit preamplifier,

(16) Eight channel EEG switching unit,
(17) Constant current transistorized lesion maker,
(18) Biphasic constant current stimulator,
(19) Automatic shut-off battery chargers,
(20) Complex control unit for micro-positioning of brain microelectrodes,
(21) Constant current stimulator modified for new functions,
(22) Pulse transformers,
(23) Burglar alarm for narcotic-containing refrigerator,
(24) Slide projector with random access feature.

E. Educational and Research Support Functions of the Department.

1. Department seminar. Twenty lectures were given in the general area of neurophysiology, neuroendocrinology, experimental psychology and neuroanatomy. The seminars were open to members of the Division, the Institute and other members of the Walter Reed Army Medical Centers clinical and research community. Each lecture series was designed to cover a broad area in each of these disciplines and attempts were made to show how the ongoing research programs contribute to the development of knowledge in the parent discipline and toward an understanding of physiology and behavior.

2. Neurophysiology lecture series. MAJ C. F. Tyner organized a series of eight lectures for the neurology residents of the Walter Reed General Hospital. Dr. R. M. Wylie, CPT F. J. Sodetz, and MAJ J. P. Mohr of the Division of Neuropsychiatry also contributed lectures to this course.

3. Neuroanatomy section.

a. Histology laboratory. In addition to its own research and training program in neuroanatomy, this laboratory supported the work of 13 investigators representing all six departments of the Division of Neuropsychiatry. Our staff, under the technical supervision of Mrs. Michie A. Vane, prepared brain or spinal cord tissues for 182 job requests. Dr. J. M. Petras and Mrs. Michie A. Vane provided technical training for off-post University technicians or investigators visiting this laboratory. These persons came from the Johns Hopkins University School of Medicine, the Harvard University School of Medicine, the Medical College of Virginia, Virginia Commonwealth University, the Museum of Natural History of the Smithsonian Institution, and Northern Arizona University.

b. Electron microscopy laboratory. Technical training in the preparation of tissues for electron microscopy and in the use and functions of the electron microscope were provided by LTC Robert Cardiff and Mr. Cyril Wingfield for 7 investigators and 8 technicians from the Divisions of Neuropsychiatry, Medicine, Communicable Diseases and Immunology, Experimental Pathology and the Walter Reed General Hospital.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 076, Brain mechanisms maintaining vital functions during surgical and medical shock: Anatomical and Physiological Correlates.

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Cole, M., and Nauta, W. J. H.: Retrograde atrophy of axons of the medial lemniscus of the cat. J. Neuropathol. Expt. Neurol. 29:354, 1970.

Fields, H. L., Partridge, Jr., L. D., and Winter, D. L.: Somatic and visceral receptive field properties of fibers in ventral quadrant white matter of the cat spinal cord. J. Neurophysiol. 33:827, 1970.

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Towe, A. L., and Tyner, C. F.: Cortical circuitry underlying the mixed receptive fields of certain pyramidal tract neurons. Expt. Neurol. 31:239, 1971.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6439	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMMARY	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
70 07 01	C. 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	61102A	3A061102B71P	08	077
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code): (U) Influence of Stress on Hormone Response, Performance and Emotional Breakdown in the Military (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 013400 Psychology /Indivi 016200 Stress Physiology 005900 Environmental Bio

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	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 71	9	260
FISCAL YEAR CURRENT 72	9	260

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME: Kotchen, MAJ T. A.
NAME: Ehle, MAJ A. L. DA

21. GENERAL USE: Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Stress; (U) Emotions; (U) Homeostasis; (U) Psychophysiology; (U) Neuroendocrinology; (U)Psychoendocrinology; (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) Principal objective is to study the integrating influences of the central nervous system in controlling and coordinating the organs of the body and their metabolic functions under environmental and emotional stresses which are likely to produce casualties due to psychiatric or psychosomatic disease.

24. (U) This involves measurement of plasma and urinary hormone levels in monkeys and humans in a variety of acute and chronic stress situations, with emphasis on the concept developed by our earlier works that we must view changes in broad, overall hormonal patterns or balance, rather than in single endocrine systems as was previously customary in the stress field.

25. (U) 70 07 - 71 06 Continued emphasis has been given in the last year to the collaborative studies of physical stress with ARIEM (Natick). Studies of endocrine responses to physical exercise before and following a six-week physical conditioning period have revealed quantitative differences in the endocrine responses with decreased epinephrine and norepinephrine responses. Increased endurance following conditioning was found to be associated with higher insulin levels during the exercise period. Studies of multiple hormonal responses to cold have revealed that this is a potent stimulus to the endocrine system but that the response is delayed when compared to psychoendocrine responses. Continued clinical studies are providing data as to possible endocrine balance differences between chronic disease groups. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 077, Influence of stress on hormone response, performance and emotional breakdown in the military

Investigators.

Principal: John W. Mason, M.D.; COL Joseph V. Brady, MSC

Associate: MAJ Theodore A. Kotchen, MC; MAJ Albert L. Ehle, MC; Edward H. Mougey, M.S.; Frances E. Wherry, A.B.; David R. Collins, B.S.; Percy T. Ricketts, B.S.; Lee L. Pennington, B.S.; Jennette Wade, B.S.

Description.

This program is concerned with the role of the central nervous system in the co-ordination of endocrine regulation. Instead of the conventional study of single endocrine systems in isolation, multiple endocrine systems are studied concurrently so that the overall balance between the many interdependent hormones may be investigated. In recent years we have learned that various forms of psychological and physical "stress" elicit broadly organized patterns of hormonal response involving many hormones in addition to those of the adrenal systems. A major goal is to define conclusively these distinctive "overall" hormonal response patterns for various stressful stimuli, including psychological stimuli, cold, heat, hypoxia, fasting, exercise, hemorrhage, dehydration, trauma, infection, and various nutritional changes. Such basic knowledge of the integrative machinery is essential as a foundation for neuroendocrine approaches to the study of clinical and field problems concerned with such parameters as endurance, fatigue, host resistance and performance. Emphasis in the past year has shifted from animal work to clinical experiments. Particular attention has been given to the hormonal correlates of endurance during various forms of physical stress. A major portion of our efforts has been the continued collaboration with the Army Research Institute for Environmental Medicine (ARIEM) at Natick, Massachusetts. A substantial amount of work on the development of new or improved hormone assay procedures has also been continued in order to provide the necessary methodological foundation for this stress research program.

Progress.

1. Hormonal Balance in Physical Stress.

a. Exercise. In collaboration with LTC Howard Hartley at ARIEM, we have continued our studies of the neuroendocrine responses to the stress of exercise. In the laboratory, hormonal responses of human volunteers have been measured to both graded exercise at carefully

measured work loads of increasing intensity and also to sustained exercise to the point of exhaustion. All of these measurements were obtained before and after an eight-week intense physical training program to determine the effects of conditioning on both exercise endurance and the neuroendocrine responses to exercise.

During graded exercise, plasma norepinephrine increased and the increase was dependent upon the intensity of exercise. Epinephrine increased significantly only after a work load of maximal intensity. Growth hormone was also elevated following exercise, although there was no apparent correlation between the intensity of exercise and the magnitude of the growth hormone response. Plasma cortisol increased slightly only after exercise at the maximum work load. Similar to the norepinephrine response, plasma renin activity increased in a stepwise fashion at work loads of increasing intensity. Plasma insulin was depressed by exercise.

The hormonal responses to sustained exercise were similar. At the point of exhaustion, catecholamines and growth hormone were significantly elevated; serum cortisol was also slightly elevated. Plasma insulin concentration continued to decrease throughout the exercise session and was at its lowest point at the time of exhaustion.

The eight-week physical training program significantly improved exercise tolerance. In response to graded exercise, and at the point of exhaustion during sustained exercise, plasma insulin concentration was significantly higher after conditioning than prior to conditioning. It was suggested that the greater exercise endurance after training might be related to this insulin response.

Also in collaboration with LTC Hartley, exercise endurance and the hormonal responses to exercise are being studied before and after a ten-day trek in Alaska, involving exposure to cold. After the exposure, exercise tolerance was unchanged; results of hormonal measurements are not yet available.

b. Altitude. In collaboration with LTC Hartley and MAJ Redmond Hogan at ARIEM, the hormonal responses to exposure to a simulated altitude of 12,000 feet are being studied. An attempt will be made to determine if hormonal changes, including changes in the renin-aldosterone system, correlate with the appearance of clinical symptoms at altitude. Preliminary results indicate a significant elevation of 17-OH steroid excretion at altitude. Other hormones are presently being measured.

c. Cold. Several experiments have now been completed on the organization of multiple hormonal responses to cold in the monkey. Gradual lowering of the temperature from 25°C to 5°C results in marked

changes in hormonal patterns. Excretion of 17-OH steroids, epinephrine and norepinephrine showed marked and sustained increases while testosterone decreases. No significant changes have been observed in plasma growth hormone or insulin levels. Plasma thyroxine, free thyroxine and TSH levels have shown a delayed increase and there is some evidence to suggest that an increase in the response of these hormones occurs during repeated exposure to cold.

d. Heat. Continued studies of chronic heat stress in the monkey have confirmed preliminary observations indicating a suppression of 17-OH steroid, norepinephrine, and epinephrine levels with heat exposure. Alternating four-week periods with temperatures up to 85°F with four-week periods of room temperature, there is a predictable, sustained, and consistent suppression of 17-OH steroid excretion during exposure to the temperature elevation.

e. Prolonged Bed Rest. The hormonal response to ten days of complete bed rest in normal volunteers is presently being measured. There are no significant changes of 17-OH steroid excretion during bed rest. The renin response to the upright posture is significantly greater immediately after bed rest than prior to bed rest.

2. Hormonal Balance in Emotional Stress.

Strikingly consistent individual responses and significant mean elevations of plasma TSH, cortisol and norepinephrine concentrations were observed during a 20-minute interval prior to exhausting exercise in eight normal young men. No detectable plasma epinephrine response was observed. These observations confirm previous work in the monkey indicating that the pituitary-adrenal cortical system is remarkably sensitive to psychological stress.

3. Hormonal Balance in Medical Illness.

a. Hypertension. MAJ Kotchen has continued to study the hormonal profile in patients with essential hypertension. A total of eight patients have been studied; each was admitted to the Metabolic Ward of the Walter Reed General Hospital for at least two weeks and daily hormonal measurements were obtained. To date, the most striking hormonal finding is a relatively constant 17-OH steroid excretion despite fluctuations in catecholamine excretion associated with stressful situations. This is in marked contrast to the elevation of both 17-OH steroid and catecholamine excretion associated with stress in the monkey and in normotensive man.

To supplement the hormonal data, each patient underwent a psychiatric interview by Dr. Myron Belfer of the National Institute of Mental Health and completed several psychological tests administered by MAJ Fred Smith, Department of Psychology, WRGH. At the completion of the

study, an attempt will be made to correlate the hormonal profile with each patient's characteristic manner of dealing with anxiety and with his unique environmental perception.

In addition, the kinetics of the renin-angiotensin system have been studied in a number of other hypertensive patients. Hypertensive plasma has been found to be deficient of a renin inhibitor. At least in some hypertensive patients, this may be etiologically related to the hypertension.

b. Colitis. Similar hormonal and psychiatric data have been obtained in a pilot study of four patients with colitis, in collaboration with Dr. Arthur McMahon of the New England Medical Center in Boston. Preliminary results suggest several unique hormonal characteristics. Plasma epinephrine tends to be suppressed and androgen tends to be elevated in the colitis patients. Attempts are being made to correlate the hormonal data with psychiatric observations.

c. Malaria. In collaboration with LTC Jerry Earll, daily catecholamine excretion was measured in six normal human volunteers before and after injection of malaria parasites. There was no significant change of catecholamine excretion even during the acute febrile illness. A more detailed description of this study will be submitted by LTC Earll in the annual report of the Department of Metabolism.

4. Hormonal Responses to Brain Stimulation.

MAJ Ehle has completed a series of experiments in chair-restrained monkeys with chronically implanted electrodes that show that stimulation of the amygdala can increase plasma levels of both cortisol and growth hormone. Preliminary experiments have demonstrated increases in urinary testosterone excretion following similar stimulations. These responses appear to be localized in the posterior portion of the amygdaloid complex. Stimulation at sites in the anterior amygdala and hippocampus have not yielded increases in these hormones.

5. Hormone Assay Methodology.

Mrs. Wherry and Mr. Pennington have set up immunoassay procedures for measurement of plasma FSH and LH. MAJ Kotchen has set up an immunoassay for plasma angiotensin. Attempts to develop an immunoassay for vasopressin are continuing. Mr. Mougey has helped develop methods for the measurement of several thyroid function studies: T_3 uptake, total T_4, free T_4. These methods are now available for routine use.

Summary and Conclusions.

We have continued to study the overall hormonal responses to a variety of different "stresses" in both man and the monkey. Our basic

studies continue to show that multiple hormonal responses occur in various physical and psychological stress situations and that these multiple responses appear to be broadly organized in a way that is distinctive for that particular stimulus. Furthermore, the neuroendocrine responses to stress may ultimately be related to the ability to function during the stress. Similarly, the neuroendocrine responses associated with a medical disease, such as hypertension or colitis, may conceivably be related to the pathogenesis of the disease.

The continued development of more sophisticated laboratory methods has greatly enhanced our ability to measure a variety of different hormone responses.

Project 3A061102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 077, Influence of stress on hormone response, performance and emotional breakdown in the military

Literature Cited.

Publications:

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4. Ehle, A.L., Pennington, L.L., and Mason, J.W.: Plasma growth hormone responses to amygdaloid stimulation in conscious monkeys. Paper presented at American Physiological Society, September 1970 (Abstract).

5. Kotchen, T.A., Hartley, L.H., Rice, T.W., Mougey, E.H., and Mason, J.W.: Renin response to graded exercise. Paper presented at Eastern Section of American Federation of Clinical Research, December 1970 (Abstract).

6. Kotchen, T.A., and Rice, T.W.: Angiotensin generation in normal, uremic, and hypertensive plasma. Paper presented at Endocrine Society, June 1971 (Abstract).

7. Ehle, A.L., Mougey, E.H., Wherry, F.E., and Mason, J.W.: Multiple endocrine responses to cold exposure in the monkey. Endocrinology, supp to Vol. 88:A-188, 1971 (Abstract).

8. Flamenbaum, W., Kotchen, T.A., Rice, T., and Oken, D.E.: Renal renin content (RRC) in experimental acute renal failure (ARF). Endocrinology, supp to Vol. 88:A-195, 1971 (Abstract).

PROJECT 3A062110A806
MILITARY PREVENTIVE MEDICINE

Task 00
Military Preventive Medicine

773

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6457	71 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 INSTRN	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	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 + I,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 71	2	25
FISCAL YEAR CURRENT 72	2	150

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, Edward L., MC

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Inst of Research
Div of Biometrics & Med Info Proc

ADDRESS: 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]

ASSOCIATE INVESTIGATORS

NAME: CROSS, ELEANOR R., M.A.

NAME:

DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Global Health Data; (U) Epidemiology; (U) Geography; (U) Potential; (U) Climate; (U) Ectoparasites; (U) Infectious Diseases; (U) Infectivity; (U) Isolation

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

(23) Unclassified health and disease data on all foreign countries is accrued, analyzed, stored and made available to approved requestors. (U) Consultation and assistance is rendered to Medical Officers and to Global Medicine Courses. (U) Disease potential is calculated for all geographic areas and for all diseases.
(24) The staff of three (2 professional and 1 administrative) makes extracts of all qualified documents relating to disease - world wide. From these extracts, concepts are formed; and after review by world authorities on various disease entities, predicted incidence of any given malady is calculated.
(25) (U) 70 07 - 71 06. The staff of three (2 professionals and 1 administrative) research for data and write extracts of documents. 1200 of these are now in the computer and a similar number are waiting for processing. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* Applicable to contractor work summary(s) entered.

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

Project 3A062110A806 MILITARY PREVENTIVE MEDICINE

Task 00, Military Preventive Medicine

Work Unit 030, Global health data

Investigators:
Principal: COL Hinton J. Baker, MC
Associates: Ann C. Fred, M.D.; Eleanor R. Cross; and Douglas L. Floyd

Description:

The extraction of the literature for pertinent material on leptospirosis, schistosomiasis, Rocky Mountain Spotted Fever, tularemia, leishmaniasis and trypanosomiasis continues. Extracts (1700), concepts (9), and potentials on leptospirosis are now in the computer as a prototype data base.

The development of an adequate storage and retrieval system has been a primary objective during the subject period. Microfiche and microfiim are being made and used extensively in the department's document collection.

Progress: The development of concepts of disease or diseases in light of geography, topography, meteorology and other physical attributes, as well as etiological factors, is closer to reality as a result of the increased input into the computer. It is anticipated that health data publications will resume as soon as the retrieval information system is well established.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DAOR 6481	71 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	N/A	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110	3A062110A806	00	032
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A (2)			

11. TITLE (Precede with Security Classification Code): (U) Pilot Study to Determine Hearing Impairment Among Retiring Army Personnel (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 012400 Personnel selection and maintenance; 007900 Industrial (occupational medicine)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 05	71 09	DA	

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 71	0	0
FISCAL YEAR CURRENT 72	.25	1

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Buescher, E. L., COL, MC
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research, Div of Preventive Med
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Kaelber, C. T., MAJ, MC
TELEPHONE: 576-2480
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: LTC C. R. Webb, Jr., MC
NAME: MAJ C. H. Llewellyn, MC DA

PII Redacted

21. GENERAL USE
Foreign intelligence not considered.

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Retirement Physicals; (U) Noise (U) Hearing Impairment; (U) Retiring Army Personnel; (U) Audiometry

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

23. (U) The specific aims of this proposed pilot project are (1) to survey a sample of retiring Army personnel for the frequency and degree of hearing impairment as recorded on separation physical examination forms, and (2) to compare the demographic characteristics available from this form for those with and those without recorded hearing impairments.

24. (U) Data will be obtained from the physical examination forms (SF 88) which are processed in PSD SGO DA. Hearing status will be evaluated from the statement of the examining physician who lists a diagnosis of hearing impairment (SF 88, Item 74) and also from the recorded results of audiometry (Item 71).

25. (U) 71 05 - 71 09 Data collection is presently underway, but no results are available yet.

*Available to contractors upon originator's approval

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

Project 3A062110A806 MILITARY PREVENTIVE MEDICINE

Task 00, Military Preventive Medicine

Work Unit 032, Pilot study to determine hearing impairment among retiring Army personnel

Investigators.
Principal: MAJ Charles T. Kaelber, MC
Associate: LTC Charles R. Webb, Jr., MC; MAJ Craig H. Llewellyn, MC

Some military environments emit noise levels high enough to produce permanent hearing impairment. While a program to conserve hearing has existed for many years, hearing loss is not uncommonly reported among active duty personnel.

Hearing acuity is routinely measured as part of the terminal length-of-service separation physical examination, and the results are forwarded to the Physical Standards Division, SGO/DA.

The specific aims of this pilot project are (1) to survey a sample of retiring Army personnel for the frequency and degree of hearing impairment as recorded on separation physical examination forms, and (2) to compare the demographic characteristics available from this form for those with and those without recorded hearing impairments.

Data are being obtained from the physical examination forms (SF 88) which are processed in PSD SGO DA. Hearing status evaluated from the statement of the examining physician who lists a diagnosis of hearing impairment (SF 88, Item 74) and also from the recorded results of audiometry (Item 71).

Data collection is presently underway, but no results are available yet

Project 3A062110A806 MILITARY PREVENTIVE MEDICINE

Task 00, Military Preventive Medicine

Work Unit 032, Pilot study to determine hearing impairment among retiring Army personnel

Literature Cited.

References:

1. Collen, Morris F. et al: Dollar Cost Per Positive Test for Automated Multiphasic Screening. New England J. Med. 283: 459-463, 27 August 1970.

2. Gloring, Aram: Hearing Levels of Adults by Age and Sex, United States 1960-1962, Vital and Health Statistics, National Center for Health Statistics, Public Health Service Publication No. 1000, Series 11, No. 11, October, 1965.

3. Roberts, Jean and David Bayliss: Hearing Levels of Adults by Race, Religion, and Area of Residence, United States 1960-1962, Vital and Health Statistics, National Center for Health Statistics, Public Health Service Publication No. 1000, Series 11, No. 26, September, 1967.

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7. Preventive Medicine in Annual Report, Surgeon General, United States Army, Fiscal Year 1959, Office of The Surgeon General, Department of the Army, Washington, D. C., pp. 45.

8. Meyer, LTC R. H.: Noise: An Increasing Military Problem, Military Medicine 133: 550-556, July 1968.

9. Noise and Conservation of Hearing, Department of the Army Technical Bulletin, No. 251, Headquarters, Department of the Army, Washington, D. C. 25 January 1965.

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 0B 6465	71 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
70 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	044
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Precede 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	g. PROFESSIONAL MAN YRS	h. FUNDS (In thousands)
FISCAL YEAR PRECEDING 71	5	29[illegible]
FISCAL YEAR CURRENT 72	9	405

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

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Bangkok, Thailand

ADDRESS:

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

NAME: Altstatt, COL L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Top, LTC F. H. Jr.

NAME: Grossman, MAJ R. A. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code) (U)Infectious Diseases; (U)Epidemiology; (U)Virus Ecology; (U)Arbovirus; (U)Japanese Encephalitis; (U)Rabies Virus; (U)Hepatitis-Associated Antigen

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 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 virus 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) 70 07 - 71 06 An epidemic of Japanese encephalitis (JE) occurred in the Chiang Mai Valley during the monsoon season of 1970. The incidence of encephalitis was estimated to be 1.5-1.8/10,000 year and the incidence of inapparent JEV infection was 200-400/10,000 of the Valley population. JE virus was isolated from C. tritaeniorhynchus, gelidus, and fuscocephala. Widespread and uniform exposure to JEV virus throughout the urban and rural areas of the Chiang Mai Valley was seen. No evidence was found that prior Group B arbovirus antibody protected against apparent or inapparent JEV infections. Cases of "summer encephalitis" occurring in U.S. military personnel in RVN were found caused by JEV. Methods for the detection of hepatitis associated antigen (HAA) were established. About 40 percent of hospitalized hepatitis cases studied in Thailand were asscciated with HAA, and the prevalence of HAA in Thai blood donors is 6 percent. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

781

*Available to contractors upon originator's approval.

DD, FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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

Task 00, Tropical and Subtropical Military Medical Research

Work Unit 044, Virus disease of man and animals

Investigators.

Principal: Franklin H. Top., Jr., LTC, MC

Associate: Ananda Nisalak, M.D.; Anong Pariyanonda, M.S.; Avudh Srisukri, M.D.; Chaninthorn Suvongse, M.D.; Robert B. Cotton, MAJ, MC; Debhanom Muangman, M.D.; Dumrong Chiewsilp, CPT, MC, RTA; Robert Edelman, MAJ, MC; Howard B. Emery, M.D.*; Marvin H. Firestone, MAJ, MC; Allen M. Glasgow, CPT, MC; Douglas J. Gould, Ph.D.; Richard A. Grossman, MAJ, MC; Robert L. Hickman, MAJ, VC; Vincent J. Ionata, CPT, MC**; Jira Sitasuwan, M.D.; Dennis O. Johnsen, MAJ, VC; Kanda Vathanophas, M.D.; Kwanyuen Lawhaswasdi, DVM; Markpol Tingpalapong; Joe T. Marshall, Jr., Ph.D.; Ronald E. Marshall, SP5; Pairatana Gunakasem, M.D.; Phanu Sitthisomwong, DDS (deceased); Pien Chiewanich, M.D.; Prathan Voodhigul, M.D.; Pricha Singharaj, M.D.; Rapin Snitbhan, M.D.; Robert M. Russell, CPT, MC**; Sittiboon Buranavej, LTC, MC, RTA; James P. Slowey, SFC; Thomas J. Smith, COL, MC (deceased); Suchinda Udomsakdi, M.D.; Michael J. Sullivan, CPT, MSC; James E. Williams, CPT, MSC; Ronald G. Wilson, M.D.*;

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* Peace Corps physician, Thailand

** Preventive Medicine Officer, 20th Preventive Medicine Unit, Long Binh, R.V.N.

Studies on Japanese Encephalitis

1. The Epidemiology (Ecology) of Japanese Encephalitis Virus (JEV) Infections in Chiang Mai.

Project Coordinator: Richard A. Grossman, MAJ, MC

Principal Investigators: Richard A. Grossman, MAJ, MC
Robert Edelman, MAJ, MC
Douglas J. Gould, Ph.D.
Marvin H. Firestone, MAJ, MC

Dennis O. Johnsen, MAJ, VC
Thomas J. Smith, COL, MC (deceased)

Associate Investigators: Ananda Nisalak, M.D.
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OBJECTIVE: To investigate the ecology of Japanese encephalitis in the Chiang Mai area of Northern Thailand, with particular referenee to aspects contributory to infection in humans. Specific areas and their objectives include the following:

1) Epidemiology: Assess the epidemiologic variables of human apparent and inapparent JEV infection; define human risk factors; determine animal reservoirs of JE virus; ascertain environmental variables of possible importance; study epidemiological interrelationships of multiple co-existing group B arboviruses in a discrete area; assess potential for JEV control measures.

2) Entomology: Determine the identity of mosquitoes involved in the transmission of JE virus and measure the seasonal density, host preferences, insecticide susceptibility status and flight dispersal characteristics of suspected vector species.

3) Neuropsychiatry: Define clinical patterns of acute neuropsychiatric defects and complications; follow mental status changes, EEG changes and neurological deficits in convalescent patients, assessing nature, duration and progression of such abnormalities; attempt to correlate antibody titer levels over time with nature of CNS changes in convalescent patients; assess the influence of home environment and school or job environment on the behavior of convalescent patients; determine if any JE patients present with acute psychosis at the major psychiatric hospital in Chiang Mai.

4) Virus Laboratory: Provide the virologic support for all of the above efforts including virus isolation, virus identification, and serology.

Improve current virus isolation and identification systems; search for more specific serologic tests in group B arbovirus infections; characterize JEV isolates; attempt to improve current sentinel animal systems; examine role of small vertebrates in the ecology of JEV; perform mosquito transmission studies.

DESCRIPTION: The background and initial methods used were described in last year's report. To briefly summarize, a large epidemic of JE had occurred in the Chiang Mai area in 1969. JE virus was isolated from a human brain, several suspect animal reservoirs had ample serologic evidence of previous JEV infection and two Culex species, known to be JEV vectors elsewhere, were found to be abundant. After the epidemic had ended in November, 1969, one urban school in Chiang Mai City and 4 scattered villages in the Chiang Mai Valley were selected as field study sites. Appropriate samples of people were selected at each site for intensive prospective surveillance.

The post-season (November 1969) bleed of the cohort in the 5 areas showed differences of group B arbovirus serological patterns between the areas, but the patterns could not be reliably interpreted from the HI test alone due to the large amount of total or cross-reactive dengue antibody present. Nevertheless in each area, the group B prevalence rose with age, which was consistent with the belief that JEV, dengue (and possibly other group B agents) were indeed endemic in the Chiang Mai Valley.

Mosquito collections for virus isolation attempts were begun in October 1969. No viruses were isolated from 1042 mosquito pools collected during the dry season from November 1969 to March 1970. Mosquito collections made between October 1969 and February 1970 indicated that mosquito breeding occurred during the winter season at a very slow rate. Males, nulliparous and parous females and larvae of Culex species were collected during this period. Over 80% of cattle, buffalo, dog and pig sera collected in November 1969 were positive for JEV HI antibody. Starting in March, 1970, JEV antibody-free pigs (aged 3 months) were placed as sentinel hosts in each of the 4 study villages. They were bled monthly, and JEV converters promptly replaced. In addition, we twice attempted to use chickens as sentinel hosts, but on neither occasion did they show serological evidence of infection. We bled the human cohort and available domestic indigenous animals 3 times in 1970: March-April (before the rains), July-August (during the rains) and November 1970 (after the rains). In addition, various wild animals, especially the house sparrow, Passer montanus, were caught and bled for serological and virological evidence of JEV infection and a possible role in the JE ecologic cycle.

The neuropsychiatric and clinical studies [illegible] 2 major Chiang Mai hospitals (Suan Dork and McCo[illegible] Mai psychiatric hospital (Suan Prung), and two [illegible] (Lamphun and Lampang). With this broad case [illegible] we believe that very few JE cases from Chiang Mai Valle[illegible] medical facility were missed. The accessible families [illegible] the Valley were visited and paired sera were collected. Confirmed JE cases will be followed monthly for one year after discharge with repeated clinical examinations, (including E.E.G., mental status exam, [illegible] psychometric testing) and by interviews with teachers or employers of the patients.

PROGRESS: A JE epidemic in humans occurred in 1970. Cases were documented from Chiang Mai, Chiang Rai, Lamphun and Lampang provinces at the 5 study hospitals. In addition, a US Peace Corps Volunteer living in Phrae province, had serologically confirmed JE. There is thus good evidence for JE endemicity in most of the valleys of Northern Thailand. Since the main purpose of this study was to define the ecology of JE in a relatively discrete area, the remainder of this report will present only results referable to the Chiang Mai Valley which includes the 2 major cities, and parts of their provinces, Chiang Mai and Lamphun.

The first rains of 1970 began in March and were followed by a steady increase in the population densities of suspected vector species of mosquitoes in the four village sites. Peaks in population densities of these species, as measured by weekly light-trap collections, occurred between May and August - varying according to the village sampled. Biting collections from domestic animals indicated that biting activity by C. fuscocephala and C. tritaeniorhynchus was maintained all night, with peaks between 0100 and 0400 hours. Biting collections and precipitin tests of engorged mosquitoes from light-trap collections indicated that C. tritaeniorhynchus, C. gelidus and C. fuscocephala fed in greatest numbers on cattle or buffalo and on pigs, in that order. All three of these culicine species showed a reluctance to enter houses. Between 1 April 1970 and 31 March 1971 a total of 391,108 mosquitoes were pooled for virus isolation attempts. These included 140,260 C. fuscocephala, 11,675 C. gelidus and 175,265 C. tritaeniorhynchus. A total of 13 JEV isolations were obtained from these mosquitoes. The first JEV isolate was recovered from a pool of C. tritaeniorhynchus collected on 27 April. During May a total of 8 JEV strains were isolated, 6 from C. tritaeniorhynchus pools and 2 from C. fuscocephala. From June to August only 1 JEV mosquito isolate (C. tritaeniorhynchus) was obtained, but 3 strains of this virus were recovered from pools of C. gelidus collected during September from 3 separate sites. No JEV isolations were made from C. gelidus collected prior to September, although greater numbers of that mosquito had been collected and tested in previous months. Insecticide susceptibility tests

made with C. tritaeniorhynchus, C. gelidus and C. fuscocephala from Chiang Mai province indicated that all three species were resistant to DDT but susceptible to malathion.

Sentinel pigs placed in the four village sites in late March were bled at the end of April, and all were negative. However, between 27 April and 30 May, 30% of the sentinels in 3 of the 4 villages had developed antibodies to JEV. The sentinel pig conversions paralleled the mosquito population indices, with a peak of 66% conversions in July, decreasing to 20% in December and 0% in January and February 1971. In at least 2 of the 4 study villages sentinel pigs converted to positive during each of the 8 successive months (May-Dec). The sentinel chicken effort was negative, for none of 100 chickens set out in villages during July and September converted to JEV or had a detectable JE viremia. Over 1100 Passer montanus sera were collected in study villages from April 1970 to March 1971. Of 70 Passer sera tested so far by PRNT, 4 sera from birds collected in July showed significant plaque reduction at a dilution of 1:19. Serological and virological results on most of the sera from small wild vertebrates is pending.

No encephalitis cases were admitted to any of the hospitals in the area between February and April. The first serologically confirmed JE case was admitted on 19 April, and several more cases appeared on 11 May. From 11 May to the end of 1970, 100 Chiang Mai Valley residents were admitted with the diagnosis of encephalitis. The number of cases admitted per month was as follows: May (23), June (39), July (22), August (3), September (5), October (4), November (2) and December (2). Although 4 of the first 5 cases in May came from one district (where 3 of the first 5 JEV mosquito isolates were obtained), there was no other evidence of spatial clustering of cases. The 100 cases came from each of the 11 districts and from 59 of the 113 subdistricts of the valley. Almost 2/3 of the cases were male, which was the sex ratio observed in 1969 and is the usual Japanese experience. The overall case fatality rate was 19%, but females had an appreciably larger fatality rate (25%) than the males (15%). The median age (about 10 years) was strikingly similar for males, females, those that died and those with serologically confirmed JEV infections. Ninety-three of 100 cases had paired sera submitted and 70(75.3%) were serologically confirmed by the HI test as JE. A small number of patients had confirmatory CF and PRNT tests performed. Convalescent patients are still under neuropsychiatric and serological surveillance and therefore followup data on these patients are not available at this time.

Serologic analysis of HI results on the 70 confirmed human cases revealed that 45(64.3%) were definite or probable primary JEV infections

while 21(30.0%) gave cross-reactive high titer antibody response to dengue serotypes and are presumably recent JEV infections in individuals with previous group B infections. Only 4 patients showed intermediate serological patterns which could not be classified as either primary or secondary infections by our criteria. The median age was slightly lower for primary (9.2) versus secondary (12.5) responders. There was no difference in the serological patterns of males and females. Attempts are underway in the laboratory to identify the previous group B arbovirus infections in patients with JE. Two or more serum specimens were collected from family members of 55 cases of JE; of the 230 family sera (minus case sera), 5(2.2%) had significant JEV antibody rises by the HI and CF tests while 3 more people had rises only in the HI test. The total of 8 individuals (3.5%) thus represents the maximum estimate of concurrent familial inapparent JEV infection, when starting from index cases.

The village and school serum samples included over 90% of the cohort for the 3 serum collections in 1970. There was little human population change in the rural villages; there was net decrease of only 7 people, between November 1969 - November 1970. There was a much larger turnover in the domestic animal populations of the 4 study villages, especially of the pigs. The overall numbers of pigs were similar in November 1969 and November 1970, but virtually every non-breeder was eaten, sold or died, and was replaced by fresh young pigs bought, or bred, locally. This uninterrupted breeding pattern continued throughout the year.

After the November 1970 sera were collected, the study area samples were run simultaneously by the HI test, starting at a 1:10 serum dilution against 4-8 units of HA antigen. The November 1969 sera had previously been run starting at a 1:20 dilution and were reported in last year's annual report. There was no evidence of significant JEV transmission between the November 1969 and April 1970 specimens and surprisingly little (<2-fold) decrease in JEV titers during this 4 month interval. The November 1969 specimens have been re-analyzed with the following results. Villages (A) and (B) had very little evidence of previous dengue exposure and all but a few (3%) positives (≥20) to dengue 1-4 and JEV had monospecific titers to JEV. The prevalence rose with age in both villages and the overall (age-adjusted) JEV antibody prevalence was 67.9% for (A) and 43.6% for (B). The geometric mean titers (GMT) similarly rose with age in both sexes. Adults aged 20-39 were 92% JEV positive in (A) and 70% in (B). The serological patterns in villages (C) and (D) and in the urban school (E) is still confusing due to the presence in most positive sera (including the 1-4 year-old age group) of elevated and broadly reactive antibody to both dengue (one or more serotypes) and JEV. The overall JEV antibody prevalence, ignoring the dengue titers, was 72.6%

in (C) and 70.3% in (D). By considering only those JEV titers that were equal to, or greater, than the highest of the dengue titers, we can reduce the overall JEV prevalence (age-adjusted) to 55.7% in (C) and 52.0% in (D). Using these corrected figures, the JEV prevalences in (C) and (D) then become quite comparable to the age and sex distributions in villages (A) and (B), and the 95% confidence limits of the JEV antibody prevalences in the 4 study villages overlap. Thus the corrected prevalence figures for villages (C) and (D) may be closer to the true background prevalence of JEV if JEV experience has indeed been similar throughout the Valley. No strong correlation was found in any village between the proportion of the family members positive for JEV in each house and the number and types of domestic animals present around the house.

Analysis of the 163 (6-8 yr. old) school children's sera obtained in March 1970 (preseason) revealed 84% positive for JEV, but 92% were positive for dengue, 9% were positive for dengue alone and only 1.2% were positive for JEV alone. Interpretation of these results is difficult. Using the correction criteria of villages (C) and (D) the JEV antibody prevalence is reduced to 53% which is still much higher than any of the villages for 5-9 year olds. Also, unlike any village, the overall GMT, (calling negatives 1:5) was higher for dengue (mean of 114) than for JE (mean of 94.5). Further attempts at clarification of these serological results are underway in the laboratory.

Over the 9 month epidemic season, inapparent JEV infections were serologically detected in 5 to 8 family members of JE cases, 7 to 22 villagers and 6 to 8 schoolchildren in Chiang Mai. As stated, these ranges reflect the minimum (CF confirmed) and maximum (CF unconfirmed) numbers of inapparent infections that can be serologically diagnosed at present. The sex ratio was quite similar for each group using either the minimum or maximum numbers. In fact, the sex ratio for inapparent infections was the same as the ratio for the clinical cases, where over 60% were males. The constancy of this ratio suggests that despite an equal sex ratio in the overall JEV antibody prevalence in each village in 1970 the males were more at risk for both apparent as well as inapparent JEV infection. The age range (1-39 yrs) and median age (10) of JEV conversions were also similar in the villagers, in the family members, and in the clinical cases of JE. These similarities between the 3 different types of human populations provides evidence for the representativeness, validity, and presumed accuracy of the sampling procedures and serological results.

Incidence rates per 10,000 population were calculated for both apparent (JE cases) and inapparent infections in order to estimate inapparent to apparent (I/A) JE ratios. The case incidence (100 cases)

in Chiang Mai Valley was 1.5/10,000, using an estimated total population at risk of 680,000. When the 5 cases admitted from the subdistricts of the 4 study villages (total population 27,000) are used for the same calculation, a similar case incidence rate of 1.8/10,000 is obtained. This provides further evidence of the representativeness of the study areas and further justifies extrapolation of study area results to the entire Valley area.

The incidence rates of inapparent infections were likewise similar between the village and case family data. They were both in the range of 200-400 infections/10,000 population. The schoolchildren had a higher incidence rate (377-503/10,000) but this rate was similar to the infection rate in the villages and case family members of a comparable age. These results suggest that: 1) Family members of JE cases are at no greater risk of acquiring JEV infection than the general Valley population; 2) Children in the urban Chiang Mai environment are being exposed to JEV infection similarly to those living in more rural areas; and 3) There was widespread and uniform exposure, presumably to infected vectors, throughout urban and rural areas of the Valley with a random rather than cluster distribution of factors leading to human infection.

The I/A ratio based on case family data is estimated as 145-235 to 1. The I/A ratio based on study village data is 137-295 to 1. These values are consistent with various previously reported estimates from other JEV areas in Asia and the S.W. Pacific. Since the sex ratios were 60:40 male to female for both apparent and inapparent infections, these I/A estimates are likewise the same for both sexes.

There is some evidence that those villagers and schoolchildren acquiring JEV infection might have had a mild illness as a consequence. Only 16% of both the villagers and the schoolchildren had a history of FUO or URI, as documented by the nurses on their weekly visits over the entire 8 month period of observation. However, 25% of the villagers and 50% of the schoolchildren developing JE infection had such a medical history during the 4 month period between bleedings in which the infection occurred. Since the time of JEV infection cannot be more precisely dated due to the 3-4 month intervals between bleedings, a direct association between these symptoms and JEV infection cannot be made.

An important question is whether the presence of pre-existing group B antibody protects against or modifies the course of subsequent JEV infection and/or illness. The following points argue against the presence of serum group B arbovirus antibody alone being important: (1) About 1/3 of the confirmed cases had a typical secondary group B antibody response; (2) Based on the village antibody prevalence data obtained before the

start of the 1970 epidemic, about 50% of the entire valley population, or about 340,000 people, has JEV serum antibody; yet an epidemic occurred with at least 100 cases and an estimated 20,000 inapparent infections. Assuming the presence of pre-existing JEV, dengue or other group B antibody in about 1/2 of those with inapparent infection, about 10,000 inapparent JE infections occurred in people with previous group B infection. Obviously there must be some host factor, other than previous group B experience, that accounts for the majority of infections (inapparent and apparent) occurring in the young since our data strongly suggests a uniform exposure of all age groups to infection. Even in the 9 confirmed JE cases in adults aged 20-49, 4 had a primary, 4 had a secondary type, and one had an intermediate serologic JEV response.

2. Virus Isolation and Identification JE Project: Chiang Mai

Project Coordinator: Robert Edelman, MAJ, MC

Principal Investigators: Ananda Nisalak, M.D.
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Robert Edelman, MAJ, MC

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Dumrong Chiewsilp, CPT, MC, RTA

OBJECTIVE: To identify viruses isolated from mosquito pools collected during the Chiang Mai JE project.

PROGRESS: Starting in April 1970 and extending thru March 1971, the Dept. of Virology processed 5,878 mosquito pools, 284 animal sera, and 4 human brains for virus isolation. Herpes simplex virus was recovered from one human brain obtained from a child dying with encephalitis in Khon Kaen, Thailand. The 3 brains obtained from encephalitis patients in Chiang Mai were negative for virus. An agent was isolated from one buffalo serum, but the agent was not identified as JEV, and it was not successfully reisolated from the original serum specimen. Pools of 9 mosquito species were pooled for virus isolation - C. tritaeniorhynchus, C. gelidus, C. fuscocephala, C. vishnui, A. linneatopennis, A. mediolineatus, A. vexans, A. albopictus, and A. aegypti. A total of 42 viral agents were recovered from mosquito pools. Thirteen JEV strains were identified among the 42 agents recovered from mosquitoes; eight JEV strains were isolated from Culex tritaeniorhynchus, 2 strains were recovered from Culex fuscocephala, and 3 strains were isolated from Culex gelidus. A variety of laboratory experiments with these 13 JEV strains indicated they share closely similar biologic and antigenic characteristics. Five viral isolates, two from C. gelidus, 2 from C. vishnui, and one from C. gelidus have been tentatively identified as Tembusu virus. Twenty-four agents have not been identified as to virus type, but none of them are group B arboviruses as determined by screening complement fixation (CF) tests. A screening CF test using group A arbovirus immune fluid against these 24 unidentified virus have produced inconclusive results. Eight of the 24 agents are resistant to ether and bile, while one isolate is ether resistant but bile sensitive. None of the 6 unknown agents that form plaques in MK2 cell cultures are neutralized by antiserum to the known Thai arboviruses. We have isolated a multiplicity of arboviruses and other viruses not previously

identified in Thailand. With the exception of A. albopictus and A. aegypti, at least one new agent has been isolated from each of the 9 mosquito species processed for virus.

Comparisons were made of the sensitivity of the two virus isolation systems, suckling mice and MK2 cell culture. The two systems were equally sensitive to JEV, but suckling mice were more efficient in the isolation of agents other than JEV. Both systems were employed in tandem whenever possible to maximize the chance of virus recovery.

3. The Transmission of Japanese Encephalitis Virus by *Culex fuscocephala*

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Dennis O. Johnsen, MAJ, VC

OBJECTIVE: To determine if *Culex fuscocephala* can be infected by and can transmit Japanese encephalitis virus.

DESCRIPTION: In 1970 JEV was isolated from 2 pools of *Culex fuscocephaia* mosquitoes collected in Chiang Mai province, when JE was epidemic in humans. *Culex fuscocephala* mosquitoes were as abundant as *Culex tritaeniorhynchus* in this epidemic area, and the feeding and breeding habits of both species were found to be similar. The purpose of this laboratory study was to determine if *Culex fuscocephala* could be infected with JEV and if *C. fuscocephala* could transmit JEV to an experimental animal host.

PROGRESS: Eggs from wild caught females were hatched in the laboratory, and 3-8 day old females were used. The results of the study show that as few as 8 plaque-forming units (PFU) of JEV in a chick blood meal can infect *C. fuscocephala*; over 80% of the mosquitoes thus exposed become infected. The virus replicates to titers greater than 10^5 PFU per mosquito within 10 days after infection, and these high virus titers persisted for at least 27 days after feeding. An experiment comparing JEV transmission by *C. fuscocephala* and *C. tritaeniorhynchus* indicated that about 16% of infected mosquitoes of both species transmitted JEV to 1 day old chicks. Transmission was measured by viremia in the chicks 4 days after being exposed overnight to infected mosquitoes. We conclude that *Culex fuscocephala* mosquitoes can transmit JEV, and that this mosquito may be an important vector in northern Thailand.

4. Plaque Reduction Neutralization Test for Japanese Encephalitis: A Statistical Evaluation

Principal Investigators: Ananda Nisalak, M.D.
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Pairatana Gunakasem, M.D.
Robert Edelman, MAJ, MC

OBJECTIVES: 1. To evaluate the efficiency, precision, accuracy and reproducibility of a plaque reduction neutralization test (PRNT) for JEV.

2. To evaluate a microtiter PRNT for JEV.

PROGRESS: Four acute-convalescent serum pairs from 4 encephalitis patients were selected to evaluate the efficiency, precision, accuracy and reproducibility of a PRNT to JEV. The method used for JEV PRNT tests is identical to that described in previous annual reports for dengue viruses. Three or four replicate determinations on each serum pair were run independently at the same time by the same person. Excellent agreement was obtained by 2 different persons in counting plaques. A valid PRNT, with good precision and reproducibility, was uniformly obtained when the following statistical requirements were met: a) control plaque counts are between 30-100, b) plaque reduction responses bracket a 50% plaque reduction, c) at least 3 responses occur between 15-85% plaque reduction. In addition 3 plaque bottles per dilution were found to be adequate. The use of 2-fold serum dilutions produced statistically more powerful results than did using 4-fold dilutions. Discrimination between 2-3 fold titer changes could be reproducibly determined when all test conditions were met. However, only half of the tests proved to be statistically valid because of the difficulty in meeting the requirements summarized above. The large amount of data that had to be discarded even when performed under standard conditions, makes the PRNT too inefficient for routine survey usage.

A micro PRNT was re-established in this laboratory for JEV, dengue 1-4, Wesselsbron, and Tembusu viruses. The tests were evaluated using techniques and statistical analysis similar to those used for the evaluation of the PRNT described above. For comparisons both the PRNT and micro-PRNT were run on the same specimens, in parallel. The results indicated that the micro-PRNT was accurate, reproducible, and precise. It requires only one-tenth the serum volume required by the PRNT, and many more sera can be tested during one work week as compared to the PRNT. Evidence so far suggests that the micro-PRNT may be a useful adjunct for large scale serological testing. Small

vertebrate sera collected in Chiang Mai are now being analyzed by the micro-PRNT.

6. Comparison of the Antigenic Variation Among Strains of Japanese Encephalitis Virus

Principal Investigators: Ananda Nisalak, M.D.
Robert Edelman, MAJ, MC

Associate Investigator: Thomas J. Smith, COL, MC (deceased)

OBJECTIVE: To detect possible antigenic differences between JEV strains isolated in S.E. Asia.

PROGRESS: Kinetic neutralization assays were used to search for possible antigenic differences existing between JEV strains isolated in Thailand and Vietnam. Four strains of JEV of the same passage level were exposed to antiserum against one of the 4 strains for 1.25, 2.5, 5.0 and 10 minutes. The tests were run with and without added serum accessory factor. The percent virus survival for each strain was plotted against time, and kinetic neutralization curves were drawn and compared. The results suggested that there are no major antigenic differences among 3 Thai strains and one Vietnamese strain of JEV tested by a one-way neutralization kinetic assay. These results should be confirmed by a two-way cross titration using homologous and heterologous antisera against each of the 4 virus strains.

6. Japanese Encephalitis in U.S. Military Personnel in Vietnam.

Principal Investigators:	Robert M. Russell, CPT, MC Vincent J. Ionata, CPT, MC Robert Edelman, MAJ, MC
Associate Investigators:	Ananda Nisalak, M.D. Suchinda Udomsakdi, M.D. Debhanom Muangman, M.D. Rapin Snitbhan, M.D. Anong Pariyananda, M.S. Thomas J. Smith, COL, MC (deceased)

OBJECTIVE: To determine the etiology of "summer encephalitis" among U.S. military personnel in R.V.N.

DESCRIPTION: During 1969, 53 cases of encephalitis with 2 deaths were reported by U.S. Army Headquarters in Vietnam. The majority of cases occurred during the summer monsoon and originated from the III and IV Corps Area. Nine of 11 paired sera obtained from patients with "summer encephalitis" showed 4-fold or greater rises in antibody to Japanese encephalitis virus (JEV) by HI testing. In addition JEV was isolated from the brain of one patient with fatal encephalitis. The purpose of this study was to determine if the encephalitis in U.S. troops in Vietnam during the 1970 monsoon season was caused by JEV.

PROGRESS: Between 15 May and 15 October 1970, patients from the III and IV Corps area with a presumptive diagnosis of encephalitis were studied. The clinical workup of each patient was performed by members of the 20th PMU unit, US, Long Binh, Vietnam, and the serological tests and virus isolation studies were performed at SMRL. A total of 33 patients with encephalitis were admitted to the 24th, 12th, and 93rd Evacuation Hospitals in Vietnam during the study period. Twenty-five of these patients had serological evidence of a recent JEV infection by the HI, CF, and PRNT tests: 20 had four-fold or greater rises to JEV and 5 had high, fixed antibody titers. Three patients had a primary antibody response to JEV, while 17 had increases in antibody to dengue serotypes as well as JEV. In addition to these standard serological tests, the IgG & IgM serum immunoglobulins were fractionated by sucrose-density gradient centrifugation and were tested for HI activity against dengue serotypes 1-4 and JEV. Using this serum fractionation technique we were able to confirm a recent JEV infection in 21 of 22 patients with cross-reactive HI, CF, &

PRN antibody to dengue. The IgM fraction in the serum of these 21 patients reacted monospecifically against JEV, while IgG reacted with JEV and all 4 dengue virus antigens. No virus was recovered from the cerebrospinal fluid of 7 patients with undetectable serum antibody to JEV. Three patients died, but their brains could not be obtained for virus studies. Thirty-eight pools of culicine mosquitoes collected on Long Binh Post, Vietnam, were negative for virus.

7. Evaluation of Biken Japanese Encephalitis Vaccine in Peace Corps Personnel in Thailand.

Principal Investigators: Howard B. Emery, M.D.*
Ronald G. Wilson, M.D.*
Robert Edelman, MAJ, MC
Richard A. Grossman, MAJ, MC

Associate Investigator: Ananda Nisalak, M.D.

OBJECTIVE: To determine the antibody response in U.S. adults immunized with Biken JEV vaccine,

DESCRIPTION: The only commercially available immunization against JEV is a formalin-killed vaccine (Biken) prepared and purified from infected mouse brain. The immune response induced by this vaccine in adults has not been adequately studied for periods greater than 4 months after immunization. During the period November 1970 through May 1971, the Peace Corps is offering the Biken vaccine to all Peace Corps Volunteers in Thailand for protection against Japanese encephalitis. The immunization of Peace Corps Volunteers permitted collaborative study involving SMRL to study the antigenicity of the Biken vaccine in U.S. adults.

PROGRESS: The vaccine has been administered to several hundred Peace Corps Volunteers on a voluntary basis. The vaccine has been given as 2 subcutaneous 1 ml. injections 1 week apart, according to the manufacturer's recommendations. When possible, a booster dose was given 4 to 6 weeks after the primary immunizations. Three lots of vaccine, obtained from the manufacturer (Research Foundation for Microbiological Diseases of Osaka University), are being examined for antigenic potency. Each volunteer was to be bled prior to immunization and at selected times after immunization up to 24 months. Sera will be tested for antibody by standard HI and PRNT tests.

Preliminary results of the antibody response induced by 2 of the vaccine lots in 38 volunteers were disappointing. Four-fold or greater JEV HI antibody rises occurred in only 5% of the immunized volunteers one month after the primary immunizations. The vaccine did not induce

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* Peace Corps Physicians

an anamnestic antibody response in the small number of volunteers with pre-existing group B arbovirus HI antibody nor in volunteers without antibody after primary immunization who received a booster immunization. The 2 lots of Biken vaccine tested appear to lack adequate antigenic potency in adults.

8. Group B Arbovirus Serology: A Search for Humoral Specificity.

Principal Investigators:	Robert Edelman, MAJ, MC Ananda Nisalak, M.D.
Associate Investigators:	Anong Pariyananda, M.S. Suchinda Udomsakdi, M.D. Dennis O. Johnsen, MAJ, VC

OBJECTIVE: To determine more specific serologic methods for the detection of group B arbovirus infections.

DESCRIPTION: Individuals develop broad serological reactivity to most group B antigens following a second infection with a group B arbovirus; this prevents reliable identification of the most recent infection by standard serological methods. The purpose of this study was to develop a serological method to specifically identify a recent group B arbovirus infection in the face of past group B experience. The information gained will be used to analyze the sera from the Chiang Mai JEV study that show secondary group B arbovirus antibody patterns.

PROGRESS: We selected 5 groups of subjects that have experienced 2 or more group B arbovirus infections. The sera from these subjects were tested by the HI, CF and PRNT tests in the expectation that one, or a combination of these serological tests, would specifically identify the recent infection. Included in the 5 groups were 1) Thai children with Japanese encephalitis, 2) Dengue-sensitized gibbons infected with JEV, 3) U.S. troops with Japanese encephalitis, 4) Thai children with dengue hemorrhagic fever, 5) Healthy adult residents of Chiang Mai Valley, Northern Thailand.

No one standard serological test, employed alone or in concert with 2 other standard tests, was found which reliably distinguished recent group B infections in individuals who have had past infection. Moreover the results suggested that the decay rate in vivo of specific and cross-reactive antibody might be similar; thus one cannot reliably diagnose past infections in currently healthy individuals using the standard arbovirus serological methods.

Several other methods were employed in a search for serological specificity. In one study we examined the effect of fresh, human, antibody-free serum on JEV and dengue virus neutralizing antibody. Fresh serum contains a poorly characterized factor (serum accessory factor) which potentiates serum neutralizing antibody titers against

arboviruses of groups A and B. We tested whether human accessory factor can increase the specificity of neutralizing antibody activity in sera containing cross-reactive antibody. We found that fresh human serum, added to immune, heat-inactivated serum obtained from JEV or dengue-infected patients increases the neutralizing antibody titers in all sera tested 2 to 25-fold against JEV and the 4 dengue serotypes. Although accessory factor increased the sensitivity of the PRNT test, it did not yield increased specificity for the most recent infection and provided no solution to the problem of antibody cross-reactivity in these group B infections.

In a second study we tested for the presence and immunospecificity of IgM in the serum of subjects with secondary group B arbovirus infections. Aliquots of whole serum were treated with 2-mercaptoethanol (2-ME) and treated and untreated serum aliquots were diluted simultaneously for HI tests run against JEV and dengue antigens. If the HI antibody titer in the treated sera was reduced to, or below, one-fourth the untreated serum, the serum was judged to contain 2-ME sensitive antibody. We tested sera obtained from 4 sentinel pigs stationed in Chiang Mai, from 2 gibbons inoculated with dengue and later JEV at SMRL, and from 8 Thai children and 12 American soldiers hospitalized with Japanese Encephalitis. We detected IgM antibody in one pig and in 6 of 7 patients showing a primary immune response to JEV; the antibody was specific for JEV and not dengue. However we were unable to demonstrate IgM in the sera of 13 patients who showed a secondary type antibody response to a recent JEV infection. Thus the treatment of serum with 2-ME did not provide increased specificity in these secondary infections.

In a third study, we fractionated IgG and IgM from whole serum by sucrose density gradient centrifugation. This procedure permitted us to test the immunospecificity of the two immunoglobulins independently of the other. Serum-sucrose fractions containing IgM or IgG were treated with 2-ME, and the treated and untreated fractions were tested by the HI test against dengue 1-4 and JEV antigens. The presence of immunospecific IgM antibody activity was shown by its characteristic position in the sucrose gradient, by determination of IgM content of each fraction, and by its susceptibility to reduction by 2-ME. IgM antibody was found in the convalescent serum of 32 of 35 patients with acute primary dengue infections and with primary and secondary JEV infections. The IgG antibody in all sera reacted to both JEV and dengue 1-4 antigens in high titer. IgM reacted monospecifically in low titer to JEV in 27 of 29 patients with encephalitis (3 primary and 24 secondary infections), and to one dengue serotype in 3 patients with primary dengue fever. Two more patients with primary dengue fever and viremia had cross-reactive

IgM antibody against one other virus antigen in addition to the causative agent. We conclude that the presence of IgM antibody reacting only to JEV confirms a recent infection by that agent in subjects experiencing their first group B arbovirus infection and in individuals showing cross-reactive serum antibody reactions to dengue. The specificity of IgM assay in primary dengue infections requires more substantiation. The IgM antibody assay does not hold promise for the identification of temporally remote arbovirus infections due to the short half life of IgM antibody in vivo. In addition, repeated infections by dengue and probably JEV induces the production of IgG antibody but not IgM. Studies are now aimed at confirming the immunospecificity of IgM in other group B arbovirus infections in animals and man. In addition investigations are underway to determine how long IgM remains detectable following clinical and subclinical infections.

9. Evaluation of Insect Tissue Culture Lines as Primary Arbovirus Isolation Systems

Principal Investigators: Pairatana Gunakasem, M.D.
Robert Edelman, MAJ, MC

OBJECTIVE: 1. To compare the growth of arboviruses recovered in Thailand in Aedes albopictus and the standard LLC-MK-2 lines.

2. To compare the efficiency of isolation of arboviruses in Aedes albopictus and LLC-MK-2 cells.

DESCRIPTION: LLC-MK-2 cells are routinely used in this laboratory as a host in the recovery of arboviruses from natural sources. Although these cells have been shown to be efficient in the isolation of dengue and chikungunya viruses, they appear to be less efficient than suckling mice for the isolation of Tembusu virus and other currently unidentified viruses which were isolated in suckling mice during the Chiang Mai JEV study. Recent publications on the growth of arboviruses in insect cell cultures led us to compare the growth of certain arboviruses recovered in Thailand in Aedes albopictus and in LLC-MK-2 cell cultures and to compare the efficiency of isolation of arboviruses from natural sources in the 2 cell lines.

PROGRESS: The Singh line of A. albopictus cells were cultured in Mitsuhashi and Marmorosch (M-M) medium. LLC-MK-2 cells were grown and maintained by standard techniques. In the experiments described, equal volumes of similar dilutions of the virus strains were inoculated onto monolayer cell cultures of A. albopictus and LLC-MK-2 cells grown in 1 ounce prescription bottles. LLC-MK-2 cells were maintained at 37°C and A. albopictus cells at 32°C after inoculation. At various intervals, half the volume of maintenance medium was aspirated from infected cultures of both cell types for titration of PFU in LLC-MK-2 cells and this volume was replaced with the same volume of fresh maintenance medium. In the first experiment only (growth of laboratory JEV strain 40783), both the cells and maintenance medium were pooled as a source of virus for titration.

The growth of JEV strain 40783 in LLC-MK-2 and A. albopictus cells

A strain of JE virus (40783), recovered from the brain of a fatal case of human encephalitis, was used at the sixth mouse passage level to infect Culex tritaeniorhynchus mosquitoes by feeding them on chicks infected at one day of age. Infected mosquito pools were diluted

serially ten-fold and inoculated onto LLC-MK-2 and A. albopictus cells. Doses of virus inoculated, determined by titration in LLC-MK-2 cells, varied from 115,000 to 1.2 PFU. Peak virus titers were obtained at all dilutions in both the cell cultures before 7 days with the exception of the 1.2 PFU dilution in A. albopictus cells when peak titers were obtained at day 11. Virus titers obtained in A. albopictus cells between 3-7 days after inoculation generally were 1-2 $\log_{10}$ higher than titers obtained in LLC-MK-2 cells.

Comparison of yields of JE virus from known infected wild mosquito pools in A. albopictus and LLC-MK2 cells.

Three wild culicine mosquito pools (BKM1022, BKM1096, and BKM977) obtained from the Chiang Mai Valley from which JEV had been isolated were used undiluted to infect LLC-MK-2 and A. albopictus cells. Peak virus titers in A. albopictus cells (9.5 and 6.6 $\log_{10}$) exceeded those obtained in LLC-MK-2 cells by 2.5 $\log_{10}$ and 1.0 $\log_{10}$ with 2 of the strains and equalled (9.3 $\log_{10}$) those obtained in LLC-MK-2 cells with the 3rd strain.

Comparison of yields of Wesselsbron and an unidentified Group A arbovirus from known infected mosquito pools in A. albopictus and LLC-MK-2 cells.

A wild mosquito (C. vishnui) pool containing an unidentified group A arbovirus (BKM705) and a wild mosquito (A. linneatopennis) pool containing Wesselsbron virus (BKM 660) were used undiluted to infect LLC-MK-2 and A. albopictus cell cultures. Peak virus titers in A. albopictus exceeded those obtained in LLC-MK-2 cells by 1.3 $\log_{10}$ for the BKM-705 strain and 2.5 $\log_{10}$ for the Wesselsbron strain.

The preceeding experiments indicated that certain low passage arbovirus strains indigenous to Thailand grew to higher titers in A. albopictus cell monolayers than in LLC-MK-2 cell monolayers. The following experiments were undertaken to compare the sensitivity of isolation of Thai arboviruses in the 2 cell lines. In these experiments, specimens containing virus were serially diluted ten-fold and each dilution was inoculated into LLC-MK-2 and A. albopictus cells. The cells were washed at 1 1/2 hours to remove unadsorbed virus in the inoculum and fed with maintenance medium. Aliquots of maintenance medium were aspirated from cultures of each dilution daily for 9 days post inoculation and inoculated undiluted into LLC-MK-2 cells for plaque assay. Dilutions showing $\geq$100 PFU in plaque assay were considered positive.

In one experiment a 20% suspension of human brain specimen containing JEV virus 40783 was used. JEV virus was isolated from undiluted through the 10^{-4} dilution of the brain suspension in LLC-MK-2 cells, while virus was isolated from undiluted through the 10^{-8} dilution of the same suspension in A. albopictus cells. In a second experiment, a C. tritaeniorhynchus pool containing JEV virus was used. The highest dilution of this pool yielding $\geq$100 PFU was the 10^{-2} dilution in both cell lines.

A third experiment employed 5 dengue virus strains of low (1 or 2) LLC-MK-2 passage. From 3 of the strains (Dengue types 1, 2, and 3) virus was isolated from 10^{-4} dilution in A. albopictus cells and from the 10^{-3} dilution in LLC-MK-2 line. No difference in sensitivity between the 2 cells was however noted with a dengue 4 strain. The 5th strain, a dengue 3 strain, failed to grow in A. albopictus cells but was isolated from the 10^{-3} dilution in LLC-MK2 cells.

These preliminary experiments indicate that A. albopictus cells support the growth of certain low-passaged arboviruses from Thailand. Generally, higher titers of virus were achieved in A. albopictus cells than in the standard LLC-MK-2 cells. Data on the relative efficiency of both cell lines in arbovirus isolation are limited by the few specimens available for testing. Further comparisons of the relative isolation efficiency of the 2 cell lines from specimens obtained in natural arbovirus infections of humans and induced JEV infections of monkeys are planned.

10. Investigations of Transmission of an Unidentified Virus in Cave Bat Populations in Thailand.

Principal Investigator: James E. Williams, CPT, MSC

OBJECTIVE: To determine the transmission of an unidentified virus, S-19-B, in cave bat populations.

DESCRIPTION: During a previous ecological and epidemiological survey for rabies virus in a cave bat population, a large number of virus strains which were not rabies virus were isolated in suckling mice from bats. The viruses grew in LLC-MK-2 cells producing a cytopathic effect. Antibody produced against one isolate, S-19-B, was found to neutralize all but one of the isolates tested. A one year serological survey of neutralizing antibody in a population of the wrinkle-lipped bat (Tardarida plicata) located at Ban Me, Thailand, is being undertaken to determine the seasonal cycle in transmission of S-19-B virus.

PROGRESS: Bat sera (60-70) collected in February, April, July and October, 1970, have been tested at 1:10 and 1:50 dilutions in standard plaque-reduction-neutralization tests using LLC-MK-2 cells. Tests of a serum collection made on 4 March 1971 have not been completed. Data in hand from the serological survey suggest that transmission of the virus varies over the year and peaks around March-April when young bats appear, which are easily preyed upon by hemophagous arthropods. Thus, baby bats are being studied to determine if they function as amplifying hosts of S-19-B virus. A collection of 150-200 immature bats is being examined for virus.

Isolations of S-19-B virus have been made from mixed pools of blooded and non-blooded bedbugs collected in the Ban Me cave. The isolations were made in both suckling mice and MK-2 tissue culture isolation systems. An attempt is being made to determine if the virus was isolated from infected insect tissues or merely from bat blood ingested by the bedbugs. In addition, samples of bedbugs collected from the cave on Khao Lom Phat, Saraburi, Thailand, where S-19-B virus was first isolated two years ago, are being examined for virus to ascertain if the virus ecology there is similar to that at Ban Me, Thailand.

11. Hepatitis-associated antigen (HAA) and Hepatitis in Thailand

Project Coordinator: Robert B. Cotton, MAJ, MC

Principal Investigators: William A. Bancroft, MAJ, MC
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OBJECTIVE: The purposes of this laboratory's initial 6 months of research in hepatitis were to:

1) establish laboratory methods for the detection of Hepatitis-associated antigen (HAA).

2) determine the prevalence of HAA in the serum of patients with hepatitis and matched controls.

3) determine the prevalence of HAA in Thai blood donors.

DESCRIPTION: These studies are based on the following premises:

1) HAA can be detected in the serum of patients with serum hepatitis. The most specific diagnostic distinction between serum and infectious hepatitis is the presence or absence of HAA in patient serum. The detection of HAA provides an epidemiologic marker for the study of serum hepatitis.

2) Serum hepatitis is infective orally, as well as parenterally. The transmission of serum hepatitis is not limited to parenteral inoculation of the virus.

3) Serum hepatitis accounts for a major proportion of sporadically occurring hepatitis cases in adults.

4) Chronic carriers of HAA exist. In general, they are asymptomatic with little or no evidence of liver disease. In the United States, at least 0.1% of volunteer blood donors are carriers of HAA. Estimates in Southeast Asia indicate that the background prevalence of HAA may be 50 times higher than in the United States.

5) Preliminary studies have indicated that predisposition to the HAA carrier state may be genetically determined by an autosomal-recessive mode of inheritance.

6) In the United States, over 50% of recipients of HAA positive blood develop serum hepatitis.

PROGRESS: 1) Laboratory detection of HAA

A microtiter complement fixation (CF) test using five 50% hemolytic units of complement and 2-4 units of antibody has been established. The test system is identical to that used by Department of Virus Diseases, WRAIR. The antiserum used is from a Thai adult (Korn) who developed antibody following transfusion with HAA positive blood. The sensitivity and specificity of this human antiserum compares favorably with guinea pig reference antiserum prepared by the Department of Virus Diseases, WRAIR. An experiment has been completed which demonstrated acceptable reproducibility in detecting HAA by the CF test between replicates in this laboratory and between this laboratory and Department of Virus Diseases, WRAIR. Over 2,400 sera were tested for HAA as of 31 March 1971.

In order to provide a complementary method of detecting HAA suitable for the testing of larger numbers of serum of small volume, an immunoelectrosmophoresis (IEOP) test for HAA was established. IEOP is a technique which accelerates the formation of immunologic precipitin reactions in an agarose gel by forcing antigen to migrate toward antibody in an electric field. In an electrophoretic field, HAA (with a negative charge) moves toward the anode and specific antibody diffuses toward the cathode. Where they meet and reach equivalence, a visible light scattering band is formed. The IEOP procedure used is similar to that employed in the Department of Virus Diseases, WRAIR. The power supply is a simple transformer-rectifier supply (Model 19, Arthur H. Thomas Co., Philadelphia) with 300 volt, 50 ma output; the electrophoresis cabinet (4937-V20, Thomas Co.) has been modified so that voltage can be measured across the wicks. Ten ml. of a 1% agarose solution (Seakem, Marine colloids, Springfield, N.J.) in 0.05M Barbital buffer, pH 8.6 is pipetted onto a clean 3 1/4 by 4 inch glass slide. After solidification of the agarose, 3 mm diameter antigen wells and 2 mm diameter antiserum wells (5mm apart center to center) are punched and aspirated. Wells are loaded brim full with serum to be tested for antigen and with antiserum. Hyperimmune rabbit antiserum R #244, prepared in Dept. of Virus Diseases, WRAIR, has been used at a 1:4 dilution. A known HAA positive and HAA negative control serum is tested on each slide. The slides are laid in the electrophoresis chamber with antigen wells closest to the

cathode, and the power is adjusted to give 12 volts across the agar which yields approximately 10ma per slide. Slides are read after 2 hours of electrophoresis.

In order to determine if HAA determined by CF or IEOP are identical to standard HAA, an agar gel diffusion (AGD) test was established. The procedure used is identical to that used in the Department of Virus Diseases, WRAIR. Three ml. of hot 1% Agarose in 0.01M tris-EDTA buffer, pH 7.6 are pipetted onto clean 1x3 inch glass microscope slides. After cooling, 7 hole well patterns with 2 mm. wells and 5 mm. center to center spacing between wells are punched and the wells are aspirated. The center well is filled with undiluted hyperimmune HAA antiserum (Rabbit #230, WRAIR) and the top and bottom wells in the pattern filled with known HAA positive serum. The side wells are filled with human serum to be tested for HAA. Slides are incubated in a closed, moist chamber at room temperature and read at 24, 48, and 72 hours.

A recent experiment using serum from hepatitis cases and controls in Thailand compared the sensitivity of the 3 techniques in detecting HAA. Antiserum used in CF tests included Korn antiserum and Rabbit #244 antiserum. Rabbit #244 antiserum, diluted 1:4, was used in IEOP tests, and undiluted rabbit #230 antiserum in AGD tests. Of the 46 sera from hepatitis cases tested, 18(39%) were positive by IEOP and 15 (33%) were positive in CF tests using both antisera. All sera positive by CF were positive by IEOP; the three sera positive by IEOP but negative by CF gave partial fixation with R244 antiserum. Only 5 sera were positive by AGD, and these sera were positive by both IEOP and CF. When tested in tandem with the standard WRAIR HAA (JF) with R230 antiserum, the 3 hepatitis sera which gave distinct precipitin lines formed a spur with JF antigen, suggesting partial identity with JF antigen.

In the same experiment, 72 sera from matched controls of hepatitis cases were run by all 3 techniques. Seven sera were positive by IEOP, 5 of which were positive by CF using both antisera. An additional serum was positive by CF using R#244 antiserum but negative by CF with Korn antiserum and IEOP. Five of the 7 sera with HAA detected by IEOP were positive by AGD: three showed spur formation in tandem with JF antigen and 2 gave lines of identity with JF antigen.

Collaborative studies with the Department of Virus Diseases, WRAIR, are planned to investigate the significance of suspected antigenic HAA variants in Thailand and to explore reasons for the differences in detection of HAA by the IEOP and CF techniques.

2) HAA in patients with hepatitis

112 hospitalized patients from Bangkok Women's and Children's Hospital and the Royal Thai Army Hospital with an admission diagnosis of hepatitis have been studied. Of the 112 patients, 69 were considered to have hepatitis on the basis of an SGOT or SGPT level exceeding 100 Sigma Units. Of these 69 hepatitis patients, 23 (33%) had sera positive for HAA by the CF test. Of 21 cases of hepatitis in the 20-29 year age range, 14 (67%) were positive for HAA.

Of the 43 patients who did not meet the criteria for a final diagnosis of hepatitis, only 1 (2%) was positive. Of 85 control cases, only 6 (7%) were HAA positive.

31 hospitalized patients from the U.S. Army Hospital, Bangkok, with an admission diagnosis of hepatitis have been studied. Of the 31 patients, 30 were considered to have hepatitis by the criteria stated above. Of the 30 patients with a final diagnosis of hepatitis, 12 (40%) had sera positive for HAA by the CF test. Of 15 control cases, only 1 (7%) was positive for HAA.

22 children with a preliminary diagnosis of hepatitis were identified in the out-patient clinic of Children's Hospital over a 10 week period ending 19 March 1971. 18 of these 22 had abnormal liver function tests compatible with acute hepatitis. There were 18 matched controls for 12 of these 18 cases of hepatitis. Of the 18 hepatitis cases, 2(11%) were positive for HAA. Of the 18 controls, none were positive for HAA.

3) HAA among Thai blood donors

Of 515 professional donors of the Royal Thai Institute of Pathology Blood Bank, 24(4.7%) were positive for HAA by the CF test. Of 689 volunteer donors of the Thai Red Cross Blood Bank, 42(6%) were positive for HAA by CF test. Of the total 1204 donors from both blood banks, 66(5.5%) were positive for HAA. Preliminary results using both CF and IEOP tests indicate that about 8% of blood donors may carry HAA.

12. Reservoirs of Rabiesvirus in Thailand

Principal Investigators: Robert L. Hickman, MAJ, VC
Kwanyuen Lawhaswasdi, DVM

Associate Investigators: James P. Slowey, SFC
Ronald E. Marshall, SP5 E5

Part I. Survey of Sylvatic Animals for Rabiesvirus Infection

The purpose of this study is to determine the prevalence of rabiesvirus infections in rodents in Thailand. A total of 303 rats was trapped in nine provinces of Thailand (four areas). The animals were identified and all brains were examined for the presence of rabiesvirus by the fluorescent antibody (FRA) technique. Approximately five percent of all specimens were inoculated into weanling mice to confirm the negative FRA results. Rabiesvirus was not isolated from any of the animals examined. These negative results differ from the results obtained in 1966 but are the same as were obtained from surveys conducted in 1967, 1968, and 1969. There is no evidence that there has been any notable change in the annual incidence of either human or canine rabies in the areas surveyed during this five year period. This fact, in addition to the consistently negative rodent results obtained during the four year period since rodents were implicated as a possible sylvatic reservoir of rabiesvirus infection, suggests that the hypothesis is false. No further surveys are contemplated at this time although the submission of rodents for rabies examination will continue to be encouraged.

Part II. Survey of Domestic Animals for Rabiesvirus Infection

The purpose was to determine the prevalence of rabiesvirus infections in asymptomatic stray dogs captured by municipal and federal authorities in Thailand. Canine specimens were obtained from two sources. Stray dog control programs were conducted by the Division of Communicable Disease Control, Thai Ministry of Health, and cooperating U.S. Air Force installations in Thailand. From 10 to 30 of the dogs collected each day during the operation of the program were submitted for rabiesvirus examination. The total number of dogs captured in the communities involved is not known. The second source of specimens was the Bangkok Municipal Health Department which operates a continuous stray dog pickup program. Each week, 10 percent of the dogs picked up on a single day were randomly selected and submitted for examination. The total figure, therefore, approximates 1.6 percent of all the stray dogs destroyed during the period of the survey (estimated 20 thousand). Only dogs not having clinical signs of rabies were submitted from both groups. All

were examined by the FRA technique. Rabiesvirus was isolated from FRA positive specimens by mouse inoculation and confirmed by serum neutralization test. A total of 531 dogs was examined during the reporting period. The examination results are presented in the Table below. Since these animals were asymptomatic at the time of euthanasia and salivary gland examinations were not done, it is not possible to estimate how many of the FRA positive animals were capable of transmitting the disease. It is assumed that all were in some stage of virus incubation and that all would have eventually died of rabies. Certainly many would have been responsible for disease transmission to other animals and perhaps to man at some time before death. During the same reporting period, 456 canine specimens were examined for routine rabies diagnosis and 213 or 46.7 percent were found to be positive. The number of isolations obtained from the two dog populations emphasizes the extent of canine rabies in Thailand and the need for more adequate control programs if the problem is ever to be eliminated.

Table I. Isolation of Rabiesvirus from Asymptomatic Stray Dogs in Thailand.

Source	No. Specimens Examined	No. of Rabiesvirus Isolation	Percent
Udorn	105	4	3.8
Korat	51*	1	2.0
NKP	47*	1	2.1
Bangkok	328	13	4.0
Combined	531	19	3.8%

* Mouse inoculation results incomplete.

Part III. Urban rabies in Thailand

The purpose was to study the canine population in an urban community in Bangkok, Thailand, in order to determine the risk potential of the dogs and human inhabitants to rabiesvirus infection. The Huey Kwang Government Housing Area was chosen as a study site. The canine population was determined and characterized. Suspect rabid animals were picked up or delivered to the laboratory for examination. The human population was obtained from 1970 census data and changes were based on population estimates furnished by local officials. The number of human dog bite victims was provided by the local medical facility and the municipal antirabies treatment center. Specific information regarding the circumstances surrounding dog bites was obtained by

interviewing the victims. The study was conducted over a ten month period. The canine-human ratio was approximately 1/15 and remained relatively constant throughout the year. However, only 65 percent of the total dog population was considered to be at risk (unconfined, unvaccinated) so the effective "stray dog" - human ratio was 1/23. Two rabid dogs were identified in the fourth month of the study period, one in the seventh and one in the ninth month. Three of these rabid dogs are known to have been unconfined, unvaccinated residents of the study area whereas the fourth is believed to have been a nonresident stray. There were several reports of "rabid" dogs during the eighth and ninth months of the study but these could not be confirmed. A total of 28 residents of Huey Kwang are known to have received treatment for dog bites during the study. Of these, 26 were given Semple vaccine and two received duck embryo vaccine. No rabies or neurologic reaction was observed in any of these patients. One rabies death did occur however. A policeman passing through the study area during the seventh month was bitten by a dog. At that time, no treatment was administered nor was the incident reported. The dog was never identified but probably was not one of the dogs diagnosed in the laboratory. Five weeks after being bitten, the patient reported to the hospital with symptoms of rabies encephalitis. He died four days later. The diagnosis of rabies was not confirmed. Data are being tabulated and evaluated and a manuscript is being prepared.

13. Serologic Effect of Duck Embryo Rabies Vaccine

Principal Investigators: Robert L. Hickman, MAJ, VC
Kwanyuen Lawhaswasdi, DVM
Ronald G. Wilson, MD*
Howard B. Emery, MD*

Associate Investigator: Markpol Tirgpalapong, DVM

This study is to determine the human serological response to duck embryo (DE) rabies vaccine given prior to exposure and after exposure to individuals who have and have not received pre-exposure immunizations. A second purpose is to compare the serologic response to DE rabies vaccine as detected by the indirect fluorescent antibody (IFRA) test and the mouse neutralization (MI) test. Sera are collected from Peace Corps volunteers receiving pre-exposure rabies immunizations with DE vaccine. Sera are also collected from U.S. military personnel and Thai nationals during and after administration of post-exposure rabies immunizations with DE vaccine. Traditionally, approximately 25 percent of the Peace Corps Volunteers in country receive post-exposure treatment during their tour. Sera will be collected from these individuals during and after treatment. The rabies antibody titers are determined by the IFRA test. Aliquots of sera are frozen for future use in MI test. Sera were collected from 56 Peace Corps volunteers, 22 U.S. Military personnel and 28 Thai nationals. During the third quarter of the reporting year, it was discovered that the reproducibility of the IFRA test results was not satisfactory. Therefore, all results were discarded and efforts to improve the test are in progress. Two sources of error have been identified. Slides with infected mouse brain impression smears cannot be stored in the frozen state after fixation for more than two weeks without resulting in lower titers. Resuspended anti-human globulin conjugated with fluorescein isothiocyanate has a shelf life in the refrigerator of less than six days. The use of new smears and freshly resuspended conjugate has eliminated the errors in reproducibility of titers determined on a single day. However, titers of a serum specimen determined on different days still occasionally vary by as much as two dilutions, thus invalidating the entire second test. Sera continues to be collected and frozen but progress on determining titers has been halted until the test has been modified to produce more satisfactory results.

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*Peace Corps Physicians

13. Ecological and Epidemiological Survey for Rabiesvirus in a Cave Bat Population.

Principal Investigator: Dennis O. Johnsen, MAJ, VC

Associate Investigators: Robert L. Hickman, MAJ, VC
William A. Neill, SP5
Naowayubol Nutkumhang, B.Sc.

The initial purpose of this study was to determine whether rabiesvirus is present among the population of bats resident in a large cave located in the Saraburi province district of Kangkoi so that the epidemiologic and ecologic aspects of this disease in bats could be studied. For a period of approximately one year both dead and live bats were collected from the cave at regular intervals and examined for the presence of rabiesvirus by both fluorescent antibody examination of their brains and intracerebral inoculation of brain and salivary gland suspension into weanling mice. Rabiesvirus was not isolated in any of the more than 1000 specimens examined, which consisted mainly of the wrinkle lipped bat, (Tadarida plicata). However, agents other than rabiesvirus were present which resulted in the death of approximately one third of all the mice inoculated. Following serial passage in mice, approximately 30 viral isolates were passaged by inoculating mouse brain suspensions onto tissue culture monolayers of MK-2 cells. Specific antisera was prepared for each of these isolates in guinea pigs, and, in several instances, rabbits, and cross neutralization tests were performed between all available antigens and antisera to determine if they were similar. On the basis of these tests, two separate antigens were identified. The first of these, virus S-19B which has been mentioned in previous reports, was identical to the agent present in all except one of the isolates; the other, 174B, was identified only once. Studies to establish the physical characteristics of the two viruses have further confirmed that they are two distinct agents. Further efforts will be made in the laboratory to determine the similarity of these two agents to other known viruses in the hope that they can be identified.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6466	71 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
70 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	045
b. CONTRIBUTING				
c. [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)
PRECEDING FISCAL YEAR 71	4.0	[illegible]
CURRENT FISCAL YEAR 72	3.4	.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

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PII Redacted

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

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

NAME: Altstatt, COL L. B.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: McMinn, CPT M. T.

NAME: Duangmani, Chiraphun IA

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) Diarrhea; (U) Pneumonia; (U) Venereal Diseases; (U) Vibrio parahaemolyticus

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) 70 07 - 71 06 Data indicates a poor correlation between the oral and pulmonary flora in lower respiratory tract diseases in Thai children. A potential enteric and systemic pathogenic bacterial species, Vibrio parahaemolyticus, has been recovered from numerous sources in Thailand. Preliminary data indicate that soft chancre may be due to synergistic infection of pre-existing lesions by normal vaginal flora. The gibbon does not appear to be a suitable animal model for infection with Neisseria gonorrhoeae. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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 68 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 045, Bacterial and mycotic diseases of man and animals

Investigators.

Principal: M. Talmage M'cMinn, CPT, MSC

Associate: Pongsom Atthasampunna, M.D.; Prakorb Boonthai, M.D.; Yupin Charoenvit, M.S.; Carleton M. Cliffords, Ph.D.; Edward W. Davis, SFC; Alexander De Paoli, MAJ, VC; Chiraphun Duangmani, M.D.; Alan Evangelista, Sp5; Harry Hoogstraal, Ph.D.; Inkam Inlao; Dennis O. Johnsen, MAJ, VC; Prakit Kanchanavatee, M.D.; Yupa Kasemsanta, P.H.N.; Panita Lakshana, B.S.; Udom Lexomboon, M.D., Ph.D.; Nongnuj Maneechai; Joe T. Marshall, Ph.D.; H. Elliott McClure, Ph.D.; M. Nadchatram; Pacharee Nawarat, B.S.; Tatsanee Occeño, B.S.; Wichiyan Paniom; Yongyuth Raengpradub, B.S.; Chantana Ratanavaraha, B.S.; Prani Ratarasarn; Chanphen Srimunta, B.S.; Harold E. Stark, Ph.D.; Michael F. Sullivan, CPT, MSC; Pramuan Sunakorn, M.D.; Prayoth Tanticharoenyos, DVM; Suthee Vallikul, M.D.; Somkiat Vongtangswad, M.P.H.

Occurrence of Vibrio parahaemolyticus in Thailand

Principal Investigators: M. Talmage M'cMinn, CPT, MSC
Pongsom Atthasampunna, M.D.*

Associate Investigator: Prakorb Boonthai, M.D.**

Assistant Investigators: Mrs. Yupin Charoenvit, M.S.
Sp5 Alan Evangelista, B.S.
Mrs. Yupa Kasemsanta, P.H.N.
Mrs. Chanphen Srimunta, B.S.

OBJECTIVE: Vibrio parahaemolyticus has been reported to be the cause of up to 70% of the "summer" diarrhea in Japan. It has been recovered from sea water, fish, and diarrheal stools in many other countries of SE Asia. This laboratory has developed the capability to culture appropriate specimens on specific media to determine if this possibly pathogenic organism could be isolated from various sources in Thailand.

DESCRIPTION: Specimens of sea water, sand, and sea fish were obtained from beaches and markets in the area. Diarrheal specimens were submitted from Bumras Infectious Hospital and from US Hospitals in Thailand. Specimens were maintained in alkaline peptone water with 3% NaCl added or were streaked directly into thiosulfate citrate-bile salts-sucrose (TCBS) media. Colonies were picked at 24 hours after 37C incubation and identified by biochemical and serological methods.

PROGRESS: Sand specimens collected along several beaches in Thailand have revealed 17 positive cultures of 50 specimens collected. Ten sea water specimens of 25 have had V. parahaemolyticus recovered from them. Sea fish cultures have produced 51 positive for V. parahaemolyticus out of 111 mollusks, 31 positive from 43 crab specimens, 59 positive from 85 fish, and 23 positive of 27 shrimp. Only 1 squid of 26 was found to harbour this halophilic organism. It is interesting to note that 4 of 5 catfish obtained from brackish water harboured V. parahaemolyticus in gills, intestine, and on the skin.

Diarrheal specimens from an infectious disease hospital were submitted to this laboratory for confirmation for 6 weeks in November and December 1970. Of approximately 300 diarrheal patients seen during that time, there were 59 isolates of V. parahaemolyticus and only 40 of Salmonella sp. or Shigella sp.

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* Dept of Health, Ministry of Public Health, Bangkok, Thailand
** Director, Bumras Infectious Hospital, Bangkok, Thailand

From 100 diarrheal specimens from children, only 1 isolate of V. parahaemolyticus was recovered. The same recovery rate of 1% was obtained for 100 non-diarrheal adults. One non-diarrheal specimen obtained from 22 youths (aged 5-15 years) revealed V. parahaemolyticus. We have identified this organism in 6 US personnel suffering from gastroenteritis seen at the US Hospital in Bangkok.

Vibrio parahaemolyticus has recently been reported to be associated with severe skin lesions resulting in gangrene and intravascular coagulation. This laboratory undertook to study these findings. Subcutaneous injections of V. parahaemolyticus into the legs of white mice has resulted in the production of progressive sloughing lesions, paralyses of the limb, and death with septicemia. Additional studies are underway.

In an effort to relate our data to that reported in the literature by Japanese investigators, growth in 10% NaCl solution and haemolysin production on Wagatsuma blood agar was studied. Japanese data indicates a lack of haemolysin in isolates obtained from sea water and sea fish. Our data indicates that 383 of 450 (85%) isolates from 'natural' sources demonstrated haemolysis on this special media. Isolates from human sources produced haemolysin with 119 of 120 isolates. Lack of growth in alkaline peptone water with 10% NaCl has been used as a criteria for V. parahaemolyticus in the Japanese reports. Unlike their data, our isolates do grow in this medium. No isolates of 113 from human sources failed to grow in this medium, while only 122 of 494 isolates from fish, sea water, etc failed to grow in 10% NaCl.

SUMMARY: There appears to be some real differences in the V. parahaemolyticus isolated from natural sea sources in Thailand and in Japan. More than one half of the isolates we have recovered are untypable against the sera obtained from Japanese cultures. This may be due to nutrients available to the organism or to water temperature differences. We are studying some possible reasons for these differences.

While we have little epidemiological evidence of pathogenicity, we suspect that this organism may be a significant factor in diarrhea in Thailand. We are currently examining several models of pathogenicity.

This laboratory plans to continue and elaborate upon the work concerned with cutaneous entry of V. parahaemolyticus into susceptible hosts and the definition of such infections.

The Etiology of Non-leuctic Soft Chancre

Principal Investigators: M. Talmage M'cMinn, CPT, MSC
Chiraphun Duangmani, M.D.

Assistant Investigators: Yongyuth Raengpradub, B.S.
Wichiyan Paniom

OBJECTIVE: Soft chancre is reported to be the second most common venereal disease among United States troops operating in SE Asia. The diagnosis and treatment of this lesion is accomplished without any bacteriological studies in almost all instances. Treatment is often long and must often be altered several times to effect a cure.

DESCRIPTION: This laboratory is currently studying the microorganisms present in these lesions. Patients are referred to this laboratory from the US Hospital in Bangkok for dark field examination and extensive culture. All lesions studied to this date are from male patients.

PROGRESS: Specimens of pus and exudate are inoculated onto Thayer-Martin media, on sheep blood agar, and on Eugonagar with 25% fresh rabbit blood. In most instances inoculations are made on fresh rabbit blood clots and on patients' blood clots. Plates are incubated at 37C aerobically and in a brewer jar. Many of the lesions are quite insignificant and are presented the first day they were noticed. In these early lesions only minute amounts of exudate can be obtained.

To date, 62 lesions have been examined. None of these have demonstated any spirochetal forms although one patient subsequently developed a positive VDRL and the characteristic skin lesions of secondary syphilis.

From the 62 lesions studied, 8 have revealed "suspected" Haemophilus ducreyi and 1 a "suspected" H. vaginalis. The most recovered organisms have been Staphylococcus epidermidis (39) and diptheroid-like bacteria (30). Alpha haemolytic streptococcus was identified in 15 lesions and S. aureus in 14. The other bacteria identified were common skin inhabitants.

SUMMARY: This study appears to indicate several facts. First, there is no adequate method of identifying H. ducreyi. It is noted in this laboratory as being a Haemophilus species that does not fit any of the biochemical characteristics of any other member of the species. Second,

a diptheroid organism is usually associated with serious lesions. Third, from histories obtained from the patients, nearly all of these lesions existed prior to intercourse, usually as a small blister, pimple, or 'rubbed raw' abrasion.

This laboratory is attempting to devise a schema to firmly identify H. ducreyi. At present an organism is designated as H. ducreyi because of its morphology, affinity for X or V factor, and inability to reproduce in any carbohydrate broth. We plan to add specific growth factors to diagnostic media and determine a biochemical and fermentative profile for H. ducreyi.

In order to evaluate the possibility of synergisms, subcutaneous inoculations of the various organisms usually recovered from lesions have been studied. Inoculations of H. ducreyi, H. vaginalis and diptheroids have produced chancre-like lesions in rabbits. Additional work on the synergisms that may be involved in this disease is being conducted.

It is interesting that nearly all patients report a pre-existing lesion prior to intercourse. None deny sexual contact. Some speak of recurring lesions that resemble those reported as due to Herpes progenitalis. It is quite possible that many of the lesions are secondary infections due to normal vaginal flora. H. vaginalis has been recovered from 5.4% of 421 endocervix cultures and from 8.9% of 279 urethral cultures in this laboratory. We have recovered this organism from 4 of 83 male patients with urethritis.

Soft chancre continues to be a serious venereal disease in SE Asia. We have some data that suggests a possible synergistic secondary infection of normal vaginal flora. Additional studies and the development of an identification scheme for H. ducreyi and H. vaginalis are necessary if directed antibiotic therapy is desired for the treatment of non-leuetic soft chancre.

Isolation of Neisseria gonorrhoeae from Females

Principal Investigator: M. Talmage M'cMinn, CPT, MSC

Associate Investigator: Prakit Kanchanavatee, M.D.*

Assistant Investigators: Mr. Wichiyan Paniom
Mr. Yongyuth Raengpradub, B.S.

OBJECTIVE: The diagnosis of gonorrhoeae in females has always posed a problem for the clinician. Female patients infected with gonorrhoeae are often asymptomatic. There is seldom a readily observable discharge as is usually presented with male patients. The Gram stain that is usually satisfactory in males is the method most often used with females as it is cheap and requires little equipment. Many clinics have been disappointed with culture techniques as the results are often ambiguous. This laboratory undertook to study the use of selective media and various specimens in the diagnosis of gonorrhoeae in females.

DESCRIPTION: We were fortunate in that selected patients seen in a venereal disease control clinic were made available for study to this laboratory by the Ministry of Health, Thailand. At this clinic females are given pelvic examinations. Those with any suggestive evidence of venereal diseases were examined further. For our studies, swabs were obtained from the cervix, urethra, and/or rectum. Smears were made from cervical exudates for Gram stains which were made and interpreted by technicians at the Venereal Disease Control Clinic.

PROGRESS: Of 424 individual specimens examined, 65 (15.33%) were positive for intra and extra-cellular Gram negative diplococci when examined by Gram stain technique. Culture in this laboratory on Thayer-Martin chocolate media with V.C.N. inhibitor and Isovitalex revealed 158 (37.2%) specimens positive for Neisseria gonorrhoeae. There were 9 (4.12%) specimens positive on Gram stain that were negative at culture.

An evaluation of various media was made during this study. Thayer-Martin chocolate media with Isovitalex and V.C.N. proved to give better recovery of N. gonorrhoeae than other media used. These included GC base with haemoglobin powder, supplement B, and 5% dextrose; Mueller-Hinton with Isovitalex and V.C.N. and GC base media with Isovitalex. The latter two media were manufactured without haemoglobin in order to facilitate the identification of 'T' strains of N. gonorrhoeae. We found this not to be a difficult problem and felt that the incorporation of

* Venereal Disease Control Clinic, Ministry of Health, Thailand

haemoglobin was important in obtaining maximum growth.

In an attempt to determine the best specimen for maximum recovery of N. gonorrhoeae, specimens were obtained from the cervix, urethra, and rectum. Greatest recovery from individual specimens occurred from the cervix with 10.2% of 422 specimens revealing N. gonorrhoeae from this site alone. Rectal specimens were positive with 1.54% of 133 specimens, followed by 0.69% of 289 specimens from the urethra. Combinations of two sites revealed 39.44% of 289 specimens with N. gonorrhoeae from both the cervix and urethra and 33.83% of 133 specimens from both the cervix and rectum.

An interesting observation regarding serum antibiotics was made in connection with this study. Serum was obtained from 79 patients and was examined for anti-bacterial activity against Bacillus cereus v. mycoides (ATCC 11778) and Staphylococcus aureus (ATCC 6538P) Of 56 patients with negative cultures for N. gonorrhoeae, one half (28) had a significant serum antimicrobial titer. Seventeen patients (30.3%) with proven gonococcal infections had serum antimicrobial activity while 6 patients infected had no indication of serum antibiotics.

SUMMARY: Our data indicates that the Gram stain of cervical exudates is a poor technique for the diagnosis of gonorrhoeae. Only 41.1% of the positive diagnosis resulted from such smears. If it is possible to culture two sites, our data indicates that the cervix and urethra are the areas most probable to harbor N. gonorrhoeae.

We believe that additional studies should be undertaken to examine the effects of threshold levels of serum antibiotics on N. gonorrhoeae. Long term use of ineffective antimicrobials of insufficient serum levels of antibiotics may prevent recovery or the organism on artificial media. Such a study is presently being designed by this laboratory.

Susceptibility of the Gibbon to Infection with Neisseria gonorrhoeae

Principal Investigator: M. Talmage M'cMinn, CPT, MSC

Associate Investigators: Alexander De Paoli, MAJ, VC
Dennis O. Johnsen, MAJ, VC
Prayoth Tanticharoenyos, DVM

Assistant Investigator: Yongyuth Raengpradub, B.S.

OBJECTIVE: A laboratory animal model for the study of gonorrhoeae infections as they occur in man is not presently available. The development of such a model would extend the opportunities to study aspects of this disease such as the development and character of antibiotic resistance and factors which affect host immunity. These aspects are beyond what is presently possible with human volunteers. Because the gibbon, Hylobates lar, has been shown to be susceptible to other human infections and numbers of these animals are readily available in the laboratory, it seemed a logical animal to study for this purpose.

DESCRIPTION: Following a small pilot study to establish the normal bacterial flora in the gibbon nasopharynx, rectum, conjectiva, the urethra or vagina, and the gingiva, 28 gibbons consisting of an equal number of males and females were inoculated topically and intramucosally by multiple routes with a heavy suspension of the virulent (T1) strain of Neisseria gonorrhoeae isolated earlier from infected humans. The conjunctival, rectal, cervical, and in the male the urethral sites of inoculation were examined daily for approximately two weeks for any evidence of clinical lesions. Bacterial cultures were also made each day from these same sites and processed in the laboratory under conditions favoring to maximize the isolation of N. gonorrhoeae.

PROGRESS: No clinical evidence of infection or N. gonorrhoeae isolates were obtained during this time.

SUMMARY: It seems apparent from these findings that the gibbon is not susceptible to infection with N. gonorrhoeae under these conditions. The normal body temperature of the gibbon is slightly higher than that of man and this factor may be significant in preventing the establishment of infections with N. gonorrhoeae, an organism that is known to be vulnerable to such increased temperatures when artificially cultivated.

Acute Pneumonitis in Thai Children

Principal Investigators: Chiraphun Duangmani, M.D.
Suthee Vallikul, M.D.*

Associate Investigator: Pramuan Sunakorn, M.D.*

Assistant Investigator: Tatsanee Occeño, B.S.

OBJECTIVE: Bacterial pathogens such as Diplococcus pneumoniae and Staphylococcus aureus have been cultured from throat and nasopharyngeal specimens from children in the absence of clinical pneumonia. This laboratory in cooperation with the Children's Hospital in Bangkok, Thailand has undertaken to interpret such findings by isolating, identifying and comparing cultures obtained from lung aspirates and throat and/or nasopharyngeal swabs.

DESCRIPTION: During the period reported a total of 53 children with positive X-ray diagnosis of acute pneumonitis have been studied. These children range in age from less than 1 month old to five years with 29 being between 6 months old to 2 years. Most of the patients, 26 (49%), were studied within three days after onset of illness although 19 (35.8%) were seen between 3 and 7 days after onset. Twenty-one (39.6%) of the children were treated before being seen by hospital physicians.

PROGRESS: Blood cultures were positive in 7 of the children and culture of lung aspirates were positive for bacteria in 15 (28.3%) patients. Organisms identified from lung aspirate cultures were D. pneumoniae (7), S. aureus (3), alpha streptococcus (2), Micrococcus sp. (2), and Escherichia coli (1). There were 9 instances in which D. pneumoniae was recovered from throat and/or nasopharyngeal specimens and was absent in cultures of lung aspirates. Twenty-two patients had S. aureus on throat and/or nasopharyngeal cultures and did not have this organism identified from lung aspirate cultures. Three patients had D. pneumoniae only in the lung aspirate culture while 2 revealed S. aureus only in the lung aspirates culture.

SUMMARY: The finding of D. pneumoniae and S. aureus in throat and/or nose swabs while negative cultures are obtained from lung aspirates may be related to the onset of the illness and treatment before admission. Considering these facts, there appears to be little or no relationship between the finding of causative agents from lung aspirates and throat or nasopharynx.

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* Children's Hospital, Bangkok, Thailand

Mycotic Diseases

Principal Investigator: Mrs. Yupin Charoenvit, M.S.

OBJECTIVE: This laboratory continues to conduct routine examination of fungal specimens. This permits us to maintain a highly trained technologist and to manufacture adequate media for any unusual outbreak of fungal diseases.

DESCRIPTION: Specimens are submitted from local Thai Hospitals and from the US Hospital in Bangkok. They are inoculated onto appropriate media.

PROGRESS: During the past year we have examined specimens from 65 patients. From these patients we have identified 22 opportunistic fungi and dermatophytes. No systemic fungal agents were isolated.

SUMMARY: Skin diseases due to fungi are a major source of lost man hours in any military operation in tropical regions. It is necessary that this laboratory maintain a capability to culture and identify these agents and to train other technicians to work in mycology laboratories.

Diarrheal Diseases

Principal Investigators: Chiraphun Duangmani, M.D.
Udom Lexomboon, M.D., Ph.D.*

Assistant Investigators: Mrs. Tatsanee Occeño, B.S.
Mrs. Chantana Ratanavaraha, B.S.
Mrs. Prani Ratarasarn

OBJECTIVE: This laboratory continues to maintain a survey of diarrhea in Thailand. This capability is maintained to insure a rapid response to any outbreak of gastroenteritis throughout the region served by this facility. During the past year we have studied several epidemics of diarrhea and have maintained an active consultation service to all hospitals and laboratories in Thailand.

DESCRIPTION: Specimens are submitted to this laboratory from local Thai Hospitals and from US Hospital in Bangkok. They usually arrive at this laboratory in Cary-Blair transport media or in alkaline peptone water. Swabs are inoculated onto SS, McConkey, and Desoxycholate agar. The methodology of Edwards and Ewing is used to identify enteric pathogens. Serological identification is carried out on all Escherichia coli isolated.

PROGRESS: During the past year 4,268 specimens from 2,438 patients were examined for enteric pathogens. 249 isolates of Salmonella sp. were recovered and 61 isolates of Shigella sp. were made. Of 2,102 Escherichia coli serotypes, 845 were identified as enteropathogens. Serotypes 0119:B14 and 0126:B16 were recovered much more frequently than other types.

SUMMARY: Our data continues to indicate that salmonellosis is more predominant among Thais than is shigellosis. The greatest number of gastroenteritis in American patients continues to be associated with Shigella sp. More isolates of enteric pathogens are recovered in the first three months of the year than at other times.

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* Children's Hospital, Bangkok, Thailand

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.

OBJECTIVE : The oriental house fly (Musca domestica vicina) is common throughout Thailand. Efforts to control this and other insect pests in Thailand through the use of insecticides have 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 were collected in the field, taken to SMRL and colonized. Reared adult females were tested for insecticide tolerance three to seven days after emergence in the F_1 generation by standard methods.

PROGRESS : The level of insecticide resistance in the oriental house flies from Udonthani and Chiengrai provinces was determined for DDT, malathion and lindane. House flies from Udonthani were susceptible to DDT, partially resistant to malathion and resistant to lindane; flies from Chiengrai were susceptible to DDT and malathion, and partially resistant to lindane.

SUMMARY : Oriental house flies (Musca domestica vicina) from Udonthani and Chiengrai provinces in Thailand were still susceptible to DDT. However, flies from the first province were partially resistant to malathion and resistant to lindane, while flies from Chiengrai were susceptible to malathion but partially resistant to lindane.

Distribution and Ecology of Ectoparasites of Vertebrates in Southeast Asia.

Principal Investigators : Carleton M. Cliffords, Ph.D. *
Harry Hoogstraal, Ph.D. **
H. Elliott McClure, Ph.D.
Joe T. Marshall, Ph.D.
Harold E. Stark, Ph.D.
Panita Lakshana, B.Sc.

Associate Investigators : M. Nadchatram ***
Edward W. Davis SFC
Inkam Inlao
Nongnuj Maneechai

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 the period of this report the tabulation and correlation of data on habitats, hosts, climatic conditions and other ecologic factors, relating to ectoparasites collected previously, were continued. A monograph on chiggers of the genus Leptotrombidium Nagayo et al., 1916 in Thailand is under

*Rocky Mountain Laboratory, Hamilton, Montana
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preparation. Three additional new species of chiggers were recorded for Thailand during this period : Neoschoengastia heynemanai Nadchatram & Upham, 1966 from Halcyon smyrnensis in Pathum Thani prov.; Walchiella lacunosa (Gater, 1932) from Rattus rattus in Pattani prov. and Gahrliepia (Walchia) erana Traub & Evans, 1957 from Rattus rattus in Nakhon Ratchasima prov.

SUMMARY : Data on distribution, hosts and ecologic factors relating to ectoparasites of Thailand were tabulated and correlated in preparation for publication. A monograph on the chiggers of the genus Leptotrombidium in Thailand was submitted for publication. Three additional species of trombiculid mites were added to the ectoparasite fauna known for Thailand.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 03 6467	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY LEVEL	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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				
XXXXXXXXX	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

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

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003600 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 71	5	300
	CURRENT 72	3.6	162

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Wilks, LTC N. E.

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PII Redacted

21. GENERAL USE

Foreign Intelligence not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U)Parasitic Diseases; (U)Malaria; (U)Host Parasite Relationships; (U)Gnathostomiasis; (U)Immunodiagnosis; (U)Pathophysiology; (U)Antimalarial Drugs; (U)Dirofilariasis

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 parasites of military importance in Southeast Asia providing estimates of the risk and consequences of infections with these parasites and describing effective control measures.

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 clinical specimens. The disciplines of clinical medicine, veterinary medicine, medical entomology, epidemiology, and parasitology are utilized to identify life cycles and the variables which influence transmission, clinical course and chemotherapy.

25. (U) 70 07 - 71 06 Preservation of parasitized erythrocytes for re-invasion of malaria parasites in an in vitro system continues to show promise. Four species of intestinal flukes of man in Thailand are described. The gibbon was found to be susceptible to Strongyloides and Dirofilaria infections with pathology similar to man; the proteolytic enzymes of E. histolytica were described in part; a screening test for antimalarial drugs in monkeys was established; studies on the life cycle, diagnosis and chemotherapy of gnathostomiasis continued to show promise; and the immunodiagnosis of parasitic infections continues to be assessed using commercial antigens, which provide satisfactory results on the whole. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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 69 (FOR ARMY USE) ARE OBSOLETE

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: Norman E. Wilks, LTC, MSC

Associate: Udomporn Chularerk, M.D.; Nipon Chuanak, Svasti Daengsvang, Med.D.; Alexander De Paoli, MAJ, VC; Carter L. Diggs, LTC, MC; Robert Gentzel, SSG; Douglas J. Gould, Ph.D.; Bruce A. Harrison, CPT, MSC; Robert L. Hickman, MAJ, VC; Richard N. Hunt, SP5; Peter K. Iber, MAJ, MSC; Dennis O. Johnsen, MAJ, VC; Pravet Lertprasert; Rapee Machimasatha, B.Sc.; George S. Manning, CPT, MSC; Ronald E. Marshall, SP5; Katchrinnee Pavanand, M.D.; Barnyen Permpanich, R.N.; Pirom Phisphumvidhi, B.S.; Prayot Tanticharoenyos, D.V.M.; Phaibul Sirichakwal, B.Sc.; Prasit Sookto; Kitti Watanasirmkit, B.S.; Paisarl Yingyourd, B.Sc.

Strongyloidiasis in the Gibbon

Principal Investigators: Alexander De Paoli, MAJ, V C
Dennis O. Johnsen, MAJ, V C

OBJECTIVE: The study was initiated to determine the incidence of Strongyloidiasis in the gibbon and to characterize the disease clinically and pathologically.

DESCRIPTION: A review of necropsy reports yielded 61 gibbons which died of natural causes between 1967 and 1970. Strongyloidiasis was the reported cause of death in 11 of these animals. The similarity of clinical history and lesions among these cases suggested that Strongyloides stercoralis in the gibbon caused a uniform syndrome with pathognomanic lesions.

PROGRESS:

A. Clinical history: With few exceptions, death was sudden with few prodromal signs of clinical disease.

B. Gross pathology: The most consistent finding was colitis varying from catarrhal to hemorrhagic. The lungs were generally described as red, congested, or hemorrhagic.

C. Histopathology: The principal lesions were present in the gastrointestinal tract with the most severe changes centered aroud the colon and distal portion of the small intestine. Here, numerous larvae penetrated the intestinal mucosa, some extending to the muscular wall disrupting intestinal glands and eliciting an inflammatory response which ranged from acute to granulomatous. Adult nematodes were generally present in the upper portion of the small intestine and pyloric portion of the stomach. Larvae were also present in other organs, the lungs being the most common site. Congestion and marked hemorrhage were the principal pulmonary changes, followed by foci of interstitial inflammation associated with migrating larvae. These pathological changes are very similar, if not identical, to those reported for the same disease in man. As in man, superinfection is the key factor in the gibbon disease. Although the nematode plays the major role in the disease, morphological and clinical evidence suggest that bacteremia and/or endotoxemia are the immediate cause of death in most animals.

SUMMARY: Strongyloidiasis, a common cause of death in the gibbon, is characterized by sudden death, entero-colitis, and pulmonary hemorrhage.

Pathology of Dirofilariasis in The Gibbon

Principal Investigators: Alexander De Paoli, MAJ, V C
Dennis O. Johnsen, MAJ, V C
Douglas J. Gould, Ph. D.

OBJECTIVE: The study was undertaken to characterize the pathology of Dirofilaria immitis infection in the gibbon and to compare the lesions in the gibbon with those produced by the parasite in the dog and man.

DESCRIPTION: Four gibbons were infected with larvae of Dirofilaria immitis and sacrificed at intervals of 6, 9 and (two animals) 14 months post infection. (Detailed information on experimental design and clinical data are given elsewhere in the report.)

PROGRESS:

A. Gross Pathology: Adult viable Dirofilaria immitis were present in the right ventricle and/or pulmonary arteries of all animals at time of necropsy. In three animals (9 and 14 month infections) remnants of decomposed nematode were lodged in branches of the pulmonary arteries. Segmental rugous endarteritis of the pulmonary arteries and their main branches was the principal gross change. The lumen of larger vessels was distended and the wall of muscular arteries was markedly thickened. Acute pulmonary infarction was present in one animal while focal pulmonary scars were noted in another gibbon.

B. Histopathology: Vascular changes were confined to the pulmonary arteries and their branches. These ranged from intimal thickening and fibrosis to marked villar proliferative endarteritis. Less frequent was a granulomatous necrotizing vasculitis which was always focal and associated with dead fragments of parasites. Focal interstitial pneumonitis and focal atalectasis were noted in areas adjoining affected vessels. Focal granulomatous hepatitis of varying intensity was present in all animals. Morphologically, the hepatic lesion is compatible with a parasitic etiology, however, multiple sections have failed to show the suspected cause.

SUMMARY: From these studies it would appear that the response of the gibbon to Dirofilaria immitis infection is similar to that of the dog, and consists principally of arteritis of pulmonary arteries.

Evaluation of Candidate Antimalarial Drugs in Monkeys

Principal Investigator: Dennis O. Johnsen, MAJ, VC

Associate Investigators: Alexander De Paoli, MAJ, VC
Robert L. Hickman, MAJ, VC
Prayot Tanticharoenyos, DVM

OBJECTIVE: Potential antimalarial drugs are to be evaluated in macaque monkeys to determine both their toxicity and therapeutic effectiveness against experimentally induced infections of Plasmodium cynomolgi.

DESCRIPTION: To determine toxic levels, each drug tested is given to a group of four monkeys by the oral route unless special drug characteristics indicate otherwise. An initial dose of 1 mg/kg is given for 2 days and then successively increased by a factor of 3.16 at two day intervals until signs of toxicity occur. The dose will then be described by the same increment until the level at which the drug is tolerated for six days is reached. Twelve monkeys are used in the test for therapeutic effectiveness, two of which are infected but untreated controls. The remainder of the ten animals are paired and given selected doses of the test drug for seven consecutive days commencing on the fourth day following their infection with malaria. Initially, the drug doses administered in the test for therapeutic effectiveness are set at the highest dose of the drug that was determined to be not toxic in the toxicity test, and if therapeutically effective, this dose is reduced during subsequent tests successively to a non-effective endpoint. In the test for therapeutic effectiveness, the drug effect on the malaria infection is compared to that of untreated control monkeys for a thirty day period, at which time animals in which parasitemias have been eliminated are splenectomized to determine whether a cure has been achieved. At the conclusion of a test each monkey is autopsied and examined grossly for the presence of pathological lesions.

PROGRESS: Testing so far has been limited to establishing consistent laboratory technique and the reliability of the test systems. Seven drugs, including chloroquine, quinine, diformyl DDS, and four unknown drugs, have been tested for toxicity and their maximum tolerated doses determined. Three tests for therapeutic effectiveness have been initiated, one of which has been completed. Each of the seven drugs have been tested for therapeutic effectiveness at least one dose level; all of them show strong evidence of antimalarial activity. The findings resulting from the testing of the three known drugs correspond with their activity as demonstrated clinically and in other test systems. These results

indicate that testing of a large number of drugs, whose antimalarial activity in primates has not been determined, may begin soon.

SUMMARY: The program for using macaquesin the evaluation of candidate antimalarial drugs is described. The three tests which have been initiated for therapeutic effects indicate that this will be a promising system.

The Gibbon as a Host for the Canine Heartworm

Principal Investigators: Alexander De Paoli, MAJ, VC
Carter L. Diggs, LTC, MC
Douglas J. Gould, Ph.D.
Dennis O. Johnsen, MAJ, VC
Prayot Tanticharoenyos, DVM

Associate Investigators: Bruce A. Harrison, CPT, MSC
Robert Gentzel, SSG
Ronald E. Marshall, SP5

OBJECTIVE: To determine the suitability of the gibbon as a model for studying transmission and clinical and pathological features of Dirofilaria immitis, the canine heartworm.

DESCRIPTION: In the United States and Japan Dirofilaria immitis, the canine heartworm, has been found in the heart, lungs, subcutaneous tissue, conjunctivae, or periorbital area of humans. Because Dirofilaria, which may infect as many as 100% of the dogs in Thailand, is transmitted by mosquitoes that also feed readily upon man, it seems likely that the risk of humans to heartworm infections may be significant in areas where heartworms are endemic and the appropriate vector mosquitoes are present. This hazard, as well as the pathological and diagnostic features of the disease this parasite causes in man, are difficult to study because there is no laboratory animal, other than the natural canine host, that has been experimentally infected with heartworms. Because the gibbon is an ape closely related to man phylogenetically, and a primate in which heartworms have been observed in natural infections, it seemed probable that gibbons would be a preferable animal to serve as an aberrant experimental host for heartworms.

PROGRESS: Four gibbons were inoculated subcutaneously with approximately 50 infective microfilaria dissected from Aedes aegypti and Aedes togoi that had fed three weeks earlier on a dog with Dirofilaria microfilaremia. Blood and serum samples for serologic and hematologic studies were collected at regular intervals for periods ranging up to 14 months following inoculation. Changes in the clinical and serological status of each animal occurred at 2 to 3 months following inoculation of the microfilaria. Skin testing with two types of Dirofilarial skin test antigen converted during this time from negative to positive. Soluble antigen fluorescent antibodies, hemagglutination-inhibition antibodies, and eosinophil counts began to rise above control values and followed an erratic, but elevated, course throughout the study. Thoracic radiographs

revealed abnormal shadows usually located in the right diaphragmatic lung lobe. Microfilaremia was never detected in any of the gibbons. One gibbon sacrificed at five months following inoculation had adult heartworms located in the right ventricle and extending along the pulmonary artery as did each of the animals sacrificed later. The number of worms present varied from approximately 3 to 9 in each animal, and included both sexes and gravid females in two instances. Although further definitive studies employing mosquitoes to inoculate infective microfilaria into host animals may be indicated, it is clear that the gibbon is quite susceptible to infection and is a worthwhile animal to consider using in future experimental work with this disease.

SUMMARY: The gibbon has been shown to be a suitable animal model for the study of D. immitis infections, producing a clinical and pathological course similar to that in the natural canine host.

Comparative Pathophysiology of Strains of E. histolytica

Principal Investigators: Norman E. Wilks, LTC, MSC
Pirom Phisphumvidhi, B.S.

Associate Investigator: Peter K. Iber, MAJ, MSC

OBJECTIVE: To investigate the comparative invasive traits of strains of E. histolytica in SE Asia to determine whether differences in proteolytic enzyme activity account for some strains colonizing in the liver rather than producing the classical colonic ulceration with typical amebic dysentery.

DESCRIPTION: It is well known that hepatic amebiasis occurs in SE Asia with an apparent absence of symptomatic amebic ulceration of the colon. Several such cases have been reported in the literature from WRAIR in troops serving in Vietnam. Cultivation of amebae has been enhanced by the development of monophasic media which permit harvesting of relatively clean populations. It is intended that strains cultured from colonic lesions, amebic abscesses of the liver and from persons serving as mere carriers be comparatively studied by spectrophotometric enzyme technics.

PROGRESS: Attempts to isolate amebae from hepatic abscesses have not been successful to date, due, it is believed, to the patients having been given chemotherapeutics which reduced the viability of the amebae. Thus, studies have been confined to obtaining the essential baseline data for the axenic strain of E. histolytica maintained in this laboratory (strain HK-9).

Following washing with physiological saline, populations of amebae are determined with a hemacytometer. Parasites are then homogenized in an ice bath, centrifuged at 12,000 rpm for 10 minutes, and the supernate is used in the enzyme assay. The following enzyme activities have been measured during the report period:

Aminopeptidase:		Dipeptidase:	
Arginine	+	Glycylglycine	+
Alanine	+	Pepsin	+
Glycine	+	Hyaluronidase	±
Leucine	+	Lactate dehydrogenase	±

Hydrolase:

Gelatin	-
Casein	+
Hemoglobin	+

SUMMARY: Invasive traits of E. histolytica are being investigated from the standpoint of proteolytic enzyme activity. An axenic strain of the amebae has been found to possess strong aminopeptidase and dipeptidase activities as well as pepsin and the ability to hydrolyze casein and hemoglobin. Hyaluronidase, lactate dehydrogenase and the hydrolysis of gelatin are very much less or absent.

Immunodiagnosis of Parasitic Infections

Principal Investigator: Norman E. Wilks, LTC, MSC

Associate Investigators: Robert Gentzel, SSG
Richard N. Hunt, SP5
Prasit Sookto

OBJECTIVE: To employ commercially prepared antigens in the SAFA test and the IHA test for screening patients with suspected amebiasis, filariasis or malaria to evaluate the test systems and to detect candidate patients for other studies.

DESCRIPTION: Under contract with the R&D Command, Parke-Davise and CO. have produced two antigens which have been standardized in preparation and in the resulting nitrogen content per milliliter of fluid. One has been prepared from axenic cultures of E. histolytica, the other from D. immitis. The antigens may be used in both the SAFA and IHA test systems. An antigen has been prepared at WRAIR intended for use in the diagnosis of P. falciparum infections. It is intended to test these antigens for specificity and sensitivity with sera from a population with a broad spectrum of infection and immunity, and to provide a reference diagnostic capability in support of other U.S. installations in SE Asia.

PROGRESS: A disparity in test results has been experienced using the Parke-Davis antigen for the diagnosis of amebiasis by the SAFA test as compared with results obtained from the IHA. Positive control sera obtained from Taipei provide reproducible and dependable results with the IGA test, but do not correlate with the SAFA procedure's results, the latter showing little or no reaction at the recommended dilution (1:20). A loss of potency in this test system may be due to a storage factor. The antigen has been useful in the IHA system to detect amebic infections in patients in Bangkok.

Sera from the 9th Medical Laboratory, RVN, which were positive by the CF test using the Parke-Davis antigen were tested by the SAFA and the IHA systems and only 5 of 39 reacted with the former method and only 11 of the 39 with the latter.

Screening of 1339 sera from troops arriving in Vietnam from CONUS detected 60 which reacted significantly with the IHA test. The SAFA has yet to be performed. Of 100 sera from cases of hepatitis in Vietnam, none reacted with the IHA or SAFA.

The SAFA test has been employed routinely as a test for filariasis. The test was found to be adequately sensitive in the studies of canine heartworm in gibbons (elsewhere, this report). Of the 1339 sera from troops arriving from CONUS who have been studied so far, 6 were found to give significant reactions. Of 638 troops departing RVN for CONUS, 3 were found to give positive reactions for filariasis. The sera from 100 hepatitis patients failed to react with the filarial antigen.

No antigen for testing for malaria by the SAFA technic has been received. Both arriving and departing personnel from Vietnam will be studied for malarial antibody by this method in the next report period.

An attempt to obtain a reactive fraction of larval and adult gnathostomes has been initiated, and a weakly reactive fraction has been obtained. The requirement for such an antigen is critical in some areas of Thailand, and this effort will be actively pursued.

SUMMARY: The SAFA test has been found to be relatively insensitive in the diagnosis of amebiasis, whereas the IHA using the same antigen has been satisfactory. The SAFA has provided satisfactory results in the monitoring of filarial infections of gibbons. No immunodiagnostic studies have been initiated in malaria.

Studies on *in vitro* Erythrocyte Penetration by *P. falciparum*

Principal Investigator: Katchrinnee Pavanand, M.D.

Assistant Investigators: Barnyen Permpanich, R.N.
Nipon Chuanak

OBJECTIVE: To explore the possibility of *in vitro* penetration by *P. falciparum* in a marked recipient cell system and to study the effect of serum from patients with a history of recurrent malaria on the *in vitro* penetration.

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 evidence of penetration *in vitro*. The culture media and technic have been described in a previous annual report (1970) and by publication (Diggs et al, 1971).

PROGRESS: Previous reports describe studies in which some degree of inhibition of fetal cell reinvasion by *P. falciparum* was found when serum from immune subjects was introduced into the culture media. Also, the inhibition of reinvasion by whole Ig globulins (half saturated $(NH_4)_2SO_4$ fraction) from 28 immune individuals has been comparably studied and reported. A complement dependent growth requirement for the *in vitro* cultivation of *P. falciparum* has been detected.

It has become obvious that collection of *P. falciparum* infected blood from patients is seasonally dependent, yet a continual requirement for this complicated experimental study. Thus, an attempt has been made to preserve and store parasitized erythrocytes. The goal is to devise a technic for retaining the viability of intact parasites for long term studies. Initially, heparinized blood from patients was incubated with 50% glucose (1:20) and stored in liquid nitrogen as blood-sand pellets. The results were not satisfactory due to complete hemolysis of red cells upon thawing. To overcome this problem, dimethyl sulfoxide was introduced as a protective agent against freezing damage to the parasitized cells. Aliquots of infected blood with different concentrations of DMSO were stored in liquid nitrogen preceded with a stepwise freezing down before reaching liquid nitrogen temperature. At different intervals, aliquots of the frozen blood were thawed in a "thawing-out"

solution at 42°C and the degree of red cell hemolysis was determined. The washed intact parasitized cells were then cultured, and the parasites grew and invasion of foetal erythrocytes occurred. Improvement has been made to achieve the least hemolysis upon thawing.

This technic has permitted the collecting of P. falciparum infected blood from malaria patients in different locations in Thailand for comparative studies. In addition, storage of P. berghei, P. coatneyi, P. cynomolgi and P. knowlesi has been similarly studied. The recovery upon thawing in each instance has been satisfactory and patent infections were established in clean recipient animals. The determination of maximum storage of infected blood while retaining viability is in progress. At this reporting, maximum storage for P. falciparum has been 150 days, and for P. berghei, 64 days.

SUMMARY: Observations of the interaction of P. falciparum and convalescent sera in vitro have continued. Improvement in the freezing and recovery of intact viable parasites has been achieved.

Ecology of Intestinal Parasites of Medical Importance in Thailand.

Principal Investigator: George S. Manning, CPT, MSC

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

OBJECTIVE: To study the life history, prevalence and pathology of two newly discovered intestinal trematodes, Phaneropsolus bonnei and Prosthodendrium molenkampi.

DESCRIPTION: Autopsy material was examined for intestinal parasites at the Udornthani Provincial Hospital (for a detailed description see 1970 Annual Report). Animal species living in and around the village of Ban Phran Muan were captured and necropsied. Life cycle studies were also carried out in Ban Phran Muan, as this site was considered to be an area where active transmission was occurring.

PROGRESS: Adult P. bonnei were recovered from the duodenum and jejunum of the first autopsy case. On a subsequent autopsy, P. bonnei and P. molenkampi were both found. To date, 24 autopsies have been examined: 15 positive for P. bonnei and 14 for P. molenkampi. Autopsy material from two cases was sent to AFIP for evaluation. There appears to be little host pathology, other than a decrease in the height of the epithelial cells situated between the host and parasite. This probably results from the pressure exerted by the presence of the flukes. However, additional study is required before we can unequivocally state that these trematodes cause no appreciable pathology.

Search for animal reservoirs has revealed Rattus rattus, Scotophilus kuhlii and Taphozous melanopogon naturally infected with P. molenkampi. No alternate hosts for P. bonnei have been found in or around the study site, but Macaca fascicularis captured from other areas in Thailand have been found to be naturally infected. This evidence suggests a rather widespread distribution of the fluke.

Ten villages, from the provinces of Udornthani and Nongkhai, were surveyed and the prevalence of P. bonnei and P. molenkampi ranged from 10-40%, based on a single stool examination. In addition, stool specimens submitted from patients in Laos have been found positive for both P. bonnei and P. molenkampi. Thus, the endemic area covers at least Thailand and Laos.

Several thousand aquatic insects were examined in the search for a second intermediate host or source of infection. Metacercariae were found in naiads of the Order Odonata. The metacercariae were fed to experimental animals and both trematode species were recovered. These immature insects are frequently eaten raw by the villagers.

In the search for a snail intermediate host, several ponds where infected naiads had been collected were extensively surveyed. The only snail species collected was Bithynia goniomphalus. Many different cercariae were shed by the snails, but specific identification was not possible. Though this is not conclusive, it strongly suggests that B. goniomphalus serves as intermediate host for both P. bonnei and P. molenkampi. Confirmation has not been possible as B. goniomphalus does not breed readily under laboratory conditions.

During the aforementioned study two additional species of intestinal trematodes were found which had never been recorded from man in Thailand. They are: Haplorchis yokogawai and Haplorchis taichui. Metacercariae recovered from three species of fish, Labiobarbus leptocheilus, Puntius sarana and P. simus were fed to dogs and adult flukes were recovered at necropsy. Other piscine hosts are probably also involved in the transmission of these two species of flukes.

Isoparorchis hypselobagri, a parasite which has been recorded from man, was found infecting fish in the province of Sakol Nakorn. Though no human infections have been found in Thailand, it is quite likely that they do occur as infection is acquired from the ingestion of raw parasitized fish, and raw fish constitute a major part of the diet for residents in the northeastern Provinces.

SUMMARY: Four species of intestinal trematodes have recently been found to be endemic in northeastern Thailand. They are: Phaneropsolus bonnei, Prosthodendrium molenkampi, Haplorchis yokogawai and Haplorchis taichui. P. bonnei and P. molenkampi are transmitted through insects of the Order Odonata. H. yokogawai and H. taichui are transmitted through small freshwater fish. Isoparorchis hypselobagri was also recorded for the first time in Thailand.

Chemotherapy of Gnathostomiasis

Principal Investigator: Professor Svasti Daengsvang, Med.D.

Associate Investigator: Udomporn Chularerk, M.D.*

Assistant Investigators: Phaibul Sirichakwal, B.Sc.
Paisarl Yingyourd, B.Sc.
Rapee Machimasatha, B.Sc.

OBJECTIVE: To determine (1) the effect of multiple subcutaneous doses of Ancylol on larval and immature stages of Gnathostoma spinigerum in infected dogs and cats, (2) the effect of oral administration of Bithionol, Thiabendazole and Niridazole on white mice previously infected with G. spinigerum larvae. These drugs have been used effectively in the treatment of certain helminthic diseases but have yet to be used in treating gnathostome infections.

DESCRIPTION: (1) Ancylol, Disophenol (2,6-diiodo-4-nitrophenol) Parenteral 4.5%.

Previous results suggest that 3 to 6 subcutaneous doses of ancylol kill many larvae and immature G. spinigerum located in the tissue (1970 Annual Report). The effect of 4-7 subcutaneous doses, of the same chemotherapeutic agent, on the migrating stage of the worm in various organs of dogs and cats is again undertaken. The drug was administered to 7 cats and 4 dogs, previously infected with G. spinigerum. Five infected cats and four dogs were used as controls. Each dose amounted to 0.1 ml of drug per pound of body weight; directions given by the manufacturer for the treatment of canine hookworm. Treated animals were given 4 to 7 weekly doses of the drug, 1 and 3 months after being transcutaneously infected with the larvae. Experimental animals were sacrificed and examined for the presence of worms in the various organs 12-22 days after the last dose was given.

(2) Bithionol or bitin, 2,2'-thiobis (4,6-dichlorophenol) oral administration.

This phenolic compound has been used effectively in treating human paragonimiasis in Japan (Yokogawa et al., 1963) and Thailand (Charoenlarb et al., 1964). Bithionol is now being studied to determine its effectiveness

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by oral administration in distilled water and/or in 10% ethanol administered through a polyethylene tube to adult white mice infected with G. spinigerum. Dosage, 40 mg/kg body weight every other day. Adult laboratory bred white mice weighing 25-38 grams were used for the experiment. Autopsies were performed on mice after completing the experiment. An electrically illuminated examination box and microscope were used to determine the presence of worms in the muscles and visceral organs.

(3) Thiabendazole (MK-360), 2-(4-Thiazolyl benzimidazole) oral administration.

This drug has been shown to be effective in treating experimental trichinosis in swine (Campbell and Cuckler, 1962) at a dosage of 86 mg/kg body weight for 7 days. Papasarathorn et al, (1964) found that thiabendazole was effective for subclinical ascariasis and trichuriasis in man when given for one day and two days and without any serious untoward reactions.

A preliminary study on the effect of thiabendazole on induced gnathostomiasis in laboratory bred white mice was undertaken. Fifteen white mice were infected orally with advanced third-stage larvae and 15 with fully developed larvae of G. spinigerum, 10 from each group were treated, the remaining 5 used as controls. Autopsies were performed on all treated and control mice to examine for the presence of worms in the tissues.

(4) Niridazole (Ambilhar, Ciba), 1-(5-nitro-2-thiazolyl)-2-imidazolidinone or Ciba 32, 644-Ba oral administration.

Niridazole was originally introduced for the treatment of schistosomiasis; it is also known to be active against Entomoeba histolytica (Lambert 1964, Powell et al., 1966). The drug was prepared fresh at 1 mg in 1 ml distilled water for administration to each experimental mouse, at a daily dosage of 25 mg per kilogram body weight. Fifteen laboratory bred white mice were orally infected, 10 were treated with the drug, the other 5 were used as controls.

PROGRESS: Table 1 summarizes the results of ancylol in 7 treated cats and 4 dogs. The results show that 5-6 weekly doses effectively cured 3 (one cat reported last year) of the 5 infected cats beginning the treatment 1 month after the infection with the larvae.

Table I. Chemotherapy of G. spinigerum larval and immature stages infecting definitive hosts (cats and dogs) by multiple subcutaneous doses of Ancylol disophenol (2, 6-diiodo-4-nitrophenol) on the basis of 0.1 ml per pound body weight per dose at 7-day intervals.

No. Animal	No. third-stage larvae penetrated thru skin/ percent	Age of worm in host before treatment	Total weekly doses of Ancylol	Autopsy findings 7-22 days after treatment			Remarks
				Survival rate of worm	No/stages of living worm	Organs found infected	
2 cats (#106,111)	42(70%), 44(85%)	1 month	5	0%,16%	7 larvae	abdominal flesh, legs, chest, diaphragm	1 dead larva in right hind leg flesh. Sacrificed 14 days after last dose Ancylol
2 cats (#117,118)	48(92%), 44(88%)	1 month	6	0%, 9%	4 larvae	diaphragm	Sacrificed 21 days after last dose Ancylol
2 cats (#125,112)	52(76%), 49(83%)	-	Control, (1 mo.) no treatment	69%,78%	1 mature female 7 immature males & females and 28 larvae in one cat, 38 larvae in liver of other	abdominal flesh, flesh of legs, chest, diaphragm, liver, stomach, lung	1 cat sacrificed 76 days the other died 43 days after first skin infection, 1 dead larva in abdominal muscle
3 cats (#126,113, 114	73(70%), 57(90%), 47(84%)	3 months	4, 7, 7	1%, 9%, 21%	16 larvae	abdominal flesh, hind leg, chest, diaphragm	1 cat died 4 days after 4th dose, other 2 sacrificed 22 days after 7th dose Ancylol
1 cat (#115)	44(88%)	-	Control, (3 mo.) no treatment	-	-	-	First egg positive stool 97 days after first skin infection, second egg positive 209 days after first infection. Kept for supplying eggs
*2 cats (#38,74)	53(62%), 46(90%)	5, 6 1/2 months	Control no treatment	51%,56%	53 immature adult males & females and larvae	stomach, diaphragm, chest wall, abdominal and back muscles, abdominal fat, omentum	Sacrificed 154, 195 days of prepatent period
2 dogs (#23,24)	67(89%), 64(80%)	1 month	5	6%, 2%	5 larvae	diaphragm, liver	Sacrificed 12 and 14 days respectively after last dose Ancylol
1 dog (# 21)	98(93%)	-	Control, (1 mo.) no treatment	25%	4 mature and immature males, 5 immature females, 16 larvae	abdominal wall, chest and back muscles, stomach, liver, diaphragm	Sacrificed 83 days after infection
2 dogs (#19,22)	71(92%), 66(88%)	1 months	7	6%,0%	4 larvae (1 doubtful living)	diaphragm, abdominal fat	Sacrificed 14 days after 7th dose Ancylol, 1 dead larva in diaphragm. Dog #19 showed moderate jaundice with loss of weight disappearing after two interruptions of treatment, one week each
1 dog (# 20)	54(95%)	-	Control, (3 mo.) no treatment	-	-	-	First ova positive stool 110 days after infection followed by 81 days of patent period. Negative since 18/12/70.
**2 dogs (#1,18)	65(79%), 44(100%)	8 1/2 months 10 months	Control, no treatment	63%,70%	86 mature males and females	stomach, lung, omentum	Sacrificed 262 and died 297 days after the infection

*From skin penetration by G. spinigerum advanced third-stage larvae (transcutaneous infection) project reported in Annual Progress Report 1969 and 1970.

** From skin penetration by G. spinigerum advanced third-stage larvae (transcutaneous infection) project reported in Annual Progress Report 1970 and in the present Report 1971.

Three of the four dogs beginning treatment 1 month and 3 months after infection showed markedly lower worm survival rates than corresponding controls. One animal was completely cured. It is apparent that Ancylol may be used with good results if administered under close and careful supervision, but no further studies are planned because of the drugs potential toxicity.

(2) Bithionol or Bitin, oral administration. The preliminary finding of bithionol chemotherapy on induced gnathostomiasis in white mice is shown in Tables 2 and 3. The drug appears to have little or no affect on the parasite, but because so few mice were studied, repeated studies using more infected mice are now being developed.

(3) Thiabendazole, oral administration. The findings indicate that no significant difference exists between the treated mice and controls. The 10 treated mice showed a total of 42 (84%) living larvae encysted in the muscles and liver. The 5 control mice killed and examined showed 23 (92%) living encysted larvae in muscles and liver.

(4) Niridazole (Ambilhar, Ciba) oral administration. This experiment on mice infected with G. spinigerum fully developed larvae in cyclops is still in progress.

SUMMARY: Three of 5 adult cats treated with 5-6 subcutaneous doses of ancylol disophenol were cured of infection with G. spinigerum; the remaining cats showed significant reductions of the worm survival rates. Similarly, all four infected dogs showed either a significant reduction in the survival rates of worms or were completely cured. Toxic effects were noted in only one animal. Oral administration of bithionol was only slightly effective in worm load reduction. Thiabendazole seems to have no therapeutic value in experimentally induced gnathostomiasis. The study on the therapeutic effect of niridazole (Ambilhar, Ciba) on white mice is still in progress.

Table 2 Preliminary finding of chemotherapy on experimental white mice infected with fully developed Gnathostoma spinigerum larvae in cyclops by oral administration of bithionol (bitin), 2, 2'-thiobis (4, 6-dichlorophenol).

Dose of bithionol per mouse	No. of mouse	No. of larvae in cyclops	No. advanced third-stage larvae found on autopsy	Organs infected	Remarks
—	4	120	32 (27%) (unencysted)	liver, chest, abdomen, hind-leg	Died before treatment began.
Drug in distilled water					
1-6	6	180	93 (52%) (unencysted)	liver, lung, abdomen, chest, back fore-hind-legs	Died after 1-6 doses.
12-20	9	270	75 (28%) (encysted)	liver, abdomen, chest, back, fore-hind-legs.	Killed 2-6 days after the last dose.
12 & 20	2	58	11 (19%) (encysted)	liver, chest, back abdomen	Died and killed 2 days after the last dose.
Drug in distilled water and 10% ethanol					
13-20	4	120	26 (22%) (encysted)	liver, abdomen, chest, back, fore-leg	Died and sacrificed 2 days after the last dose. 1 mouse negative.
20 (control-no drug)	2	60	16 (26%) (encysted)	liver, abdomen, back, fore-hind-legs	Killed 2 days after the last dose. 1 mouse negative.
Drug in 10% ethanol					
20	6	90	28 (31%) (encysted)	skin, abdomen, chest, back	Killed 1-2 days after the last dose.
20 (control-no drug)	2	30	12 (40%) (encysted)	liver, back	Killed 1-2 days after the last dose.

Table 3 **Preliminary finding of Chemotherapy on experimental white mice infected with Gnathostoma spinigerum advanced third-stage larvae obtained from other infected white mice by oral administration of bithionol (bitin), 2, 2'-thiobis (4,6-dichlorophenol).**

Dose of Bithionol per mouse	No. of mouse	No. of all advanced third-stage larvae fed	No. of all advanced third-stage larvae found on autopsy	Organs infected	Remarks
Drug in 10% ethanol					
20	7	45	19 (42%) (encysted)	liver, chest, back, hindleg	Killed 1 day after the last dose.
13-20 (control-no drug)	3	17	13 (76%) (encysted)	liver, back, hindleg.	Died or killed 1-2 days after the last dose.

Studies of New Experimental Hosts, Life Cycles and Modes of Transmission of Gnathostomes.

Principal Investigator: Professor Svasti Daengsvang, Med.D.*

Assistant Investigators: Phaibul Sirichakwal, B.Sc.
Paisarl Yingyourd, B.Sc.
Rapee Machimasatha, B.Sc.

OBJECTIVE: To locate new experimental host animals susceptible to Gnathostoma spinigerum, G. hispidum, and G. doloresi and to determine the life cycle of G. vietnamicum.

DESCRIPTION: Fresh water crabs were tested as possible intermediate hosts of G. spinigerum in feeding experiments using infected cyclops for some and third-stage larvae from laboratory mice for others. A Macaca irus fed larvae of G. spinigerum nearly 4 years previously was examined for infection. Laboratory determinations for paratenic hosts of G. hispidum continued.

PROGRESS: The results of experimental feeding of fully developed Gnathostoma spinigerum larvae in cyclops and advanced third-stage larvae from experimental mice to fresh water crabs were as follows:

Number of crabs	Larvae fed and source	Autopsy results
3 Potamon smithanus 3 Paratelphusa sexpunctatum	22-43 Larvae in cyclops	Neg. at 2-25 days.
1 P. smithanus 7 P. sexpunctatum	7-30 Larvae from mice	Neg. at 3-34 days.
1 P. sexpunctatum	3 Larvae from mice	Pos. 1 living, 1 dead at 1 1/2 hours.
2 P. smithanus 2 P. sexpunctatum	8 Larvae from mice	Pos. 6 living at 38 days.
1 P. sexpunctatum	50 (2 feedings) from mice	Pos. 16 living at 34 days.
1 P. smithanus 6 P. sexpunctatum	None (controls)	Negative.

It is concluded that fresh water crabs can act as a source of infection for G. spinigerum.

The monkey examined 1112 days after receiving one feeding of 17 G. spinigerum larvae yielded 5 encysted living larvae in the muscles. The larvae measured 5.0 x 0.5-0.6 mm which is slightly larger than those found in other paratenic hosts.

The feeding of G. hispidum larvae in cyclops to a variety of animals showed that snake headed fishes, fighting fishes, a common small fresh water fish (Trichogaster trichopterus) and toads could be so infected, but that 3 catfish and a giant lizard were not susceptible during this particular series of experiments. The list of susceptible animal hosts for the larval stages of this gnathostome has been broadened to include the following:

Snake headed fish (Ophicephalus striatus, and O. gachua).
Catfish (Clarias batrachus)
Fighting fish (Trichopsis vittatus)
Toads (Bufo melanostictus)
Frogs (Rana rugulosa)
Pla kadi (Trichogaster trichopterus)
White mice (Mus musculus musculus)
White rats (Rattus norvegicus var. albinus)
Roof rats (Rattus rattus)
Polynesian rats (Rattus exulans)
Tree shrew (Tupaia glis)

The third-stage larvae of G. hispidum obtained from white mice and a toad were found to survive feeding a second time to mice, thus establishing the mouse as a suitable paratenic host. Fish, amphibians, and mammals have thus been found to serve as transmitting hosts for this parasite.

The studies on G. doloresi and G. vietnamicum were without progress during the reporting period.

SUMMARY: Fresh water crabs were successfully infected with third stage larvae of G. spinigerum, but not with larvae from cyclops. Larvae of this species were found to survive at least 1112 days in a monkey. The list of intermediate hosts for G. hispidum was broadened to include white mice and toads as paratenic hosts. Amphibia, fishes and mammals now are known to harbor this larval parasite.

Infectivity of Gnathostoma hispidum Larvae from Cyclops and of Advanced Third-stage Larvae from Cold-blooded Animals for Primates.

Principal Investigator: Professor Svasti Daengsvang, Med.D.

Assistant Investigators: Pisatbul Sirichakwal, B.Sc.
Faisarl Yingyourd, B.Sc.
Rapee Machimasatha, B.Sc.

OBJECTIVE: To determine whether or not infection in primates with Gnathostoma hispidum fully developed larvae from cyclops is possible, and whether or not the advanced third-stage larvae obtained from cold blooded animals will infect primates.

DESCRIPTION: An adult monkey (Macaca irus) was fed 500 fully developed G. hispidum 12-day-old larvae, another adult monkey (Macaca irus), and one adult gibbon (Hylobates lar.), were each fed 53 and 50 G. hispidum advanced third-stage larvae respectively. These larvae were obtained from experimentally infected fresh water fish Ophicephalus striatus (snake-headed fish) Trichopsis vittatus (small fighting fish) and toads (Bufo melanostictus). The feeding was done via a polyethylene tube. The primates were subsequently killed and examined after 2-3 months for larvae with the use of an illuminated examination box.

PROGRESS: An autopsy of the monkey 64 days after being fed with 500 fully developed larvae in cyclops was negative for gnathostome larvae. The gibbon when necropsied 98 days after being fed with the advanced third-stage larvae obtained from the cold blooded animals was also negative. However the monkey fed with the advanced third-stage larvae obtained from the cold blooded animals was found infected on necropsy 93 days after the first feeding, and 57 days after the last, with 1 encysted living G. hispidum advanced third-stage larva, with a thin fibrous cyst wall, in the back muscles. This larva after being removed from the cyst wall was seen actively moving and its size and morphology were still that of the advanced third-stage larva.

SUMMARY: This study demonstrated that monkeys were not infected by feeding them with G. hispidum larvae in cyclops, but G. hispidum advanced third-stage larvae obtained from cold blooded animals would infect Macaca irus.

Transcutaneous Infection by Gnathostoma spinigerum

Principal Investigator: Professor Svasti Daengsvang, Med,D.

Assistant Investigators: Phaibul Sirichakwal, B.Sc.
Paisarl Yingyourd, B.Sc.
Rapee Machimasatha, B.Sc.

OBJECTIVE: To characterize experimental gnathostomiasis induced by the recently discovered transcutaneous route.

DESCRIPTION: Three cats, three dogs, one adult civet cat (Viverricula indica) and one palm civet cat (Paradoxulus hermaphroditus canus), were exposed to transcutaneous infection through the shaved intact abdominal skin with G. spinigerum advanced third-stage larvae removed from experimentally infected white mice. Stools were examined weekly after infection for the presence of gnathostome ova. Autopsies were performed on the animals after a specified time to examine for the development and migration of the worms in various organs.

PROGRESS: The results of observations on the 3 cats, 3 dogs, 1 civet cat and 1 palm civet cat were as follows:

On autopsy, cats number 83 and 91 showed no infection with the parasite. The remaining cat (#84) was kept for further infection with G. spinigerum larvae.

On autopsy, dogs number 2 and 10 showed no gnathostome infection. The remaining dog (#18) died of an unknown cause on day 66 during the patent period. On necropsy, 3 small gastric tumors at the greater curvature of the stomach yielded 45 (70% worm recovery rate) living mature adult males and females.

In Tables 1 and 2, a summary of the experimental study on transcutaneous infection by G. spinigerum advanced third-stage larvae through the healthy intact skin of 19 common definitive hosts of the worm (10 cats and 9 dogs) shows that the successful rate of skin penetration of the larvae varied from 39-100% with not more than 2-hour exposure. The prepatent period of the parasite was found to be 60-310 days in cats, thus the life cycle of the worm in cats could be completed in 2-10 months. The prepatent period in dogs was 96-247 days, or the time required to complete the life cycle about 3-8 months. It was also found that the

Table 1. Skin penetration by Gnathostoma spinigerum advanced third-stage larvae in 10 adult domestic cats up to end of reporting year 1971.

Animal No.	Source of larvae	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 36	white mice & snake-headed fish	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	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	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	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	61 (100 %)	310	89	-	-	-	Sacrificed 385 days after the last positive stool. Autopsy negative
Cat 84	white mice	44 (66.7%)	127	100	-	-	-	Ova negative stool 537 days after the last positive up to end of reporting year. Kept for further infection
Cat 87	white mice	18 (100 %)	22 +	-	17 larvae (94.4%)	larvae	liver, skin, diaphragm abdominal fat	Died perhaps of vaccination on 1 April 69 (day 22 of prepatent period)
Cat 89	white mice	45 (75%)	22 +	-	38 larvae (84.4%)	larvae	liver abdominal muscle abdominal fat	Died perhaps of vaccination on 1 April 69 (day 22 of prepatent period)
Cat 91	white mice	59 (93%)	60	357	-	-	-	Sacrificed 107 days after last positive stool. Autopsy negative.
Cat 97		10 (100 %)	60 +	-	7 larvae (70%)	larvae	liver	Died on 6 Oct 69 (day 60 of prepatent period)

Table 2. Skin penetration by Gnathostoma spinigerum advanced third-stage larvae in 9 adult domestic dogs up to end of reporting year 1971.

Animal No.	Source of larvae	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	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	76 (100%)	247 (from first skin infection)	257	-	-	-	Sacrificed 143 days after the last positive stools. Autopsy negative
Dog 9	white mice	192 (81.4%)	222 (from first skin infection)	58	9 larvae (4.7%)	larvae	hind leg muscle costal muscle abdominal muscle, abdominal fat	Sacrificed with ova negative stools, 26 Sept 69 (57 days after last positive stool)
Dog 10	white mice & snake	139 (69.6%)	236 (from first skin infection)	228	-	-	-	Sacrificed 144 days after the last positive stools. Autopsy negative
Dog 11	white mice	33 (97.1%)	1 +	-	28 larvae (85.0%)	larvae	skin abdominal flesh	Died on 5 Sept 68 with ova negative stool (day 1 of prepatent period)
Dog 12	white mice	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	64 (38.6%)	112 (from first skin infection)	329 +	-	-	-	Discharged on 17 Oct 69 from the study for being uncontrollable
Dog 14	white mice	68 (46.2%)	96 (from first skin in-tion)	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	64 (100%)	231	66 +	45 (70%)	Mature adult males & females	Stomach	Died of unknown cause day 66 of patent period

larvae, after completing the penetration through the skin of the animals, were found infecting many organs. Sometimes mature adults were found in the stomach wall; concurrently, the larvae and or immature worms were found in other organs of the host. This finding seems to indicate that the larvae do not develop at the same rate in the host after completing the transcutaneous phase.

The civet cat was never found to pass ova and on necropsy showed 15 living larvae (14 unencysted and 1 encysted larvae) located in the muscles of the abdominal wall, back, and fore-and hind-legs. One mature adult male was located in the right costal muscles. The 11 larvae were only slightly larger than before the experiment. The palm civet cat never became ova positive and at necropsy there were 11 living larvae (6 encysted and 5 unencysted) in the abdominal muscles. The results indicate that the civet cat could potentially act as a natural definitive host because the site near the stomach wall was found infected with 1 mature adult male. The palm civet cat may perhaps be considered as the paratenic host because only the larvae could be found infecting the animal.

SUMMARY: Continued observations of transcutaneous infection with G. spinigerum advanced third-stage larvae on 3 cats, 3 dogs, 1 civet cat and 1 palm civet cat showed that the cats had prepatent periods ranging from 60 to 310 days and patent period of 89-357 days. In 3 dogs the prepatent periods ranged from 231 to 247 days and the patent periods from over 66 to 257 days. The larvae, after completing the skin penetration, were found developing to immature adults at different rates before becoming mature adults in the stomach wall. The civet cat was considered to be a potential definitive host, but the palm civet cat was thought to be a paratenic host (transmitting host).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8b. DISS'N INSTR'N	8c. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	047
b. CONTRIBUTING				
c. [redacted]	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:
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FISCAL YEAR PRECEDING 71	2.5	148
FISCAL YEAR CURRENT 72	2	90

19. RESPONSIBLE DOD ORGANIZATION
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PII Redacted

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Veterinary Medicine; (U) Gibbon; (U) Leukemia; (U) Encephalopathy; (U) Hepatic Failure; (U) Hyperammonia

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) 70 07 - 71 06 The maintenance of a large experimental animal colony provides an opportunity to observe these animals for diseases that are not deliberately induced experimentally. The gibbon, in particular, has been studied because of its close phylogenetic relation to man and because of its availability in Thailand. Infectious diseases (bacterial and parasitic) are the leading cause of death in the animal colony. Chronic granulocytic leukemia has occurred in 3 gibbons. In order to promote gibbon breeding in captivity, their reproductive physiology is being studied. Gibbons are observed from birth to established growth and development norms. Fatal acute encephalopathy occurs frequently in Thai children. Hepatic failure with hyperammonia has been established as the cause of the encephalopathy. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* Applicable to contractors under arrangements

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

Project 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: Robert B. Cotton, MAJ, MC

Associate: Dirck L. Brendlinger, MAJ, VC: Alexander DePaoli, MAJ, VC; Kamnual Dhiensiri, M.D.*; Allen M. Glasgow, CPT, MC; Robert L. Hickman, MAJ, VC; Dennis O. Johnsen, MAJ, VC; Kwanyuen Lawhaswasdi, DVM; Walter W. Noll, MAJ, MC; Prayot Tanticharoenyos, DVM; Markpol Tingpalapong, DVM

*Khon Kaen Provincial Hospital, Thailand

Encephalopathy Due to Ammonia Intoxication in Hepatic Failure

Principal Investigator: Allen M. Glasgow, CPT, MC

Associate Investigators: Robert B. Cotton, MAJ, MC
Kamnual Dhiensiri, MD*

OBJECTIVE: Investigate the possibility that the encephalopathy of Reye's syndrome is secondary to hepatic failure.

DESCRIPTION: Blood ammonia (BA) levels were studied in 40 cases of Reye's syndrome.

PROGRESS: Blood ammonia was elevated (above 0.1 uM NH_4^+ per ml) on admission in 32 cases. In 22 cases, the admission BA was above 0.2 μM/ml and in 17 above 0.3 μM/ml. BA levels ranged from .025 μM/ml to .095 μM/ml in 20 hospitalized controls.

There were eleven survivors. Seven of eight patients with a normal admission BA and an additional patient with only marginal elevation survived. Of the 3 survivors with definite elevation of BA, 2 regained consciousness immediately after receiving intravenous glucose.

The highest BA levels were found in the most deeply comatose patients. In all instances where serial determinations were possible, the level of BA fell following admission. Many patients expired even though the BA level fell to normal or nearly normal levels.

In thirteen patients treated with glucose-saline cleansing enemas, no effect was demonstrated either on BA level or survival.

SUMMARY: This study demonstrates a relationship between BA and encephalopathy in Reye's syndrome. These observations indicate that the encephalopathy of Reye's syndrome is probably secondary to hepatic dysfunction.

* Khon Kaen Provincial Hospital, Thailand

Laboratory Animal Disease in Thailand: Its Occurrence and Importance to Comparative Medicine.

Principal Investigator: Dennis O. Johnsen, MAJ, VC

Associate Investigators: Alexander DePaoli, MAJ, VC
Robert L. Hickman, MAJ, VC
Kwanyuen Lawhaswasdi, DVM
Prayot Tanticharoenyos, DVM
Markpol Tingpalapong, DVM

OBJECTIVE: The objective of this study is to detect and investigate spontaneous metabolic and infectious diseases of laboratory animals for the purpose of recognizing and developing animal models for research studies as well as to define and improve the health of laboratory animals maintained in Thailand.

DESCRIPTION: In order to accomplish the objective, a program of continuous surveillance of the health status of the animal colony has been developed. Four areas are emphasized in this program: the disease screening program conducted in the laboratory animal breeding colony, the recurring clinical and laboratory examination of animals housed in the laboratory including those procedures performed during the quarantine of newly purchased animals, complete post mortem examination of each animal that dies in the colony, and the development of standards for operation and quality control that are indicated by the resulting findings. When indicated by the findings, experimental studies are initiated to explore the problems that occur in detail.

PROGRESS: In the breeder disease screening program and from rodents that were autopsied because of spontaneous death, 155 mice, 60 rats, 165 hamsters, and 110 guinea pigs were examined for spontaneous lesions and bacterial flora. No bacteria considered to be common pathogens were isolated from either the lungs or intestines. Pathological lesions in the mouse consisted chiefly of chronic pneumonia in about 5% of the animals examined with a smaller number showing multifocal hepatitis probably due to nematode parasites. Two neoplasms, one a teratoma, occurred in the mouse colony. The only pathological lesions detected in the inbred Fischer strain rat colony were two tapeworm cysts, probably Cysticercus fasciolaris. Pulmonary congestion appeared in about 5% of the hamsters examined,

and in an equal number liver pathology, consisting of congestion, fatty infiltration, periportal hepatitis, tapeworm cysts, and other nonspecific hepatitis was observed. In contrast to the other rodents, there was also a significant amount of kidney pathology consisting of congestion, pyelonephritis, and cortical scarring present in the hamsters. Like the other rodents, pneumonia was the most consistently occurring lesion observed in the guinea pig. Two cases of interstitial pneumonia, one of which was caused by Toxoplasma, one case of chronic bronchopneumonia, and one case of lipid pneumonia were found. Two guinea pigs had liver lesions consisting of multifocal necrosis and fatty degeneration. Acute cystic necrotizing colitis and coccioiosis were found in the intestine of one animal. Five macaque monkeys died from non-experimental causes during the year, the most frequent cause of death being pneumonia, with enteritis accounting for the rest of the deaths. Parasitic infections, consisting of oesophagostomiasis, whipworms, and hookworms occurred randomly in the macaques in that order of frequency. A significant number of the macaques were also naturally infected with malaria, nearly all of which was identified as Plasmodium inui. Microfilaria were discovered in the blood of three animals, and adult filariae were found in the mesentery of one; an identification of this parasite was not made. Ten non-experimentally induced deaths occurred among the gibbons. Although pneumonia was apparently the leading cause of death, intercurrent pathological processes in other organs as well often complicated a clear diagnosis. Strongyloidosis and lymphoproliferative diseases, which are described elsewhere in this report, were involved in seven cases of death or terminal illness, and are considered significant pathological observations.

Gibbon Growth and Development Study

Principal Investigators: Dennis O. Johnsen, MAJ, VC
Prayot Tanticharoenyos, DVM
Dirck L. Brendlinger, MAJ, MC

Associate Investigator: Markpol Tingpalapong, DVM

OBJECTIVE: The production of gibbons from the gibbon menstrual cycle and 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 by body weights, dentition, whole body radiographs, and sexual development evaluated at quarterly intervals. During the report period 11 quarterly observations have been made among the four baby gibbons that were available for study.

PROGRESS: In addition to having whole body radiographs available through the age of 3 years in one animal, the birth of a gibbon in the breeding program made it possible to obtain the first skeletal radiographs taken at the time of birth. The identifiable differences in skeletal development between 2 years and 3 years are mostly subjective. The long bones and the epiphyses assume a more sculptured appearance that more closely corresponds to their configuration at maturity. During this time the ossification of the sesamoid process of the metacarpophalangeal joint begins. The upper and lower permanent second incisors erupt during this time as well, and more than 1 kg is gained to bring the body weight to approximately 3 kg.

Gibbon Menstrual Cycle and Breeding Study

Principal Investigator: Markpol Tingpalapong, D.V.M.

Associate Investigators: Robert L. Hickman, MAJ, VC
Dennis O. Johnsen, MAJ, VC
Prayot Tanticharoenyos, D.V.M.

OBJECTIVE: In this study the reproductive cycle of the female gibbon and semen of the male gibbon are 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: Data characterizing the female menstrual cycle has been obtained by daily vaginal swabbing and visual observation for the presence of blood and measuring the degree of vulvar eversion in females believed to be reproductively normal.

PROGRESS: The degree of variation in the length of the menstrual cycle and the duration of menstrual bleeding confirms previous observations that regularity in the gibbon is a yet to be discovered exception to the rule that the gibbon has a completely irregular menstrual cycle. By broadly generalizing, it appears as if the duration of the cycle is commonly 19-23 days, with the menstrual flow occupying from 2-4 days of this cycle. Periods of amenorrhea, lasting up to 116 days, seem to be multiples of the basic cycle time interval. These periods of amenorrhea also occurred most frequently during the months of April through September, so it may be that gibbons tend to be more reproductively quiescent during these months. Two baby gibbons were born during the report period from a total of eight breeding pairs present in the laboratory. One of these babies was born prematurely and did not survive.

Leukemia in The Gibbon

Principal Investigators: Alexander De Paoli, MAJ, V.C.
Walter W. Noll, MAJ, M.C.
Dennis O. Johnsen, MAJ, V.C.

OBJECTIVE: The study was undertaken to characterize clinically and pathologically chronic granulocytic leukemia in the gibbon.

DESCRIPTION: During this reporting period three cases of chronic granulocytic leukemia occurred in the gibbon colony. Striking similarities in time of onset, clinical course and hematologic values were noted in these animals. In addition to the leukemia, unusual solid periosteal masses composed of foamy histiocytes and mature granulocytes were present.

PROGRESS: The three animals have recently been autopsied. Clinical, hematological and pathological data are presently under evaluation.

SUMMARY: Three cases of chronic granulocytic leukemia have recently occurred in the gibbon colony. Clinical and pathological data on these cases are presently under study.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6469	71 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
70 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				
c. CONTRIBUTING	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 71	0.5	31
FISCAL YEAR CURRENT 72	0.1	41

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

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: Bodhidatta, Flight Lt P., RTAF

NAME: Sankasuwan, MAJ V., RTA DA

PII Redacted

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.

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).

25. (U) 70 07 - 71 06 Preliminary results suggest that the bandicoot, Bandicota indica, may be an important reservoir host of the Q-fever rickettsia in central Thailand. Man and the potential vectors are being investigated to better determine the ecologic cycle as well as human disease potential and significance in the area. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon soldiator's approval

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
	Cheodchai Chuenchitra, B.Sc. [Med Tech]
	Bupha Manetese, B.Sc. [Med Tech]

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:	Cheodchai Chuenchitra, B.Sc. [Med Tech]
	Bupha Manetese, B.Sc. [Med Tech]

OBJECTIVE: To determine the distribution and seasonal variation of Q-fever and spotted fever group rickettsiae at Lopburi; identify arthropod vector[s], mammal reservoirs and alternate hosts[s]; serve as consultative laboratory.

DESCRIPTION: Search is being made for evidence of *Coxiella burneti* and spotted fever infections in man and rodents at Lopburi, central Thailand. Standard rickettsial identification, isolation and serologic techniques are being used.

PROGRESS: Trips to Lopburi were made in December, 1970 [winter season] and March 1971 [summer season]. Six strains of *Coxiella burneti* had previously been isolated from trapped bamboo rats [*Bandicota indica*] in Lopburi. In December, 2 *C. burneti* were isolated from 61 *B. indica* and 7 sera were positive to Q-fever

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* Chief Investigator [Thai Component]
** Investigator, Division of Research [Thai Component]

CF antigen. No spotted fever rickettsiae were recovered and 2 sera were positive to spotted fever group CF antigen in these bandicoots. In March, 79 B. indica were trapped. Isolation results are pending; 16 of the sera were CF positive [Q-fever]; no spotted fever agents were isolated and 2 sera were CF positive [spotted fever group]. No isolations or positive sera were obtained for either CF or spotted fever group in 6 Rattus rattus trapped in December and 17 trapped in March.

A total of 106 Hemophysalis and Amblyomma ticks were removed from these rats. Results are pending on rickettsial isolation attempts from these arthropods.

A total of 150 sera were collected from people living in and around the study areas. Results are pending on rickettsial serology.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 03 6470	71 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
70 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				
c. [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 71	2	118
FISCAL YEAR CURRENT 72	1.9	86

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: US Army Medical Component, SEATO

ADDRESS: Bangkok, Thailand

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

NAME: Altstatt, COL L. B.

TELEPHONE

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Firestone, MAJ M. H.

NAME: DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with security classification code)

(U) Psychiatry; (U) Human Behavior; (U) Neurological Diseases

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 variables in the host environment that may adversely affect the performance of duty, principally through alterations in human behavior.

24. (U) American and Thai psychiatrists, working with trained technicians using systematic observation of human behavior and mental status testing, observe the impact of such diseases as Japanese encephalitis and indiscriminate drug usage upon the immediate and long-term performance of individuals in their natural environment or in some cases, in an alien environment.

25. (U) 70 07 - 71 06 A one-year follow-up study of the acute and chronic effects of Japanese encephalitis in survivors is nearly complete. Preliminary data suggest significant residual neurological and psychological deficits. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

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: Marvin H. Firestone, M.D., Major, MC

Associate: Sanchai Bunlungpoh; Vivat Chavengchaiyong; Pien Chiowanich, M.D.; Harry C. Holloway, M.D., LTC, MC; Tien Jaiboonma; Winaisuk Kattipattanapongse; Apakorn Kuntatun; Verl M. Lackey, SFC; Surasak Laosuwan; Boonarb Panpanya, P.H.N.; Utaiwan Pongsukree; Thawon Ramayothin; Kriangsak Rittaporn; Jonathan J. Russ, M.D., Major, MC; Phon Sangsingkeo, M.D.; Pricha Singharaj, M.D.; Chira Sitasuwan, M.D.; Cherdchalong Sivasomboon, R.N.; Siriwat Sothornwit; Umneuy Sriroongroj; Avudh Srisukree, M.D.; Ladda Srithumma, R.N.; Chantana Sukavajana, M.D.; Sukree Tumrongrachaniti, R.N.; Prathan Voodhigul, M.D.; Chinda Witayarut, P.H.N.

Psychiatric Evaluation of North Thai-Lua' People and How this Evaluation Is Influenced by Experience and 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, M.D. Apakorn Kuntatun, M.D. Kriangsak Rittaporn, M.D. Tien Jaiboonma, M.D. Umneuy Sriroongroj, M.D. Sanchai Bunlungpoh, M.D. Vivat Chavengchaiyong, M.D. Surasak Laosuwan, M.D. Siriwat Sothornwit, M.D. Winaisuk Kattipattanapongse, M.D.
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 of the affectual reaction medical student observers experienced during interviews on diagnoses they offered;

5. To provide to 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.

DESCRIPTION: A previous cooperative research project surveying disease morbidity and culture in the Mae Sariang area 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.

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: Data as reported in the previous Annual Report has been ordered, and analysis of several of the data has been completed during this year. Write-up of data and publication is anticipated during the coming fiscal year.

SUMMARY: A transcultural psychiatric study of a segment of a predominantly North-Thai Lua' community was accomplished utilizing North Thai medical students, Thai psychiatrists, and American Psychiatrists. Comparisons of diagnoses and recommended treatment, and correlation with the results of standard mental status examinations and psychological tests were made. Analysis and write-up for publication will be completed during the next fiscal year.

Drug Abuse in Servicemen and Dependents

Principal Investigator: Marvin H. Firestone, M.D.
Major, MC

Associate Investigator: SFC Verl M. Lackey

OBJECTIVES AND DESCRIPTION: We have been initiating steps to develop a drug abuse research program in Thailand, and three segments of the population have been considered for study. Meetings have been held with the key personnel who would share the responsibility for such a study in their respective communities.

(1) The Provost Marshal at the JUSMAG and the GI of SUPTHAI;

(2) The Commanding Officer and Chief of Psychiatry at the U.S. Army Hospital;

(3) The school authorities at International School, Bangkok, where about 90% of the military school-age dependents attend classes.

All of these personnel are interested in cooperating with us in research efforts that might include a systematic survey of their particular segment of the population. With JUSMAG and SUPTHAI, we would be working mainly with law enforcement agencies such as CID; with the Army Hospital, we would be dealing mainly with the clinic population; and with the school population, the military dependents.

Any research that we do here would be complementary and supplementary to work being conducted by the Division of Neuropsychiatry at WRAIR. This research would contribute to understanding of the drug problems which occur in an overseas environment. Some of the questions which we will attempt to answer are (1) what drugs are being abused, and in what quantity, and by whom? (2) what are the psychological and sociological variables which correlate with or contribute to drug abuse? (3) what factors appear to correlate with lack of drug use (i.e., how do users and non-users of drugs differ?) (4) what suggestions can be made to others who would treat drug abusers? (5) what factors have contributed to the success or failure of drug treatment programs? The ultimate goal of all work in this area would be to develop a body of knowledge which will permit adequate command and medical response to drug usage,

a response which will decrease ineffective performance and human suffering.

COMMENT: One of the major problems which has retarded progress in the development of this project is inadequate staffing. Due to the departure of a Medical Corps Officer without another officer assigned to replace him, we have been hesitant to embark on a long-term study of drug abuse. It is anticipated that the assignment of a research psychologist will be accomplished by the latter part of this fiscal year. This addition to the staff will enable us to initiate a long-term, multi-faceted program of research into drug abuse in Thailand. We have, instead, attempted to elicit the cooperation and participation of the personnel working closely with the populations under consideration, in order to develop several independent studies. The Department of Neuropsychiatry has been acting as coordinator and has provided consultation to projects developed, in exchange for access to and utilization of any data collected in such studies.

PROGRESS:

(1) Starting 1 October 1970 arrangements were begun with the U.S. Diplomatic Mission Medical Unit and U.S. Army Hospital, Bangkok, to evaluate patients with drug usage difficulties. Patients referred to us from these sources are evaluated by the Spitzer Mental Status Examination to help determine factors relating to drug usage.

(2) Two technicians and three nurses have been trained in the administration and interpretation of the Spitzer Mental Status Examination.

(3) A library of books, journals, and reprints devoted to drug abuse research has been started in the Department of Neuropsychiatry as a source of reference materials.

(4) Additional files are being kept of (a) clippings from newspapers reflecting governmental policies and actions (including local, national, and international agencies) toward drug abuse; and (b) reports of research projects which have been planned or conducted by various agencies and which provide information on the prevalence and the social and psychological variables related to drug usage and abuse.

(5) Consultation and cooperation has been sought with the Department of Pathology and Department of Biochemistry regarding urine and serum analyses for chemical determination of drug use among examinees.

(6) With the cooperation of psychologists at International School, Bangkok, a survey of the high school population was accomplished in March, 1971. This survey has attempted to determine the extent, pattern of, and reasons for drug abuse in this population. The data are currently being analyzed. Analysis will be completed in the next few months. Much effort and time was necessary to design and administer the questionnaire. A cooperative arrangement to provide consultation services to the school staff required additional professional time.

(7) Consultation with ISB students and active-duty soldiers to aid in understanding the psychiatric aspects of drug abuse has been accomplished throughout the period covered by this report.

(8) We have been consulting with the JUSMAG Surgeon, the Air Force Psychologist at Korat, the psychiatric staff at the U.S. Army Hospital, and the physician and social work staff at Camp Samae San.

(9) We are currently making arrangement for computer support to aid in statistical analysis of data collected in future studies. Arrangements have been made for the utilization of key-punchers and verifiers at the Thailand School of Public Health. Other necessary equipment has been placed on order.

(10) Additional survey questionnaires are in the development phase, These will aid us in determining the extent of drug abuse in Thailand in both the American and Thai communities. Both urine and blood analysis will be utilized in any such surveys, which will aid in the assessment of the validity of examinee responses.

SUMMARY: A formal drug research program has been initiated in Thailand. At this time a survey of ISB (dependent) children has been completed to determine patterns of drug use. A great deal of time is currently being utilized making area contacts and developing the tools for a broad drug research program. It is anticipated that a wide range of areas will be investigated during the coming fiscal year.

Community Attitudes Towards Chronic Schizophrenic Patients in Thailand

Principal Investigators: Professor Phon Sangsingkeo, M.D.
Harry C. Holloway, M.D., LTC, MC
Jonathan J. Russ, M.D., MAJ, MC
Chantana Sukavajana, M.D.

Associate Investigators: Boonarb Panpanya, P.H.N.
Cherdchalong Sivasomboon, R.N.
Sukree Tumrongrachaniti, R.N.
Chinda Witayarut, P.H.N.
Ladda Srithumma, R.N.

OBJECTIVE: To describe and analyze the interaction of the hospital, the rural 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 occurring within and under the control of Srithunya Hospital, Nonthaburi, Thailand.

DESCRIPTION: Srithunya Hospital is a large (approximately 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 administration of 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.

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 responsible to the director, the section chiefs have considerable autonomy. 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 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%, and outpatient visits and re-admissions increased about 500% each during the same period. During this time the physician staff only doubled. The large ratio of professional staff size to patient load 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).

Srithunya was found to be a successful organization in that 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).

PROGRESS: During the period of this report attention was given to the short-term prognosis of chronic schizophrenic patients. Twenty male and eighteen female chronic schizophrenic patients who were admitted to Srithunya Hospital during a four-week period were followed using standard techniques of mental status examination and psychological testing. Special attention was given to the influence of social and treatment factors on the length of hospitalization. This work augments data on the organizational analysis of Srithunya Hospital obtained previously (see earlier Annual Reports).

Information was also collected pertaining to modality of treatment given, accuracy of predictions of the patients' stay in the hospital made by the patients' relatives and hospital staff, and condition of the patient prior to admission (obtained by history given by the responsible relative).

On 3 September 1970, data collection on all patients was completed. On 24 September, preliminary plans for analysis were made. On 25 September, Major Jonathan J. Russ, principle investigator, returned to WRAIR where further analysis and write-up for publication will be accomplished in collaboration with LTC Harry C. Holloway during this fiscal year.

SUMMARY: Tentative and partial results of an organizational study of Srithunya Hospital are reported. Special emphasis was given to describing admission, treatment, and discharge of schizophrenic patients.

A Follow-up Study of Japanese B. Encephalitis in Northern Thailand

Responsible Investigator: Marvin H. Firestone, M.D.
Major, MC
Chief, Department of Neuropsychiatry
SEATO Medical Research Laboratory

Principal Co-Investigators: Phon Sangsingkeo, M.D.
Jira Sitasuwan, M.D.
Avudh Srisukri, M.D.
Pien Chiowanich, M.D.
Prathan Voodhibul, M.D.
Pricha Singharaj, M.D.

OBJECTIVES: The study is designed so that answers to the following questions were approached:

a. How many cases of Japanese B. Encephalitis presented as acute psychoses to the major psychiatric hospital in the Chiang Mai area? How are these cases evaluated and to what extent was there misdiagnosis to these cases?

b. What are the mental status changes in the encephalitis convalescent cases? How do these mental status changes affect the functioning of the convalescent patient, and what is the progression of recovery, extent of recovery, and duration of abnormalities in the convalescent encephalitis patient?

c. What are the nature of the EEG changes in convalescent encephalitis patients? What is the duration of these changes?

d. What are the nature, duration, and progression of improvements in neurological deficits manifested by convalescent encephalitis patients?

e. What antibody titer changes occur in the convalescent encephalitis patient? Are there any correlations between these changes and the clinical changes in the convalescent encephalitis patient?

f. What is the effect and reaction of home environment on the convalescent encephalitis patient? How does the home environment influence the behavior of the convalescent encephalitis patient?

g. What is the effect and reaction of school (job) environment on the convalescent encephalitis patient? How does the school (job) environment influence the behavior of the convalescent encephalitis patient?

DESCRIPTION: In the 1969 encephalitis epidemic in Northern Thailand, several hundred cases of encephalitis were reported of which 20-25% died. Of the surviving convalescent cases, less than 20 were followed for any significant length of time. After reviewing these cases several tentative impressions were offered by Chiang Mai physicians:

1. return to school (job) at an early stage of convalescence seemed to be associated with more rapid improvement in psychological as well as neurological symptoms;

2. when the symptoms of encephalopathy abated in adult patients, agitated depressive symptoms often followed;

3. among children, the disturbing patterns of motor deficiency (spasticity and rigidity), dyslexia, dysarthria, memory defects, blocking of speech, temper tantrums, retardation, emotional lability and nightmares were common.

4. the E.E.G. changes were typical of any encephalitis, and these abnormal patterns persisted much longer than clinical signs and symptoms.

Ecological studies of Japanese B. Encephalitis as reported in other parts of this report were in the planning phases at the time this project was considered. The study of the clinical aspects of the acute encephalitis during hospitalization, as well as a follow-up of the sequelae of this illness were felt to be an important aspect of the complete surveillance of this disease in the Northern Thailand area. The Department of Neuropsychiatry was particularly interested in studying the neuropsychiatric complications in this disease and we were fortunate in developing a cooperative study with the major Northern Thailand hospitals to accomplish this survey.

PROGRESS: Excellent cooperation was obtained from the local medical and lay community in doing this clinical survey. Therefore, we were successful in following approximately 111 confirmed cases of encephalitis in the Chiang Mai area utilizing both home interviews by field team as well as return follow-up physical examinations, including E.E.G., mental status examinations, psychometric testing and complete

neurological examinations during each follow-up visit.

Auxiliary data has also been obtained on the follow-up patients by interviewing teachers and employers of the patient at the school and place of work.

Presently the study is still in the data collection phase. Some of the data collected during the acute phase of hospitalization has been partially analyzed. It is planned that the complete analysis of the data will begin during the latter part of this fiscal year. The following is a breakdown of the progress made during this fiscal year in each of the work areas of the project.

Physical and Neurological Examinations

Acute Illness:

During the fiscal year under consideration in this report, 195 cases of suspected encephalitis were admitted to the four general hospitals cooperating in the study (Chiang Mai University Medical Center - 79; McCormick Hospital - 60; Lamphun Provincial Hospital - 23; Lampang Provincial Hospital - 33). Of these 195 cases, 49 patients expired during the acute phase and 136 patients were discharged in an improved condition. All of the patients admitted in these four hospitals with the suspected diagnosis of acute encephalitis received complete physical and neurological examination by the cooperating physician investigators at the various hospitals. This examination included assessment of complete past and present history of illness, general physical examination including physical measurements and vital signs; general appearance and systems examination; neurological examination, including tests for cerebral function, meningeal irritation, cranial nerves, cerebellar function, motor system, sensory system, and reflex status. The physical and neurological examinations were done at the time of admission, two weeks after admission, and at the time of discharge on all cases of suspected acute encephalitis. All data forms were in both Thai and English, and a standardized system of recording results was adopted by all of the doctors. Approximately 1620 professional man-hours have been used to evaluate and record the findings necessary for this aspect of the survey up to this time.

Follow-up Data Collection:

During the acute phase 111 serologically confirmed cases of encephalitis were followed at intervals of one month during the first three months, two months during the next four months and three months

throughout the remainder of the one year follow-up after discharge from the hospital. If the patient was discharged from either Chiang Mai University Medical Center, Lamphun Provincial Hospital, or Lampang Provincial Hospital, he received his follow-up examinations at Chiang Mai University Medical Center. If he was discharged from McCormick Hospital he would be examined by the Chief Pediatrician of McCormick Hospital during follow-up examinations. Follow-up examination includes an interval history taken by the physician and a repeat physical and neurological examination which is recorded on the standardized forms maintained from his hospitalization. Duplicate copies of all examinations are kept in file at the Department of Neuropsychiatry office established on the Pediatric Ward of Chiang Mai University Medical Center.

E.E.G.
Acute Illness:

An electroencephalogram was done on all admitted cases suspected of having acute encephalitis during the hospitalization period. Over 100 EEG records were done during this period and the abnormal records could be classified into five patterns.

Pattern I: This is the largest group; this pattern was found in approximately 30% of the patients evaluated during the acute phase. The record has the slow background activity of 4-6 cycles per second, intermixed with large slow waves of higher amplitude. This pattern was seen throughout the record, but locally in some of the records.

Pattern II: This is the second largest group. The EEG showed generalized large slow waves of very high voltage. The electrical frequency is 1 1/2 to 3 cycles per second, amplitude, 200-300 micro volts. There was asymmetry of the records in some cases. Twenty percent (20%) of the patients were found to have this type of record.

Pattern III: There were approximately 10% showing this pattern of focal or generalized sharp wave or spike-like activity. This abnormality was mainly seen in generalized form.

Pattern IV: Four percent (4%) of the total patients studied during the acute phase were found to have this abnormal record, which is a mixture of large slow wave and sharp wave or a spike-like activity.

Pattern V: Five percent (5%) of the patients were classified in this group. The record has a burst of large slow waves interrupted by a flattened record.

Approximately 1/3 of the suspected cases of encephalitis showed normal EEG during the acute phase of the illness. Study numbers of all cases were correlated with the EEG patterns. Of the cases of suspected encephalitis on whom EEG were done during the acute phase, approximately 72% of those on which serologies were done showed positive titers for Japanese B. Encephalitis virus. It is clear now that the abnormal patterns in the cases of positive serology for Japanese B. virus are mainly in patterns I and II, which indicate a generalized involvement of the brain in the cases of acute encephalitis.

Follow-up Data Collection:

During each follow-up examination visit, an electroencephalogram is done. Results of the electroencephalographic readings are presently being ordered and analyzed. These are expected to be reported during the next fiscal year, along with other data collected during the follow-up phase.

Mental Status Examination

Acute Illness:

Mental status examination was done by the Director of Suan Prung Psychiatric Hospital on all patients suspected of having acute encephalitis during their period of hospitalization. Report was made of the patient's general appearance, consciousness, speech, affect, mood, thought process, thought content, perception, memories, judgment, and insight. Most commonly found during acute illness was disorientation, apathy, lack of insight, and blocking of speech. All mental status examinations were accomplished either in the general hospital, or at Suan Prung Psychiatric Hospital.

Follow-up Data Collection:

Mental status examinations are done on all cases of convalescent encephalitis at Suan Prung Psychiatric Hospital by our cooperating Thai psychiatrist as part of the follow-up examination.

The results of the mental status examinations during follow-up have not been analyzed, and will be reported in the future.

Psychometric Testing
Acute Illness:

Psychometric data were collected on approximately 100 patients during the acute phase of the illness. Testing was carried out by five psychologists at the Suan Prung Hospital. Adults were administered (1) the Zung test for the measurement of depression; (2) the Bender Gestalt Visual Motor Test; (3) the Performance Sub-Tests from the WAIS; and (4) the Organic Integrity Test to measure subjects' tendency to solve problems by "immature" color matching. For children the Goodenough Draw-a-Man test was substituted for the Zung test.

General findings indicated disturbances of two main types; (1) subjects exhibited passive, hypokinetic motor activity and clouding of consciousness. This group was also negativistic, and showed regressive behavior such as crying; and (2) hyperkinetic subjects, who showed impulsive, agitated, aggressive behavior. Self-depreciation and destructive behavior in this group was common.

Follow-up Data Collection:

Test scores have also been collected at the time each patient returns to the hospital for follow-up visits (6 per year). These data are still being collected.

Analysis of these data is expected to commence in June 1971.

In addition, the psychologists plan to continue collecting psychometric data on all new acute encephalitis patients admitted to the hospital during the 1971 rainy season.

Field Team

A field team consisting of two public health nurses and a driver visited homes, places of employment, and schools to conduct interviews to assess the post-hospitalization course of the patients in these environments. After the patients are discharged from the hospital, the field team will have completed more than 500 home visits on the patients who were discharged from the four hospitals and more than 100 visits to schools and places of employment. Signs and symptoms that were frequently reported during the early phases of follow-up by the families included irritability, memory difficulties, and tremors.

During home visits it was noted by the public health nurses that most of the patients with encephalitis came from poorer families, lived all together in small bed rooms, and sometimes did not have enough mosquito nets. Some of the cases reported that they stayed up late at night watching their farms and live-stock.

It was further reported that the encephalitis seemed to be spread over a large geographic area rather than many cases in each village. The following is a break-down, geographically, of the cases admitted to the four cooperating hospitals:

Chiang Mai Province:	60 cases, with the highest number reported from Amphur Muang, and Amphur Doi Saket
Lamphun Province:	25 cases, the largest number coming from Amphur Muang
Lampang Province:	25 cases, the largest number coming from Amphur Muang
Chiang Rai Province:	2 cases

The low number of cases reported from Chiang Rai indicates merely the way in which cases were selected for this study. The majority of cases from Chiang Rai Province were admitted to the provincial hospital in Chiang Rai, which was not one of the cooperating hospitals, and therefore the low number of cases reported in our study does not reflect the actual number of cases from this province.

At the time of discharge from the hospital, there were different recommendations to the families regarding the time to return to school or work, in an attempt to study the influence of this variable on the convalescent encephalitis patient's functioning. The effect of these different recommendations for return to school will be analyzed at the completion of the follow-up study during the next fiscal year. Data acquired from the teachers and work supervisors during visits shortly after the patients were discharged from the hospital, revealed the following: The most frequent symptoms reported were poor school and work performance, poor memory, decreased intellect, slurring of speech, and general irritability. Further analysis of the entire body of data collected during the follow-up study in the field must await completion of the year of study.

Laboratory Support

Serological testing including hemagglutination inhibition tests and compliment fixation tests was done on blood samples drawn on patients at the time of admission and two weeks after hospitalization. Presence of Japanese Encephalitis was determined if there was a four-fold change of antibody titer in the patient's serum. There were several difficulties regarding serological confirmation in that 20% of the cases could not be confirmed serologically because of either of the following reasons:

(1) Failure to get paired sera due to the expiration of the patient;

(2) Failure to get paired sera due to misunderstanding on the patient's part, and/or loss of the second sample because of the patient not following instructions;

(3) Failure to get the first blood specimen early enough during the acute illness, due to late admission of the patient;

(4) Collection of the second blood specimen too early, rather than allowing two weeks interval between the first and second blood-letting.

Despite difficulties in the collection of the sera, 84% of the sera sent for serology testing were positive for Japanese Encephalitis. From these results we may assume: (a) clinical diagnoses made by the physicians were quite accurate and reliable, and (b) for the patients whose sera did not show a four-fold change in antibody titer, there may have been other possible agents causing their encephalitis syndromes.

Besides serological testing, other laboratory diagnostic procedures were available to this study through the services of the Department of Virology at SEATO Medical Research Laboratory. Virus isolation from brain specimens of patients who had expired during the acute phase were attempted, although none were isolated. The negative results of these specimens probably were due to technical errors in collection, storage, and transportation of the specimens. Since the viremia stage in man is less than a day or two, the virus probably had already died by the time the patient expired.

Supervision and Coordination During Data Collection Phase

Supervision of the entire project was carried out by the Department of Neuropsychiatry, including Major Marvin H. Firestone, Chief of the

Department; Dr. Phon Sangsingkeo, Special Consultant to the SEATO Medical Research Laboratory and Dr. Pricha Singharaj, Public Health Physician in the Department of Neuropsychiatry. Supervision and coordination were accomplished by spending alternate periods of time in the Chiang Mai area, making arrangements for the study and refining the data collection procedures in the hospitals and field. Time requirements of the supervisors included a total of approximately 2,000 professional man-hours during the one year. A full-time secretary-clerk in the office at Chiang Mai University Medical Center was utilized for secretarial services in all aspects of the project.

SUMMARY: A clinical survey of the cases of Japanese Encephalitis who presented for treatment to four large general hospitals and one large psychiatric hospital in the Chiang Mai Valley during the 1970 epidemic is being accomplished. This study included hospitalization and a projected one-year follow-up study of the convalescent phase. Facilities and staff of the cooperating hospitals were utilized to accomplish both aspects of the study. One hundred and ninety-five patients ranging from age 1-69 years were studied during the acute hospitalization period, and 111 cases of confirmed Japanese Encephalitis are being followed for a one-year period at home, school, and work as well as with repeat medical and psychological examinations. Some of the findings during the acute phase and early convalescent phase of the illness are reported. The remainder of the data will be analyzed during the next fiscal year and reported in the near future.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6459	71 06 30	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
70 07 01	E.Termination	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	304
b. CONTRIBUTING				
c. [illegible]	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	71 04	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

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TELEPHONE: [illegible]-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: Shields, LTC Charles E.

TELEPHONE:

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

PII Redacted

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 medical and surgical problems in Vietnam having effects on military activities of the US Army personnel and their allies. Special areas of study included trauma, dermatological lesions, plague and cholera surveillance, enteric diseases, immunological changes associated with clinical hepatitis, and evaluation of new antimalarial drugs.

24. (U) Standard epidemiological procedures were used by multiple teams to collect samples which were tested in base laboratories. Tests were standardized where possible.

25. (U) 70 07 - 71 04 Surgical studies included research on peptic ulcers occurring in patients with acute trauma, injury patterns in helicopter accidents, evaluation of volume/pressure resuscitation for chest wounds and concomitant effects on oxygen transport and relief of shock. Medical research involved epidemiology of hepatitis, enteric diseases and general occurrence of disease in incoming and departing troops. The role of immunological factors in hepatitis was studied. Identification and determination of the role of Vibrio parahemolyticus in diarrheal disease was carried out. Testing of various routes of administration of quinine in the treatment of malaria and the use of two new experimental drugs for malaria was performed. The active portions of these programs were shifted to the US Army Medical Component, SEATO, Medical Research Laboratory Bangkok, Thailand, and the general activities in Vietnam terminated 30 Apr 71. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

896

*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 304, Military medical research program SEA, United States Army Medical Research Team, Vietnam

Investigators:

Principal: LTC Charles E. Shields, MC

Associates: Maj James A. Shaw, ANC; CPT Michael W. Benenson, MC; CPT David A. Neumann, MC; CPT Joseph M. Ballo, MC.

From 1 July 1970 until closure of the unit on 30 April 1971, several programs were completed. Remaining projects were transferred to the U. S. Army Medical Component, SEATO, Medical Research Laboratory, Bangkok, Thailand. These programs were in three major areas: medicine, surgery, and activities of the attached photography team.

General Medical Research.

1. Over the past few years, the continued occurrence of chronic and severe dermatological lesions aggravated by climatic and environmental conditions has decreased the combat effectiveness of troops stationed in many areas. Previous studies indicated that most lesions were mycotic. Spreading and secondary infections were particularly prevalent in U. S. troops working in wet field conditions, which contrasted with the low degree of pathology observed in local nationals. Since most lesions were of mycotic origin, a study was established to determine whether pretreatment use of griseofulvin would prevent the pathology.

Combat units were observed by the team to determine the pre-test baseline. Active medication was then given to one group; placebo to another. Skin biopsies were taken over the course of treatment. Evaluation was not considered complete unless each man completed at least eight weeks of treatment. Unfortunately, it was readily apparent that many individuals did not fully follow the daily regimen.

Thus, after comparing the incidence of skin infection between treatment and placebo groups and finding little difference, it was not possible to determine if it was because of a failure of the drug or a failure of the individual to follow the therapy regimen. Attempts to measure the drug level in the skin based on biopsies were incomplete, and, therefore, the study was inconclusive at this point.

2. Diarrheal disease was studied in two programs. Generally, diarrhea occurred sporadically in all types of units, and, as expected, it was difficult to isolate the etiologic agent or to characterize the epidemiology. The program was designed to consolidate data obtained from patients from various units while in civilian and military hospitals. Acute outbreaks in military units were studied when the occasion arose. Association of enteric disease with other illnesses, particularly hepatitis, was attempted as another part of the program.

Unit Preventive Medicine Officers notified the team of diarrheal outbreaks. The mobile unit was then sent to the area to gather epidemiological data, obtain samples for culture, and to interview patients.

Samples were processed by standard tests in the Enteric Lab, Institute Pasteur. Other special tests as part of the next project were included. Despite these efforts, acute outbreaks were not shown to have any definite pattern, nor to be related to any major public health hazard. The special testing for hepatitis associated antigen (HAA) became very difficult, as followup samples for titers were frequently not obtained because of troop movements, and the results were considered incomplete.

In a second study, samples from all types of diarrheal patients were routinely processed to determine common enteric pathogens as part of the cholera control program and public health surveillance. Because recent work in the Orient, particularly Japan, indicated that Vibrio parahemolyticus might be a possible pathogen, tests for this organism were added to the routine program, and samples were cross-checked by the SEATO Medical Research Laboratory.

Identification of V. parahemolyticus required special media. Additional tests of food, particularly fish, and water from local wells, the Saigon harbor, and the South China Sea at Cam Ranh Bay, as well as tests on ice and other sites of food preparation, were examined for V. parahemolyticus.

Routine processing of the samples indicated the usual common pathogens. However, the local native adult population from the civilian hospitals was found to have a 8.5 per cent incidence of V. parahemolyticus. The organism was also found in two U. S. adults exhibiting diarrhea. Patients of the local children's hospital did not demonstrate the organisms. Based on the positive cultures from patients, it would appear that V. parahemolyticus can be a pathogenic in the Vietnamese adult.

Correlation with hepatitis associated antigen studies was attempted, but results are incomplete.

3. Identification of the hepatitis associated antigen (HAA) in patients with clinical icteric hepatitis was carried out in U. S. troops in major hospitals in the Third Military Region. Interviews were used to identify possible sources, clarify the clinical disease pattern, and ascertain possible cross-relationships, such as drug usage, prior exposure to hepatitis, blood products, etc. The samples were also checked by another method by the U. S. Army Medical Component SEATO Medical Research Laboratory.

The study included 175 patients from fire bases, outlying units, and from Saigon units. Clinical history and laboratory findings were anlyzed for the acute and recovery phases.

The gel diffusion diagnostic technique was used in Vietnam, and the positives were confirmed in every instance by the complement-fixation technique in Bangkok. The latter test detected other positives, and, based on titer results, appeared to be more sensitive than the gel test. A greater number of positives was found in patients during the early part of their clinical course. Present findings indicate a higher incidence of HAA positives than would be anticipated in comparable U. S. groups. A slightly higher incidence of viral hepatitis in the Negro population was apparent when compared to total population at risk, but HAA incidence did not appear unusual. Correlation with drug usage or other infection sources was minimal.

4. A study on the protection of pseudomonas vaccine in battle casualties was prepared; however, the vaccine was not released for use during this period.

5. An assessment was made of various forms of veneral disease reported by soldiers to local dispensaries and hospitals. Unfortunately, patient response was too erratic for adequate study.

6. To ascertain the general occurrence of various diseases of interest in the tropics, over 1000 incoming and departing troops were surveyed by interview and various serological tests. The testing program involved several different procedures, some performed by the WRAIR Team at the 9th Medical Laboratory and at the SEATO Medical Research Laboratory, Bangkok. This study was transferred to the SEATO MRL for completion.

7. Studies on malaria were carried out in three programs over the year. Two concerned treatment of falciparum malaria; the third was an anthropological study of the native population designed to relate malaria susceptibility to biological traits and findings.

The approved treatment of relapsed falciparum malaria is intravenous administration of quinine, together with two other agents. The effectiveness of the route of administration was considered the basis for the study comparing two other forms of quinine. These were administered orally, as a tablet or a capsule.

Patients not considered too ill for the study were divided into three groups by the clinical staff, and, based on random choice, they received one of the three forms of quinine therapy. During the ten-day treatment period, those on oral therapy who exhibited clinical intensification or other complications were changed to intravenous therapy and excluded from the study. Those able to complete the one course were observed for fourteen days for possible relapse.

Fewer than fifty patients completed the required treatment. Consequently, it was not possible to demonstrate differences between the three regimens of treatment. There was only one possible instance of relapse after cessation of treatment. The most common problem involved in switching modes of administration was complaints of gastric symptoms.

A special study was undertaken by LTC Craig J. Canfield, MC, to test two new oral agents in treatment of falciparum malaria. The patients tolerated both drugs well, and had only four recrudences and one failure to clear parasites in the thirty days following treatment of fifty-one patients. This recrudescence rate is equivalent to the present treatment with three drugs, but the drugs were superior by virtue of absence of side-effects and one of them was faster-acting than standard therapy.

Blood samples from local Vietnamese and primitive tribes were correlated with the prevalence and relapse of malaria. As previously reported, the general blood group distribution pattern of the people in Vietnam was different from the general U. S. troop distribution. Incidence of malaria infection did not appear to correlate with the different blood groups, and susceptibility to relapse or recurrent infection did not show a relationship to blood groups.

8. Public health reports from the Long Binh post had shown a mild increase in encephalitis cases which led to a study jointly handled by the WRAIR Team, the 9th Medical Laboratory, the 20th Preventive Medicine Unit, and the SEATO Medical Research Laboratory in Bangkok. Serological specimens were drawn during acute and recovery phases. Though the number of cases was fewer this year, suggesting that the late monsoon and spraying had reduced the infective agent, the serological findings indicated that the majority of cases were Japanese B. type encephalitis.

General Surgical Research.

1. During this report period, a significant drop in battle casualties, partially assisted by a simultaneous decrease in military strength, led to a significant decrease in clinical material for surgical projects. Previous programs were brought to conclusion, and departing investigators were not replaced.

2. An active program was involved with oxygen transport mechanisms as related to altered oxygen and 2,3-diphosphoglycerate levels in patients after various types of injuries and after large volumes of blood were transfused. Since these were involved in the treatment of shock and such sequalae as renal shutdown, the program was studied in several phases. The overall program involved establishing a laboratory to measure blood gases, electrolytes, and associated chemical tests, and to monitor physiological changes during various stages of treatment and recovery.

The measurement of 2,3-diphosphoglycerate (DPG) was undertaken by another laboratory. Samples from patients receiving bank blood were divided into those being massively transfused (receiving over six units) and those with more moderate volumes being compared to patients using other types of fluid during the procedure. Blood gases and pH were measured at intervals during surgery and recovery. Those already on oxygen were excluded from this portion of the study.

During the initial planning stage, it was anticipated that 1 - 3 patients a week would be studied. By the time procedures were ready, patient input was too low to permit completion of the study prior to the departure of the staff.

3. Patients with acute chest trauma were studied as part of the oxygen system study. Findings indicated the value of volume over pressure type respirators. Long-term follow up was precluded since the majority of these patients were evacuated out of country.

4. Patients with multiple transfusions were further evaluated when the "wet lung" syndrome ensued. Evidence of pulmonary edema was detected clinically. Treatment with diuretics was used and the results correlated in terms of oxygen levels, recovery, and general complications. Such early use of diuretics appeared to be of clinical benefit to these patients.

5. Acute trauma involving the large viscera, particularly the liver, was clinically evaluated in terms of treatments used, recovery, and complications. These observations were made primarily by chart review of the operative

procedures, course, and evidence of complications. However, the paucity of cases and turnover of general surgical staff did not provide a large enough pool of material for study.

6. Severely injured patients receiving large amounts of bank blood were found to have cardiac instability and deranged physiology that appeared to be related to the decreased body core temperature induced by the cold blood. Other attempts to mechanically warm the blood unit prior to transfusion were considered either impractical for large volumes or caused serious changes to the stored red cells. The present device available for study was designed for diathermy-type heating. In actual use, the device did not appear to function satisfactorily, and tended to have heating effects on the red cells.

7. Standard coagulation tests were used to evaluate bleeding tendencies of patients following large transfusions. Individuals were divided between those receiving ten or more units in twenty-four hours and those receiving five units or less. Patient input rapidly decreased during the study period, and little evidence was obtained from the standard tests as to the nature of the clotting defect.

8. Study of peptic ulcer disease in patients with acute trauma was continued, using more detailed chemical tests, biopsies, and pathological specimens. The blood samples were sent to a contract laboratory for analysis, and final correlation of data was being prepared by the investigators after their return to WRAIR.

9. Aircraft accidents have remained a major cause of traumatic injury and fatality. Access to autopsy reports for the past year provided a source of data to be used to study patterns of injury, type of accident and aircraft. These findings were considered possible sources for correction of possible hazards in aircraft or operational procedures. Analysis of findings was complemented by review of body x-rays, and the final report is being prepared by the investigator upon return to WRAIR.

Photographic Team Projects.

Assignment of photographers to the team provided a unique capability to film many medical activities, including field research. Documentation of findings, procedures, and general activities provided material for demonstrations, education, and historical review. Some projects were linked with ongoing research or special activities of field medical units. Documentation was provided of the procedures involved in cleaning cargo prior to its return to the United States as an example of supply economy and of public health measures taken to

control spread of disease. A special project officer from USAMEDCOM was assisted in documenting with films and pictures of the current combat surgical procedures. Along the same lines, the use of the water-pik as a means to clean war wounds was filmed. Various field units in related medical fields had procedures documented by film and included those in optometry, as well as the training and care of war dogs for the veterinarians. Skin lesions being treated in the dermatology programs were photographed to show temporal progress and illustrate the pathology. The Preventive Medicine Officers designed and prepared a film for use in training future officers entering the field of preventive medicine, and to illustrate common procedures and problems likely to be encountered in this area.

The U. S. Army Medical Research Team (WRAIR) Vietnam reverted to inactive status on 30 April 1971, WRAIR G.O. 9, 13 April 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	3. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6462	71 06 30	DD-DR&E(AR)636

2. 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
70 07 01	H.Termination	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 (Precede 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)
PRECEDING FISCAL YEAR	70	3.5	60
CURRENT FISCAL YEAR	71	2.5	45

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL, E. L.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

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Division of Veterinary Medicine
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
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TELEPHONE: 202-576-5376
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Rogul, Ph.D., M.
NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Precede 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. Precede 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) 70 07 - 71 06 Studies were continued to develop means for recognition and differentiation of varied cultural forms of Pseudomonas pseudomallei and other pseudomonads causing infections in troops. Differentiation of select pseudomonads by cultural and biochemical tests has limitations. Serological characteristics of specific pseudomonads in the "pseudomallei complex" and other clinically important members of this genus appear to be distinct and may afford a means of differentiation. The various physiological types of P. pseudomallei reflect disclosed variations in the electron transport and respiratory chain. These variations may bear on strain virulence. The high therapeutic efficacy of ampicillin for leptospirosis was affirmed by more critical in vivo tests. An outbreak of jaundice in ROK troops in Vietnam appeared to be caused by Leptospira on the basis of serological findings. This work unit is now being incorporated with work unit 170, Task 00 of Project 3A061102B710 - Militarily Important Diseases Transmissible Between Animals and Man (09). For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

DD FORM 1498

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: A. D. Alexander, Ph.D.
Associate: M. Rogul, Ph.D.; D. Haas, Ph.D.; L. Evans, B.S.; J. Brendle, B.S.; S. Carr, M.S.

Description.

Studies are conducted on real or potential militarily important diseases transmissible from animals to man in SEA with emphasis on melioidosis and leptospirosis. Studies are directed toward improvement of laboratory diagnostic procedures, development of suitable treatment and control measures, and determining natural history and occurrences of these diseases.

Progress.

1. Melioidosis

a. Bacteriological differentiation of Pseudomonas pseudomallei, from other bacteriologically similar pseudomonads. Pseudomonas cepacia (Syn. P. multivorans) has been increasingly recognized in clinical specimens.[1-4] It has emerged as a potential pathogen that can cause wound, skin, and systemic infections. Its importance and role in human infections may have been masked because it is not readily identified by conventional selection and differential bacteriological schema.[3-4] The biological properties of P. cepacia are not remarkably different from those of P. pseudomallei.[1-5] In fact, strains of P. cepacia isolated from "foot rot" infections in soldiers were erroneously identified to be P. pseudomallei. Like P. pseudomallei, P. cepacia has been found in soils of Vietnam, is relatively resistant to antibiotics and is a potential infection hazard in war wounds and burns, It was therefore deemed important to determine if schema developed for identification of P. pseudomallei would also serve to differentiate it from P. cepacia. The comparative reactions of reference strain P. pseudomallei and 10 strains of P. cepacia are shown in Table 1. Five of the P. cepacia strains were from the American Type Culture Collection; the others were isolated from soldiers with skin infections.

Table 1. Comparative cultural and biochemical characteristics of *P. cepacia* and *P. pseudomallei*

Test	*P. cepacia*	*P. pseudomallei*
Oxidase	+	+
Motility	+	+
Growth on Crystal Violet	+	+
Growth on VCN Agar	+	+
Growth on MacConkey's Agar	+	+
Growth on CTAB Agar	-	-
Growth on SS Agar	-	-
Growth in 4% NaCl	-	-
Growth in 2% NaCl	+	+
Growth in Blood Azide Agar	+	-
Growth in EMB Agar	+	+
OF Media Glucose, Butt	+	+
OF Media Glucose, Surface	+	+
Nitrate Reduction	+	+
Gelatin Liquifaction	+	+
Indole Production	-	-
H_2S Production	-	-
Penicillin	Resistant	Resistant
Chloramphenicol	Sensitive	Senitive
Tetracycline	Variable	Sensitive
Polymyxin	Variable	Resistant
Kanomycin	Sensitive	Sensitive
Novobiocin	Sensitive	Sensitive
Dihydrostreptomycin	Resistant	Resistant
Erythromycin	Resistant	Resistant

With the shown scheme, strains of P. cepacia could be differentiated from P. pseudomallei by differences in nitrate reduction and susceptibility to sodium azide. Gilardi[3] has recently shown that strains of these two species differ in reactions on O-nitrophenyl-B-D-galactopyranoside test, arginine dihydrolase and lysine decarboxylase activity.

Serological cross-reaction between P. cepacia and P. pseudomallei were examined. In addition tests were conducted with the following other phenotypically similar pseudomonads in the "pseudomallei complex:" P. mallei, P. marginata, P. allicola, and P. carophylli.[1] Tests were conducted with 1 strain P. mallei, 4 strains of P. pseudomallei, 6 strains of P. cepacia, 1 strain each of the remaining strains. Antigen were prepared from smooth colonial growth on nutrient agar, and consisted of washed suspension of cells in 0.3% formalinized physiological salt solution. The concentration of cells was adjusted spectrophotometrically to approximately 9×10^8 cells per ml. Antiserum was prepared in rabbits by injection of increasing amounts of 0.5, 1.0, 2.0 and 4.0 ml. of antigen at 6- day intervals intravenously. Animals were usually bled for serum processing 1 week after last inoculation. Sera were diluted two-fold from 1:40 to 1:1280 and dilutions were distributed in 0.5 ml amounts to hemagglutination tubes to which were added equal amounts of antigen. Antigen-serum mixtures were shaken, incubated at 37° for 18 hours, then read. Titers are the highest final detection in which cells were distinctly agglutinated. A summary of test findings is shown (Table 2). Non-reciprocal cross reactions were found with P. pseudomallei antiserum and P. cepacia strains. These cross-agglutinations were heat-labile and could be removed by boiling the antigen for 2 hours. Otherwise the serological characteristics of pseudomonads in the pseudomallei "complex" appeared to be distinct. The cross- reactions of P. marginata and P. allicola are consistent with recent report that these were identical species.[1]

Table 2. Cross Agglutination Reactions of Strains in the "Pseudomallei Complex" of Pseudomonads

Antisera		Reciprocal of Titer* with Antigens													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
P. mallei 3873	1.	2560	640	160	640	80	-	-	-	-	-	-	-	-	-
P. pseudomallei 8202	2.	2560	2560	1280	2560	2560	-	-	640	1280			-	-	-
" 4845	3.	2560	2560	1280	1280	1280	-	-	80	160			-	-	-
" 7820	4.	2560	2560	1280	2560	1280	160	80	320	160			-	-	-
" 295	5.	1280	2560	2560	2560	1280	-	-	640	1280			-	-	-
P. cepacia 17460	6.	-	-	-	-	-	1280	160	160	-			-	-	-
" 17759	7.	-	-	-	-	-	320	2560	2560	2560			-	-	-
" 4a	8.	-	-	-	-	-	1280	2560	2560	2560			-	80	-
" 5a	9.	-	-	-	-	-	-	320	2560	2560			-	-	-
" CJ69	10.														
" FB96	11.														
P. marginata 702	12.	-	-	-	-	-	-	-	-	-			640	320	-
P. alliicola 19302	13.	-	-	-	-	-	-	-	-	-			640	320	-
P. caryophylli 721	14.	-	-	-	-	-	-	-	-	-			-	-	280

* - = < 1:80; titers of 1:2560 were equal to or greater than 1:2560

b. Metabolic differences among strains of P. pseudomallei. The purposes of this study were to determine the physiological and biochemical basis for smooth and rough colony formation in pseudomonads, to apply these findings to the type of treatment and laboratory identification of pseudomonas diseases. Our previous investigations (WRAIR annual reports 1969-1970) have shown, how certain strains of P. pseudomallei mimic coliform organisms, why certain maintenance media are detrimental to their prolonged cultivation, and that colony appearance and physiological characteristics are highly correlated. We have found that all members of this species produce ammonia, however, some strains neutralize its potential toxicity by coupling ammonia with oxalic acid. This would indicate that some cases of melioidosis are complicated by ammonia toxicity. It is necessary to determine the pivotal reasons for physiological differences within the species P. pseudomallei. Once these basic mechanisms are defined, it is anticipated that we will be afforded a more rational approach to laboratory identification and disease treatment. Our first questions asked whether the natural transition of smooth to rough colonies was genetic, adaptive or both. Since many of the correlates of smooth and rough characteristics occurred only under highly aerobic conditions, we assumed that knowledge of the cytochrome content and electron transfer chain might elucidate important regulatory mechanisms. P. pseudomallei strains 165 L (smooth) and 7815C (rough) were grown on 3% glycerol in Difco brain heart infusion agar (glycerol agar) for two days at 37 C.

The resulting growth was washed in phosphate buffer and processed as previously described. The bacterial fractions were separated by centrifugation into components which were sedimented at 78,480 x g or remained in the supernatant fluids.

These components were examined in the Cary 15 spectrophotometer at room and liquid nitrogen temperatures. The oxidized, reduced, difference spectra, heme and carbon monoxide spectra were recorded. Protein determinations were assayed by the Lowry method.

These same strains were grown in deep cultures of Difco nitrate broth and examined in a similar manner.

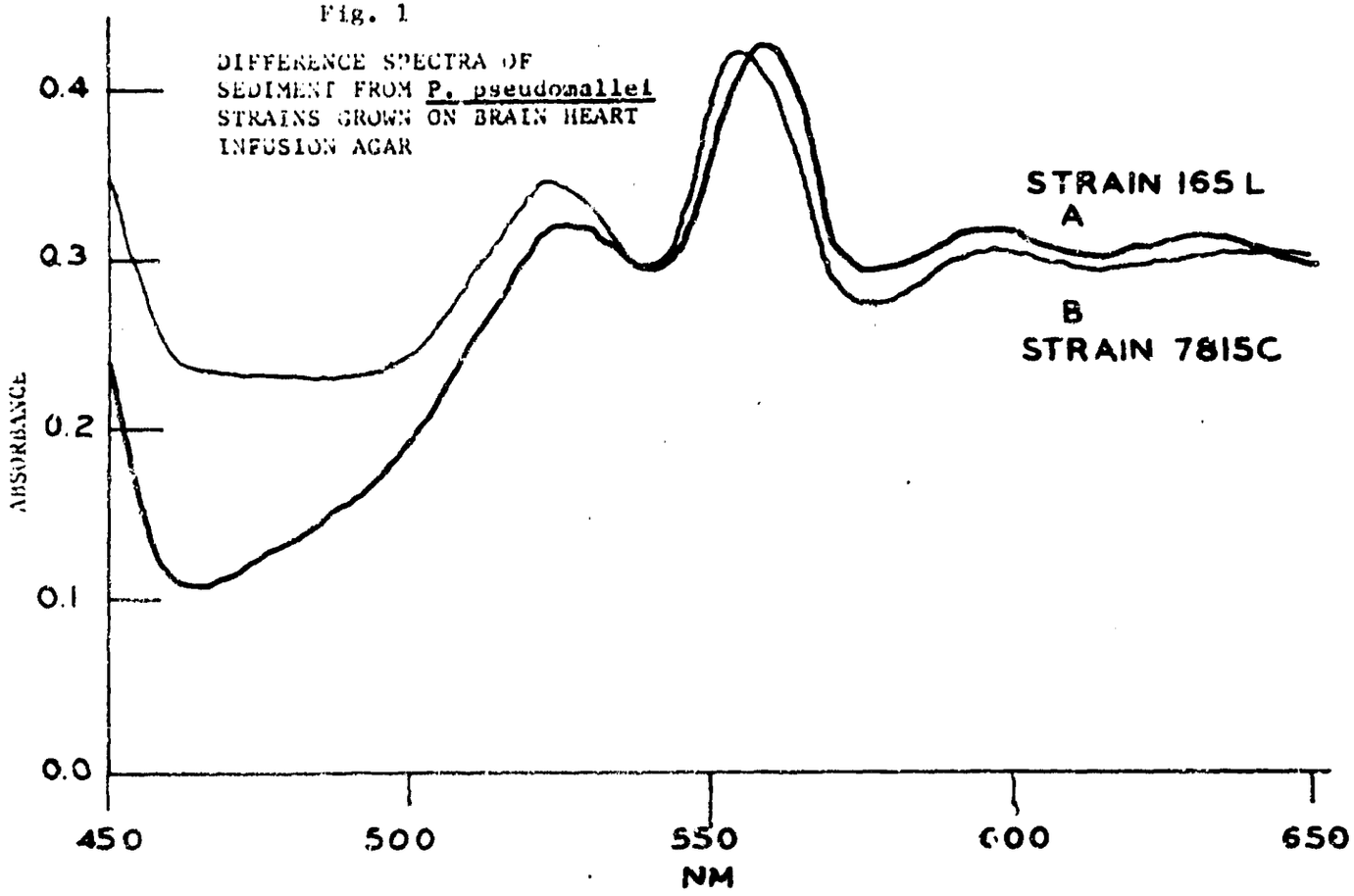
The cytochromes listed in Table 3 and were determined by spectrophotometry. The soluble cytochrome system of strain 165L definitely contained a cytochrome c. It was difficult to determine whether this cytochrome had masked the optical spectra of any b or a cytochrome. The reactions with carbon monoxide (CO) indicated an oxidase activity associated with an a type cytochrome. The particulate cytochrome system was definitely composed of a, b and c type cytochromes.

Table 3. Qualitative Cytochrome Content of P. pseudomallei Strains Grown on Glycerol Agar or in Nitrate Broth as Determined by Spectrophotometry

	Strain 165L	Strain 7815C
Glycerol Agar		
Supernatant Fluid	c type cytochrome b type cytochrome (.) a type cytochrome oxidase (?)	c type cytochrome (oxidase?)
Sediment	c type cytochrome b type cytochrome a type cytochrome	c type cytochrome b type cytochrome a type cytochrome
Nitrate Broth		
Supernatant fluid	c type cytochrome b type cytochrome a type cytochrome	c type cytochrome (oxidase?) b type cytochrome (oxidase?) a type cytochrome oxidase
Sediment	c type cytochrome b type cytochrome a type cytochrome oxidase	c type cytochrome b type cytochrome (oxidase?) a type cytochrome oxidase

In strain 7815C we only found a c type of cytochrome in the supernatant fluid. It appeared that a small amount of this cytochrome reacted with CO. It is possibly an oxidase but more likely the reaction is due to some slight denaturation. The sedimented fraction contained a, b and c types of cytochromes. The a type demonstrated oxidase activity. Table 3 also shows that each cytochrome type was found in both the supernatant and sedimented fractions of cells grown in nitrate broth. The a type cytochrome demonstrated oxidase activity in all fractions. In strain 7815C the b and c type cytochromes may have had oxidase activity (marked by "?") but it is also possible that these components may have denatured or reacted with nitrate in such a way as to appear as an oxidase. The difference spectra in Figures 1 and 2 illustrate that the two strains are qualitatively similar in their cytochromes content but quantitatively quite different. The amounts of cytochrome b and c from strains 7815C and 165L appeared to be proportionately greater in nitrate broth than the amounts of these cytochromes from these same strains on glycerol agar. On agar, strain 165I appeared to contain much more flavin than 7815C (comparative depth of flavin notch between 460-500 nm). In nitrate broth both strains had a shoulder peak between 475-480 which might be indicative of an oxygenase.

Fig. 1


DIFFERENCE SPECTRA OF SEDIMENT FROM P. pseudomallei STRAINS GROWN ON BRAIN HEART INFUSION AGAR
STRAIN 165 L
A
B
STRAIN 7815C
ABSORBANCE
0.4
0.3
0.2
0.1
0.0
450
500
550
600
650
NM

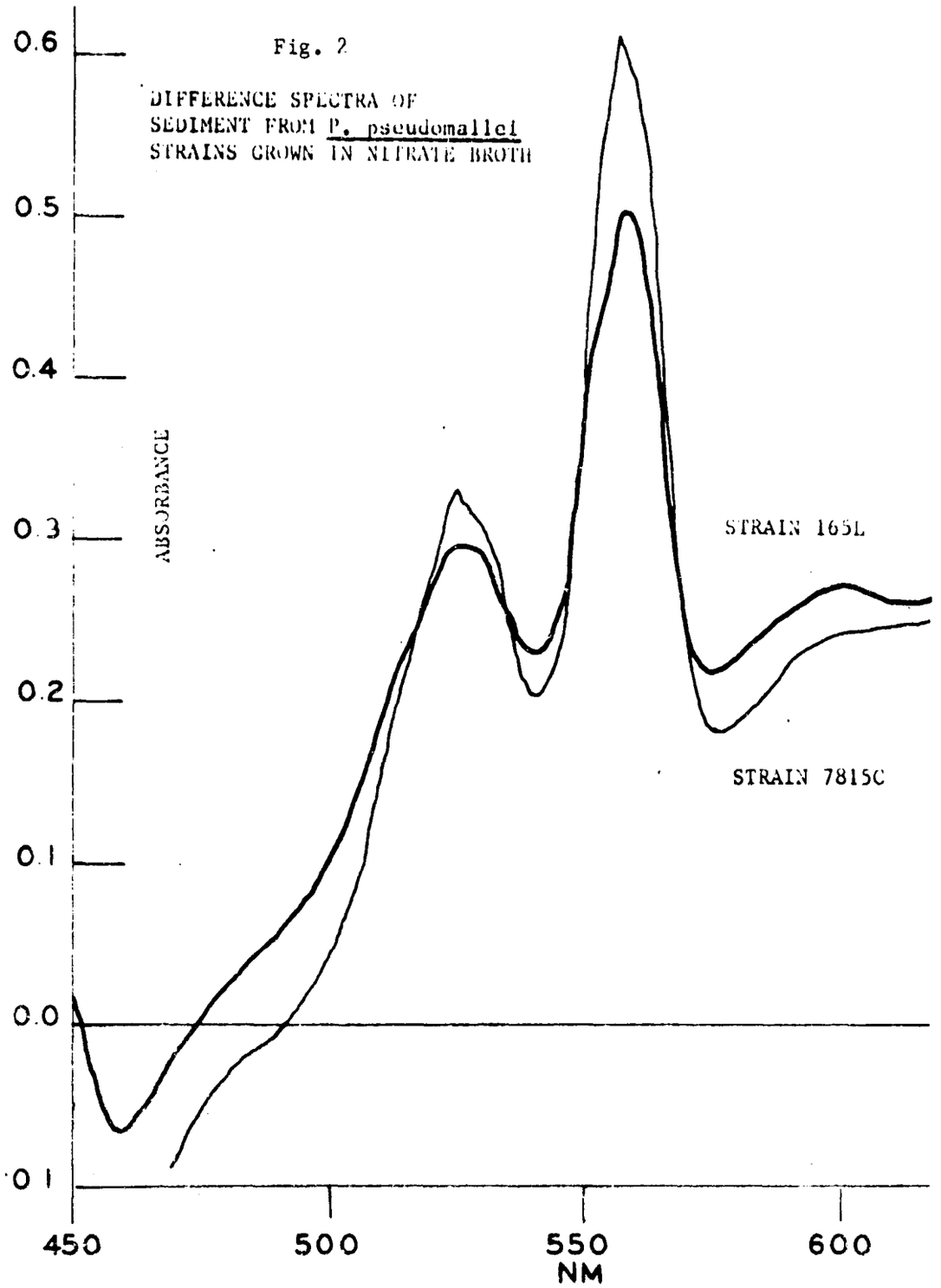


Fig. 2
DIFFERENCE SPECTRA OF SEDIMENT FROM P. pseudomallei STRAINS GROWN IN NITRATE BROTH
ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
0.0
.01
STRAIN 165L
STRAIN 7815C
450
500
550
600
NM

2. Leptospirosis

a. Therapeutic efficacy of ampicillin in experimental hamster infections. Penicillin, streptomycin, tetracycline, macrolide antibiotics are usually effective for treatment of leptospirosis if they are first given on the first or second day of disease. Their efficacy is questionable if treatment is started on 3rd or 4th days after disease onset. When treatment is started after the 4th day of disease, the consensus is that none of the antibiotics alter the course of disease. There is still a need for a chemotherapeutic agent that can be effective after the 4th day of disease especially when severe signs are manifest.

Specific attention was given to ampicillin which was previously found to have high antileptospiral activity (in vivo) in comparison to penicillin G, chlortetracycline, and tylosin. The efficacy of the drug was evaluated in adult hamsters experimentally infected with a strain of serotype bataviae (1415). In previous in vivo tests to measure drug efficacy when treatment was delayed, the infectivity dose was lighter than anticipated (See Annual Report 1970). It was deemed advisable to repeat the experiment with the use of larger challenge dose. For this purpose adult hamsters (131 to 150 gms.) were inoculated i.p. with a concentration of leptospiras which produced a fatal 7-day course of infection. In various groups of hamsters treatment at dose levels of either 6 or 3 mg/kg/day was delayed from 3 to 6 days after inoculation of leptospiras. In addition to ampicillin, tests were done on penicillin G and chlortetracycline for comparative purposes. Treatment was given s.c. in divided dose 2 times daily for 5 days. The regimen used for 3 antibiotics was previously demonstrated to be non-toxic for hamsters. The results of tests are summarized in Table 4. The findings affirmed that ampicillin had greater curative properties than penicillin G or chlortetracycline under the test conditions. Ampicillin afforded greater protection against death. Its efficacy in curing leptospiruria was again demonstrated. Only 2 of the 36 surviving hamsters treated with ampicillin had leptospiruria whereas 27 of 30 surviving hamsters treated with penicillin G and 16 of 33 hamsters treated with chlortetracycline were carriers.

Table 4. Effect of Delay in Treatment on Chemotherapeutic Efficacy of Ampicillin, Penicillin G and Chlortetracycline for Hamsters Infected with Serotype Bataviae (1415)[a]

Days treatment delayed	Drug dose Mg/Kg/day[b]	Ampicillin		Penicillin G		Chlortetracycline	
		D[c]	L[c]	D	L	D	L
3	6	0/5	0/5	1/5	4/4	0/5	0/5
4		0/5	0/5	1/5	4/4	0/5	0/5
5		0/5	0/5	0/5	5/5	0/5	1/5
6		2/5	0/3	3/5	0/2	4/5	0/1
3	3	0/5	0/5	1/5	4/4	0/5	5/5
4		0/5	1/5	1/5	4/4	0/5	4/5
5		0/5	1/5	0/5	5/5	0/5	5/5
6		2/5	0/3	3/5	1/2	3/5	1/2

[a] Controls: 35/35 deaths; mean survival time 7.0 days.

[b] Drugs given subcutaneously 2 x daily for 5 days.

[c] D = No. dead/total; L = Leptospiruria in survivors.

b. Leptospirosis in ROK troops in VN. During the spring of 1959, a purported outbreak of severe leptospirosis occurred in a group of 200 to 240 ROK soldiers. Serum samples from 73 ROK soldiers who were involved in the outbreak were submitted for more definitive serological tests with the microscopic agglutination technic. Samples were also tested with 6 pooled slide test antigens. Significant antibody titers were demonstrated with the microscopic agglutination technic in 46 cases, of which 43 were also positive on slide tests. Seven additional sera were slide test positive but gave partial or negative reactions on microscopic agglutination tests. The distribution of seropositive sera by predominant serotype reactions and titer is shown in Table 5. High titer reactions of 1:1600 or 1:6400 demonstrated in 14 of 46 seropositives would seem to indicate that leptospiral infections had occurred within a short time after samples were taken and might be related to the outbreak. It is also apparent from the distribution of reactions with various antigens that infections in this group were produced by a variety of serotypes.

Table 5. Distribution of Leptospiral Agglutinins in Sera from ROK Soldiers by Predominant Serotype and Titer

Predominant serotype	No. of Sera with Titer				Total
	1:100	1:400	1:1600	1:6400	
grippotyphosa	1	3	2	1	7
grippotyphosa and other	2	2			4
pyrogenes - alexi	1	3	3		7
djasiman	1	1			2
pyrogenes & djasiman			1		1
autumnalis		1			1
autumnalis - australis	1		1		2
australis			1		1
bataviae			2	1	3
icterohaemorrhagiae		1	2		3
canicola - celledoni	1				1
multiple	1				1
patoc - butembo	10	3			13
Total	18	14	12	2	46

Summary and Conclusions.

1a. P. cepacia resembles P. pseudomallei in cultural characteristics, biochemical activity, resistance to antibiotics, and in its natural occurrence in soils. The two species may be differentiated by ability to reduce nitrate, susceptibility to sodium azide and reportedly[3] reactions in ONBG test, production of arginine dihydrolase and lysine decarboxylase. Serologically some strains of P. cepacia contain heat-labile antigens which agglutinate P. pseudomallei antiserum. A larger number of strains should be tested to affirm these differential attributes.

1b. At the present time, spectrophotometry suggests that the cytochrome systems of P. pseudomallei strains 165L and 7815C are qualitatively similar but differ quantitatively under varied growth conditions. Kinetic and oxygen consumption studies on the oxygraph are now being performed to further elucidate the electron transport chain.

2a. Ampicillin was found to have better curative properties than penicillin G and chlortetracycline for experimental leptospirosis in hamsters in repeat tests with a greater infectivity dose. Its value in management of human or animal infections when given relatively late in course of disease merits additional observations. It has potential usefulness for chemoprophylaxis of leptospirosis.

2b. An outbreak of severe disease in ROK troops appeared to be caused by Leptospira on the basis of serological test findings. A variety of serotypes appeared to be responsible for the agglutinin reactions.

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

Tast 00, Tropical and Subtropical Military Medical Research

Work Unit 305, Military medical research program, SEA, WRAIR - zoonoses

Literature Cited.

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Publications:

1. Alexander, A. D., Huxsoll, D. L., Warner, A. R., Jr., Shepler, V., and Dorsey, A. Serological diagnosis of human melioidosis with indirect hemagglutination and complement-fixation tests. Appl. Microbiol. 20: 825-833, 1970.

2. Alexander, A. D. and Williams, L. C. In vitro susceptibility of strains of Pseudomonas pseudomallei to rifampin. Appl. Microbiol. 22: 11-12, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6526	71 06 30	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. ORG'N INSTR'N	8A. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
70 07 01	H. Terminate	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				
c. [redacted]	CDOC 1412A(2)			

11. TITLE (Procede 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: 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	0	1	40
FISCAL YEAR CURRENT	71	1	40

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3365
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

PII Redacted

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 human serum gamma globulin is effective in decreasing the prevalence of endemic hepatitis among soldiers stationed overseas, to determine the period of immunity and the dose required.

24. (U) 107,803 U.S. soldiers who arrived in Korea between May 1967 and August 1969 received an injection containing either 2 ml, 5 ml or 10 ml of 16 per cent gamma globulin or a placebo solution upon arrival and 65 per cent received a similar injection 5 to 7 months later. Injections were given in a double blinded manner based upon the last integer of the soldier's service number. The incidence of hepatitis in each group was enumerated by surveillance of all hospital admissions.

25. (U) 70 07 - 71 06 Gamma globulin diminished the incidence of icteric endemic hepatitis by fifty per cent. The 5 ml dose seemed to produce maximal protection. Protection was provided for only six months after injection. Similar protection was observed against both HAA positive and negative hepatitis. HAA positive hepatitis was observed in 12 per cent of icteric cases of endemic hepatitis. Patients who received gamma globulin had a diminished history of chills, fever and icterus, decreased albuminuria and a lower peak mean serum bilirubin value than control subjects. Prophylactic injections of gamma globulin did not significantly alter the incidence of other commonplace infectious diseases observed in the soldiers of this study. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

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

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 May 1967, a controlled study was initiated in U.S. soldiers assigned to Korea to evaluate the effectiveness of gamma globulin produced from U.S. donated blood in the prophylactic prevention of icteric hepatitis. Between May 1967 and August 1969, 107,803 soldiers arriving in Korea received a 10 ml injection containing either 2 ml, 5 ml or 10 ml of 16 per cent human serum gamma globulin or 10 ml of an albumin-sucrose-potassium glutamate solution. Sixty-five per cent of these soldiers received a second injection of the same material five to seven months later. Other than transitory syncope, no adverse reactions were observed in the subjects of this study.

The materials used in this study were manufactured by Hyland Laboratories and were prepared by Cohn fractionation of ACD or citrated plasma from recently bled paid donors in various geographic areas of the United States. Assays of this gamma globulin showed little fragmentation (< 5 per cent); large amounts of IgG (16 g/100 ml); small amounts of IgA (100 mg/100 ml), IgM (110 mg/100 ml) and albumin (16 mg/100 ml) and high antibody titers for measles, poliovirus type II and diphtheria. Immunoelectrophoresis of the albumin sucrose potassium glutamate solution used as a placebo solution in this study contained no detectible IgG, IgA or IgM as measured on immunoplates. Biologic materials were shipped and stored under monitored conditions to insure they were maintained properly refrigerated until they were used.

The various dosages of gamma globulin and placebo solution were bottled in vials labeled with a single digit identification number. An equal number of vials were labeled with each possible integer, 0 through 9. Vials containing the three dosages of gamma globulin were each assigned one of two integers and the control solution was labeled with one of the four remaining integers. Each soldier was injected with the contents of a vial labeled with an integer selected to match the last integer of his army service number. This permitted random selection of subjects and test materials. The code was not known to members of the study and people involved in analysis of data or care of patients until after completion of the investigation.

Soldiers with symptoms or physical findings suggestive of hepatitis were hospitalized at either the 121st Evacuation Hospital or the 11th Evacuation Hospital. The diagnosis of icteric viral hepatitis in these soldiers included an abnormal serum bilirubin concentration (1.5 mg/100 ml), abnormal serum transaminase determinations (SGOT and/or SGPT, 200 units) and lack of evidence of infectious mononucleosis. A liver biopsy specimen was obtained from 82 per cent of these hepatitis patients and was used to provide confirmation of the diagnosis. Only five patients were hospitalized during the period of this study with well documented anicteric hepatitis; they were not included in the analyses.

There were 467 documented cases of icteric viral hepatitis in the subjects of this study with an incidence of 5.67 cases per thousand among the control subjects who received albumin, and 3.39 (10 ml), 2.90 (5 ml) and 4.04 (2 ml) cases per thousand among soldiers injected with the various amounts of gamma globulin. Analyses of these data showed that 2 ml of gamma globulin provided significant protection but less than was observed with the 5 ml injection. A larger dose of gamma globulin did not produce a greater reduction in the incidence of icteric viral hepatitis. Groups who received either 5 or 10 ml doses of 16 per cent gamma globulin had one half the hepatitis rate observed in the control groups for six months after injection. Subsequently, there was no significant difference in the hepatitis rate in either the gamma globulin protected subjects or the soldiers who received the placebo solutions. Studies of the occurrence of other infectious diseases which occurred in these soldiers showed no significant change in incidence among the gamma globulin protected group when compared to the control group. This included upper respiratory infections, bacterial pharyngitis and bronchitis, pneumonia, infectious mononucleosis, influenza, gastroenteritis, meningitis, bacterial and fungal skin infections, urinary tract infections and the venereal diseases.

Acute sera from 211 patients admitted to the 121 Evacuation Hospital with icteric viral hepatitis was examined for Australia antigen (HAA)

by both the complement fixation and agar gel immunodiffusion method. Following distribution of these patients into groups which had received gamma globulin or placebo solution, statistical analysis showed that gamma globulin provided similar protection against both HAA positive and HAA negative endemic hepatitis. The protection observed against HAA positive hepatitis by gamma globulin in this study seems to differ from other recently reported data in which gamma globulin did not prevent HAA positive hepatitis after blood transfusions. However, most of our patients were probably infected with a smaller number of viral particles by the oral route and received gamma globulin which was derived from a paid donor population.

To determine whether gamma globulin affected the severity of illness, we compared the hospital records of patients who received 5 or 10 ml doses of gamma globulin with those who were injected with placebo solution. Patients with viral hepatitis who received gamma globulin had a diminished history of chills, fever and icterus; decreased albuminuria and lower peak mean serum bilirubin (total and direct reacting fraction) values than control subjects. Other comparisons to quantify severity including the duration of hospitalization; other symptoms, physical findings and laboratory tests showed no significant difference between groups.

In this study, we believe most patients were infected by the oral route. Only a few patients had received blood transfusions within six months before the development of icterus and there was little evidence by history or physical examination of the parenteral use of narcotics or habituating drugs among these hospitalized soldiers. It seemed unlikely that immunizations or dental work contributed significantly to the prevalence of hepatitis because disposable hypodermic needles and syringes and pressure sterilization have been used in all medical facilities during recent years. Further, surveillance is maintained over shipments of vaccines so that outbreaks of disease could rapidly be traced to their source. In addition, in this study there was a similar history of dental work, immunizations and the use of medications among patients with hepatitis and soldiers who did not develop this disease.

A comparison was made between the 467 patients who developed hepatitis and 4,000 soldiers who did not develop hepatitis during their military tour in Korea to ascertain which factors were more frequently observed in the hepatitis patients than the normal soldiers. On a per capita basis, the highest risk group was a white enlisted man between 20-29 years of age with 3-9 years of military service who had more years of education than his peers and was reared in a nonurban environment. We believe these data indicate that individuals reared in an urban environment are more likely to have developed immunity to hepatitis in childhood and that junior noncommissioned officers have a greater exposure to the native environment than either young enlisted men, senior noncommissioned officers or commissioned officers.

Results.

Gamma globulin diminished the incidence of icteric endemic hepatitis by fifty per cent. The 5 ml dose seemed to produce maximal protection. Protection was provided for only six months after injection. Similar protection was observed against both HAA positive and negative hepatitis. HAA positive hepatitis was observed in 12 per cent of icteric cases of endemic hepatitis. Patients who received gamma globulin had a diminished history of chills, fever and icterus, decreased albuminuria and a lower peak mean serum bilirubin value than control subjects. Prophylactic injections of gamma globulin did not significantly alter the incidence of other commonplace infectious diseases observed in the soldiers of this study.

Conclusions and Recommendations.

Human serum gamma globulin should be administered intramuscularly in a 5 ml dosage every six months to military populations where it is important to produce a 50 per cent reduction in the prevalence of disease. The reduction in incidence of HAA positive hepatitis among gamma globulin protected soldiers is probably caused by obtainment of the original plasma from plasmapheresis of prisoners and a higher titer of HAA antibody than in conventionally available gamma globulin. Purchases of gamma globulin for prevention of HAA positive hepatitis should include specifications which insure that hyperimmune biologic materials are obtained. The use of gamma globulin to attempt to reduce infectious diseases other than those for which it has been shown to be effective or in patients with hypogammaglobulinemia should be discouraged. The population of this study should be followed in collaboration with the Veterans Administration for 10-20 years for detection of cases of postnecrotic cirrhosis. This might be helpful in determining whether anicteric cases of hepatitis develop postnecrotic cirrhosis unlike icteric cases and delineate whether the protection with gamma globulin enhanced the incidence of cirrhosis.

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.

Publications.

1. Conrad, M., Young, A., Conrad, P., Park, R., Bancroft, W., and Bernier, G.: Prophylactic use of gamma globulin to prevent endemic viral hepatitis. J. Clin. Investment 50:21a, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6463	71 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
70 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	310
b. CONTRIBUTING				
c. XXXXXXXXX	CDOG 1412A12			

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: 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 71	1.5	35
FISCAL YEAR CURRENT 72	1.5	35

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ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

DA

PII Redacted

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) Endemic viral hepatitis has been a major cause of morbidity in military populations. A collaborative effort was established to attempt to isolate the causative agent(s) of 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 and shown to be infectious in volunteers in collaboration with Northwestern University. Aliquots of these materials are distributed to investigators attempting to isolate the causative agent, demonstrate hepatitis associated antibodies and identify a susceptible laboratory animal.

25. (U) 70 07 - 71 06 In three virologic laboratories, a parvovirus has been isolated from infective sera which measures 20 mu and whose growth is stimulated by adenovirus. However, no antibodies have been demonstrated in convalescent sera to this agent. Double blinded studies have convincingly demonstrated that marmosets develop hepatitis following injection of infectious sera. The dose and route of administration of infective sera were shown to be important in the causation of clinical hepatitis in humans. For technical reports see Walter Reed Army Institute of Research Annual Progress Reports, 1 Jul 70 - 30 Jun 71.

*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 68 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 310, Etiology of infectious hepatitis

Investigators.
Principal: COL Marcel E. Conrad, MC
Associate: MAJ Allen L. Ginsberg, 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.

Material obtained 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 from one of these patients caused icteric hepatitis in five of ten volunteers when administered orally in a dose of 0.1 ml. Convalescent sera from these patients failed to show a reaction of identity by agar gel immunoelectrophoresis or complement fixation methods against either the initial infective sera or against sera obtained from the recipients at the time they were acutely ill. It was postulated that this was caused by

either small quantities of antigen or antibody or both. An attempt was made to increase the antibody titers of these volunteers by re-exposing them to the initial infective material. They received 0.1 ml of the infectious sera subcutaneously. None of the volunteers who developed icteric hepatitis previously became ill from the re-exposure. However, two of four who failed to develop hepatitis previously developed icteric HAA negative hepatitis from the first subcutaneous exposure. This suggests that the dose of infective material and the route of administration are important in the causation of clinical illness. Presently, the convalescent sera of volunteers repeatedly exposed to HAA negative hepatitis has not shown serologic reactivity with acute infective materials. Plans are in progress to attempt to concentrate the acute infective material by ultracentrifugation and on cesium gradients before retesting with convalescent sera and various preparations of gamma globulin which are known to protect humans against HAA negative hepatitis.

Infectious sera has been supplied to eight virologic laboratories to attempt to cultivate the etiologic agent of infectious hepatitis. Using Detroit 6 tissue culture materials and methods, three laboratories have isolated a parvovirus which is similar but not antigenically identical to the H3 rat virus. The isolate has been shown by electron microscopy to be approximately 20 mμ in diameter and growth in tissue cultures can be increased one hundredfold by the addition of adenovirus to the media. Antibodies have not been identified in human convalescent sera which react serologically with this agent. Obviously this makes it highly possible that the parvovirus is a contaminent and not the causative agent of viral hepatitis. Presently, experiments are in progress to ascertain if tissue culture materials containing the parvovirus produce hepatitis in marmosets. If the animals develop no disease, they will be challenged with acute infective sera to show that they remain susceptible and have not been immunized with an attenuated virus.

Six coded groups of specimens have been forwarded to Presbyterian-St. Luke's Hospital for examination in marmosets. In each of these experiments, the marmosets have developed hepatitis when exposed to infectious sera but not to control sera. The results of these experiments were reviewed by an assigned subcommittee of the Liver Advisory Committee to USAMRDC who became convinced that the animal model was useful in detecting infectious materials. It was recommended that animal studies be utilized for testing of candidate agents and to detect various strains of HAA negative hepatitis.

Conclusions and Recommendations.

There is a continued requirement for the maintenance of pools of infectious material which are known to produce hepatitis in man. It is believed that this undertaking is the most reasonable approach to provide materials for use in laboratories attempting to cultivate the etiologic agent of hepatitis. There is a continued requirement to attempt to establish serologic methods to identify the causative agent of hepatitis; these studies are in progress.

Literature Cited.

Publications.

1. Boggs, J. D., Melnick, J. L., Conrad, M. D., Felsher, B. G., Caldwell, E., Burkhardt, M. S., and Cossitt, B. A.: Viral hepatitis. Clinical and tissue culture studies. J.A.M.A. 214:1041, 1970.

PROJECT 3A062110A821
COMBAT SURGERY

Task 00
Combat Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6466	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. OMSP'N INSTR'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	3A062110A821	00	120
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Provide 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. 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)
PREVIOUS FISCAL YEAR 71	3	180
CURRENT FISCAL YEAR 72	2.5	125

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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NAME: Rosenthal, MAJ A. Ralph DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Immune Mechanisms; (U) Rejection; (U) Skin Grafting; (U) Antilymphocyte Serum; (U) Enhancement; (U) Intraocular Foreign Body

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

23 (U) Better understanding of the immune mechanisms involved in the rejection of allografts and foreign bodies. The ultimate goals are enhancement of skin and bone grafts in combat casualties, clinical acceptance of renal transplantation in the Army Transplant Program, and the provision of better criteria for guidance in the management of retained intraocular foreign bodies.

24 (U) Utilizing the uptake of tritiated thymidine as an indication of DNA synthesis (lymphocyte reactivity) in one-way mixed lymphocyte cultures, a large variety of experiments are underway. Models include transplanted skin, kidney and heart, the latter two in the neck. An intracardiac balloon has been developed to measure ventricular contraction force as an index of rejection. A variety of antilymphocyte sera and enhancement antisera are being studied, as is the role of the granulocyte. Foreign body studies have been restricted to copper (bullet jacket) fragments retained in the rabbit's vitreous.

25 (U) 70 07 - 71 06 Thymus dependent versus bone marrow dependent lymphocyte reactivity has been delineated by specific antisera. These antisera have been shown to prolong skin graft survival while others have been specific antibody inhibitors. An IgG has been demonstrated to occur post-transplantation which inhibits MLC reaction and is a positive reflection of enhancement of organ acceptance by the host. This has led to a new in vitro method to determine the success of clinical renal transplantation at WRGH. Copper aqueous [illegible]vels and the location of the FB have shown to be of value in arriving at the decision to operate on retained vitreous foreign bodies. Both the transplantation and FB studies are performed in connection with active programs at the Walter Reed General Hospital. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

*Available to contractors upon authorization approval

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

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 120 Wound healing

Investigators.
Principal: COL Robert T. Cutting, M.C.
Associate: LTC Joshua Miller*, M.C.; LTC Brack G. Hattler*, M.C.; MAJ A. Ralph Rosenthal, M.C.; COL Budd Appleton, M.C.**

1. Transplantation -- Mechanisms of Enhancement, Cellular Immunity, and Allograft Rejection

a. Statement of the problem: (1) Facilitation of permanent acceptance of skin and bone graft in combat casualties and (2) elucidation of better guides to renal transplantation and follow up.

b. Background: The current research program is partially in direct support of the newly established Organ Transplantation Service, Walter Reed General Hospital. During the past year six successful transplants have been accomplished, each in patients on tri-weekly dialysis, at a saving of approximately $25,000 a year per patient.

c. Approach to the problem: Current studies involve (1) the studiy of enhancement (the specific suppression of immunity by humoral antibodies), (2) mechanisms of cellular immunity, and (3) humoral and cellular mechanisms involved in allograft rejections.

d. Results and discussion of the results: Utilizing the one-way mixed lymphocyte culture model and the uptake of tritiated thymidine as an index of reactivity, lymphocytes of the dog, human, and inbred mouse were studied. An immunoglobulin G isolated from dog serum was discovered to occur in the serum of recipients during unmodified allograft rejection; the IgG rises as early as 5 days after transplantation. Specific antilymphocyte sera have been prepared to include thymic-derived as well as bone marrow derived lymphocytes. Using a cardiac allograft rejection model immunologic alterations have been correlated with physiologic changes such as ventricular contraction, coronary blood flow, and with electron microscopy. The role of the granulocyte is being investigated utilizing a system of pure granulocyte preparations and anti-granulocyte sera.

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* Organ Transplantation Service, Walter Reed Army Medical Center
** Ophthalmology Service, Walter Reed Army Medical Center

e. Conclusion: Additional understanding of the factors involved in enhancement and rejection has been obtained.

f. Recommendation: Further studies along similar lines are necessary, including collaborative studies with the National Institutes of Health to include tumor enhancement and rejection as a model.

2. Studies on Intraocular Foreign Bodies

a. Statement of the problem: (1) To describe the early and late gross changes when a copper foreign body (CFB) is placed in various positions of the vitreous. (2) To demonstrate those factors associated with elevated copper levels in the aqueous as a guide to operative management. All bullet jackets are made of copper which is nonmagnetic and difficult to remove surgically.

b. Background: We have demonstrated the marked inflammatory response following implantation of either a pure or alloyed CFB against the wall of the vitreous cavity. We also showed that vitreous copper levels as determined by atomic absorption spectrophotometry were elevated 48 hours after the implantation whereas aqueous copper levels did not elevate until 60 - 90 days post-implantation.

c. Approach to the problem: Pure and alloyed (60% Cu, 40% Zn) CFBs were placed in various locations in the rabbits' vitreous and the response was regularly observed with the indirect ophthalmoscope. Aqueous taps were performed periodically. Correlation of aqueous copper levels, appearance, and the duration of the CFB was made.

d. Results and conclusions: The position of a CFB in the vitreous determined the response: When placed anteriorly behind the lens in the midvitreous, it could remain there indefinitely without eliciting an inflammatory response. The development of a blackened surface indicating corrosion was observed from 2 - 8 months with little or no vitreous reaction and migration to the surface of the retina immediately eliciting an inflammatory reaction.

If a CFB lay against or near the wall, a vitreous abscess with encapsulation usually occurred. If the FB was pure copper, encapsulation was complete by 4 - 6 days. Breakdown of the abscess might occur with release of the foreign body into the vitreous cavity between 3 and 9 months after encapsulation. The likelihood of this occurring was greater with the alloy (73%) than with the pure CFB (10%).

Aqueous copper levels in the normal rabbit were determined in 100 rabbits' eyes in which no operative procedures had been performed and in 109 eyes in which a pure iron foreign body had been placed in the vitreous.

Analysis of the aqueous of eyes containing a foreign body in the vitreous revealed that only 36% of such eyes yielded elevated copper levels. Factors associated with higher levels were duration and corrosion on the surface.

e. Conclusions: The most important fact determined from these experiments was that pure copper can remain suspended in the mid-vitreous without eliciting an inflammatory response indicating a need for caution in the clinical management of cases since the danger of removal might be greater than the damage done by the foreign body.

f. Recommendations: (1) The efficacy of corticosteroids in the treatment of the inflammatory response to an intravitreal CFB should be studied. (2) The usefulness of penicillamine therapy in the lowering of elevated aqueous copper levels in eyes with an intravitreal CFB should be evaluated. (3) Determine if the alloy composition affects the reactivity of the foreign body.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 120 Wound healing

Literature Cited.

Reference.

Rosenthal, A.R., Appleton, B., Hopkins, J.L.: Studies on intraocular copper foreign bodies. (In preparation)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6467	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	3A062110A821	00	121
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Provide with Security Classification Code)

(U) Responses to trauma (0°)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008800 Life Support 012200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 09	CONT	DA	C In-House

17. CONTRACT GRANT 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 71	5	125
FISCAL YEAR CURRENT 72	3	75

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Fischer, LTC R. P. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide each with Security Classification Code)

(U) Stress Ulcer; (U) Restrain Model; (U) Shock; (U) Ulcerogenesis; (U) Vagotomy

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

23 (U) Elucidate the pathophysiology of stress ulcer, a delayed complication which causes massive gastrointestinal bleeding in 3% of severely wounded soldiers and which has a mortality of 40% regardless of mode of therapy.

24 (U) The search for a better animal model continues. The restrained rat model has given reproducible results. A variety of larger quadrupeds and primates in whom shock has been induced by hemorrhage or endotoxin have also been investigated.

25 (U) 70 07 - 71 06 Sophisticated uni- and bidirectional flux studies have successfully characterized the changing patterns of electrolyte movement across the gastric mucosal barrier in large animals with vagally denervated, internally drained, gastric pouches. Striking drops in gastric potential differences have been observed following shock. Electron microscopy and the use of nonpermeable molecules have permitted further conclusions as to the anatomic and physiologic changes occurring in these models. Vagotomy failed to protect the restrained rat from ulcers induced by hemorrhage; however common bile duct ligation enhances ulcerogenesis which is prevented by vagotomy, supporting the hypothesis that multiple mechanisms are involved. Further studies involve urea and the inhibition of oxidative phosphorylation.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

*Available to contractors upon originator's approval

DD FORM 1498 MAR 68

Project 3A062110A821 COMBAT SURGERY

Task 00, Combat Surgery

Work Unit 121, Responses to trauma

Investigators.
Principal: COL Robert T. Cutting, MC
Associate: MAJ Wallace P. Ritchie, Jr., MC; LTC Ronald P. Fischer, MC; MAJ John C. Bowen, MC; MAJ John F. Mullane, MC; CPT Arthur I. Kobrine, MC; CPT Neven A. Popovic, VC

1. Effect of Endotoxemia on the Gastric Mucosal Barrier

a. Statement of problem: Hemorrhage from stress ulcers occurs in 3% of moderately and seriously wounded soldiers. Mortality approximates 50% regardless of therapy. The incidence of non-bleeding stress ulcers is unknown but may be tenfold higher.

b. Background: Although the etiology of post-traumatic stress ulcer is unclear, a variety of mechanisms have been proposed including hypotension, jaundice, sepsis, and hypoxia. Current interest affirms that, whatever the predisposing factors, the end result in an organ which elaborates a highly caustic acid and powerful proteolytic enzyme is an inability to maintain a high intraluminal concentration of hydrogen ion.[1]

c. Approach to the problem: The lipopolysaccharide of the cell membrane of E. coli contains an endotoxin which, in dogs, produces many of the characteristics of septic shock in man.[2] This study assessed the effects of an LD_{80} dose of endotoxin on the ability of the gastric mucosa to maintain (1) a normal electrical gradient (PD) across the mucosa and (2) a normal barrier to ionic diffusion (including HCl).

d. Results: During the initial bout of hypotension, the PD fell to 50% of control levels but returned rapidly to normal as the systemic arterial blood pressure rose. Thereafter no change was noted for at least 3 hours, even though systemic pressure gradually fell to shock levels. Pretreatment with volume expanders and alpha adrenergic blocking agents vitiated the initial systemic arterial response but did not alter the fall in PD. Associated changes are noted below:

Flux Rates ± SEM/100 cm^2 mucosal area/15 minutes
((-) = Stomach loss; (+) = Stomach gain)

	Net H_2O	Net Na^+	Na Out_1	Na In_2	Net K^+	Net Cl^-	Net H^+
Control	+1.4±0.2	+192±24	11±7	211±30	+8±3	+164±27	-63±16
Endotoxin	+1.1±0.2	+148±12	21±6	175±17	+20±2	+104±20	-106±7

1. Lumen to blood
2. Blood to lumen

e. Conclusion: These results indicate that mucosal integrity is interrupted during the initial hypotensive phase of endotoxin shock and that this disruption is probably not a function of systemic arterial hypotension.

f. Recommendations: Assess the long-term effect of endotoxin shock; compare the effect of endotoxin and live E. coli in both dog and primate.

2. Effect of 2,4 Dinitrophenol on Gastric Mucosal Integrity

a. Statement of problem: Hemorrhage from stress ulcers occurs in 3% of moderately and seriously wounded soldiers. Mortality approximates 50% regardless of therapy. The incidence of non-bleeding stress ulcers is unknown but may be tenfold higher.

b. Background: Disruption of the gastric mucosal barrier has been postulated to be the fundamental defect preceding the development of stress ulcer disease in man. The molecular events associated with such disruption are not understood. Insight into these events might enhance our understanding of the disease.

c. Approach to the problem: A classical method for studying physiologic function is to attempt to inhibit it with anti-metabolites. In this study, the effect on gastric mucosal integrity of perfusing the mucosa with 2,4 dinitrophenol was assessed by measuring the net fluxes of H_2O, H^+, Na^+, K^+, and Cl^- and the bidirectional fluxes of Na^+. In addition, the transmucosal potential difference (PD) was monitored. 2,4 dinitrophenol prevents the reduction of NADH to NAD and therefore the incorporation of $PO_4^=$ into ADP, essentially disrupting oxidative phosphorylation and depriving tissues of energy derived from pre-formed high energy $PO_4^=$ bonds. The results were compared to data obtained when the gastric mucosa was perfused with 4 M urea, a substance which disrupts the mucosal barrier, resulting in enhanced "back diffusion" of H^+, and increased influx of H_2O, Na^+, and K^+.

d. Results:

PD & Flux Rate (±SEM)/30 minutes/100 cm^2 Mucosa
((-) = Pouch loss; (+) = Pouch gain)

	PD	Net H^+	Na Out	Na In	Net K^+	Net Cl^-	Net H_2O
Control	-44±7	-156±29	39±15	292±11	+15±1	+184±15	+1.6±0.4
Urea	-6±3	-402±20	141±15	756±46	+30±3	+133±12	+3.3±0.5
DNP	-12±4	-394±45	148±32	663±115	+22±4	+137±17	+2.3±1.6

e. Conclusion: These results suggest that classical disruption of the gastric mucosal barrier results from an inability of the affected tissue to carry on oxidative phosphorylation.

3. Effect of Graded Hemorrhage on Gastric Mucosal Permeability

a. Statement of problem: Hemorrhage from stress ulcers occurs in 3% of moderately and seriously wounded soldiers. Mortality approximates 50% regardless of therapy. The incidence of non-bleeding stress ulcers is unknown but may be tenfold higher.

b. Background: Clinical data on severely wounded combat casualties suggests that hemorrhagic shock, in some way, is related to the development of "stress ulcers" in these patients.[3] Recent experimental work has shown that the gastric mucosal barrier undergoes permeability changes after hemorrhagic shock in rabbits and that similar changes are frequent in patients prone to develop "stress ulcers."[1] Altered permeability results in increased "back diffusion" of hydrogen ion similar in magnitude to that observed when urea, aspirin and bile are applied topically to the gastric mucosa.[4] These studies form the basis of a theory of the pathogenesis of "stress ulcers" which states that the permeability change which permits enhanced "back diffusion" of hydrogen ion initiates mucosal changes which result in "stress ulcers."

c. Approach to the problem: This study was designed to determine the effect of hemorrhagic shock on gastric permeability in the dog, selected because it has been the experimental animal used in most experimental studies of gastric mucosal permeability and because the canine physiologic response to hemorrhagic shock has been well delineated. Mucosal integrity was determined by measuring the net flux rates of water, H^+, K^+, Na^+, and Cl^- and the bidirectional flux rates of Na^+ and by determining the transgastric electric potential difference (PD). Control studies were performed in dogs who had been previously prepared with an internally drained fundic pouch and then

repeated 2 hours after hemorrhagic shock. An additional group of dogs underwent control studies and then 2 hours later were subjected to the topical application of 2 M urea. The urea study allows for the comparison of the results of hemorrhagic shock with urea which produces classical disruption of the gastric mucosal barrier.

d. Results:

PD & Flux Rates (+SEM)/30 minutes/100 cm^2 Mucosa[1]
((-) = Pouch loss; (+) = Pouch gain)

Group	PD (mv)	Net H_2O (ml)	Net H^+ (μEq)	Na Out (μEq)	Na In (μEq)	Net Cl^- (μEq)
Control	-48±4	+1.3±0.2	-138±27	46±8	334±60	+06±47
Shock (10 dogs)	-17±7[2]	+0.9±0.5	-241±39[2]	158±34[2]	237±27[2]	+91±17[2]
Control	-44±6	+1.4±0.2	-151±22	69±13	330±22	+226±56
Urea (7 dogs)	-6±2[2]	+3.0±0.6[2]	-311±39[2]	91±23	680±115[2]	+211±59

1. Standard test solution (80 mM/L HCl, 80 mM/L NaCl, 4-5 μC/L ^{24}Na)
2. P = to or < 0.05 vs control

e. Conclusions: These results suggest that during early hemorrhagic shock mucosal permeability is not increased, as is the case with urea. Rather, as a consequence of reduced net Cl^- flux, the electrical gradient is altered (↓PD), permitting intraluminal cations to "diffuse" out more rapidly (e.g., net H^+, Na^+ Out) and extraluminal cations to "diffuse" in less rapidly (e.g., Na^+ In), in accordance with the Ussing equation. If enhanced absorption of H^+ by the gastric mucosa is important in the etiology of "stress" ulcer, then measurement of the PD may be a useful way of assessing this modality clinically.

f. Recommendation: Clinical studies are in order to determine the validity of our assumption that PD measurements may provide a useful assessment of gastric mucosal integrity in patients at great risk to develop "stress ulcers."

4. Studies on the Etiology and Treatment of Stress Ulcers: Effects of Hemorrhagic Shock, Transfusion and Vagotomy

a. Statement of problem: Hemorrhage from stress ulcers occurs in 3% of moderately and seriously wounded soldiers. Mortality approximates 50% regardless of therapy. The incidence of non-bleeding stress ulcers is unknown but may be tenfold higher.

b. Background: Restraint-induced ulcers in the rat resemble post-traumatic "stress ulcers" in man in that they are acute and multiple, occur mainly in the stomach, are confined to the oxyntic cell area, and are not associated with gastric acid hypersecretion.[5,6] In combat casualties, hemorrhagic shock frequently precedes the development of "stress ulcers." It was determined, therefore, to study whether or not non-lethal hemorrhagic shock in the rat affected the frequency of restraint-induced gastric ulcers.

c. Approach to the problem: Once it had been determined that the incidence of gastric lesions increased in the rat restraint model following hemorrhagic shock, a study was performed to see if vagotomy offered protection under these circumstances. In addition a study was performed to see what effect transfusion with non-Hbg containing volume (saline + albumin) would have on the same model.

d. Results:

Treatment	No. Animals	Lesions (± SEM) Animals	Ulcers (± SEM) Animals	Erosions (± SEM) Animals
Restraint Alone	15	4.3±1.0	0.3±0.2	4.1±1.0
Restraint + Hemorrhage	15	12.8±2.0	1.8±0.4	11.0±1.9
Restraint + Hemorrhage + Transfusion	12	4.7±1.2	0.4±0.3	4.3±1.1
Restraint + Hemorrhage + Vagotomy[1]	14	13.2±1.6	3.4±0.6	9.7±1.5

1. Chronic truncal vagotomy + pyloroplasty

e. Conclusions: These results indicate that sublethal hemorrhage markedly enhances ulcerogenesis due to restraint ($p < .05$), and that this enhancement can be prevented by transfusion. Truncal vagotomy affords no such protection.

5. Electron Microscopy Studies of Gastric Permeability

a. Statement of problem: Hemorrhage from stress ulcers occurs in 3% of moderately and seriously wounded soldiers. Mortality approximates 50% regardless of therapy. The incidence of non-bleeding stress ulcers is unknown but may be tenfold higher.

b. Background: It has been shown experimentally that the topical application of urea disrupts the integrity of the gastric mucosa,[4] resulting in increased mucosal permeability, enhanced "back diffusion" of H^+, and hemorrhage. Similar ionic flux changes have been reported in critically ill patients prone to develop "stress ulcers."[1] The manner in which the permeability of the mucosal membrane or the mucosal cell, per se, is altered is unknown. One explanation is that the tight junctions between cells are destroyed. This study was designed to assess this problem directly by electron microscopy performed following urea instillation and after hemorrhagic and endotoxin shock in dogs.

c. Approach to the problem: Intraluminal lanthanum nitrate is not normally diffusible into gastric cells nor will it pass through the gastric tight junctions into the intracellular border spaces.[7] Since lanthanum nitrate is demonstrable by electron microscopy, changes should be demonstrable in either the permeability of the gastric mucosal cells or in the tight junction between cells following urea application, hemorrhagic shock or endotoxin shock.

d. Results: This project is only partially completed. Electron microscopic studies of the stomach before and after urea application have been performed, and confirm the fact that, under resting conditions, lanthanum nitrate does not traverse the tight junctions nor does it appear within mucosal cells. However, attempts to perform lanthanum nitrate studies following urea instillation have not been successful because urea causes a profuse outpouring of mucus in which the lanthanum precipitates. It is anticipated that this problem can be overcome by using mucosal strips in an *in vitro* perfusion chamber. Lanthanum nitrate will be applied to the serosal rather than to the mucosal surface of the membrane. In the presence of competent tight junctions, lanthanum nitrate will be blocked after diffusing to these structures from this direction. The *in vitro* perfusion chamber has been constructed but neither these experiments nor the studies after hemorrhagic or endotoxin shock have as yet been performed.

Study is continuing.

6. Increased Phenolsulfonphthalein Absorption Following Disruption of the Gastric Mucosal Barrier by Urea

a. Statement of problem: Hemorrhage from stress ulcers occurs in 3% of moderately and seriously wounded soldiers. Mortality approximates 50% regardless of therapy. The incidence of non-bleeding stress ulcers is unknown but may be tenfold higher.

b. Background: It has been postulated, but not proven, that disruption of the gastric mucosal barrier is the result of destruction of the tight junction between surface epithelial cells, thereby

increasing the effective pore area and resulting in increased mucosal permeability. Increased absorption of hydrochloric acid by the gastric mucosa has been implicated in the etiology of erosive gastritis and benign gastric ulcer. The gastric mucosal barrier which normally limits the "back diffusion" of H^+ can be altered by the topical application of urea.

c. Approach to the problem: Proof that disruption of the gastric mucosal barrier is the result of destruction of the tight junctions between surface epithelial cells requires as a first step the demonstration of absorption by the gastric mucosa of a lipid insoluble substance with a large molecular radius to which the stomach is not normally permeable. Accordingly, in the present study, the absorption of phenolsulfonphthalein (PSP; MW 354; mol. radius = 6A°) from canine Heidenhain pouches before and after the topical application of 4M urea was determined. In addition, the associated changes in the net fluxes of H_2O, H^+, K^+, and Cl^- and the net and bidirectional fluxes of Na^+ were assayed to document disruption of the mucosal barrier.

d. Results: PSP absorption before urea instillation averaged 0.13 ± 0.4 uM of PSP/30 minutes/100 cm^2 mucosal area. Following urea an average of 0.63 ± 0.06 uM/30 minutes/100 cm^2 mucosal area was absorbed. Associated ionic and water flux rates demonstrated significantly increased "back diffusion" of H^+ and influx of Na^+, K^+, and H_2O.

e. Conclusion: The observation of over a 4 fold increase in PSP absorption after urea instillation indicates that mucosal permeability is increased and suggests that increased effective pore area may be responsible.

f. Recommendation: PSP absolutely must not be used as a volume marker in studies in which the integrity of the gastric mucosal barrier is being assessed.

7. The Use of Dexamethasone in Experimental Closed Head Injury

a. Statement of problem: Effective treatment of severe closed head injuries continues to be a major problem among civilian and military neurosurgeons alike. Brain swelling, secondary pulmonary changes, and altered cerebral blood flow are some of the associated problems which need further intensive study.

b. Background: To study these problems accurately, a pathologically similar and reproducible experimental model of severe closed head injury has been needed With the development of this model different therapeutic regimes as well as the pathophysiology of head injury itself can be studied.

c. Approach to the problem: During the past year we have developed such a model and successfully used it to study the efficiency of dexamethasone in the treatment of closed head injury. Our model consists of an impact sled, constructed at WRAIR, and propelled by a weight and pulley arrangement, with varying resultant accelerations and velocity at impact up to 25 ft/sec. This type of model was selected because it has been shown that severe head injury most readily occurs when the head is moving at the time of impact, and the sudden deceleration results in a differential of velocities of skull and brain, producing shearing forces within the brain.

d. Results: Following a series of pilot studies we found that at an impact velocity of 18 feet/sec, 2.0 - 2.9 Kg Rhesus monkeys would sustain a head injury thereby leading to marked brain swelling and death in 6-18 hours. The marked brain edema was nicely demonstrated by staining with Evans Blue, a vital dye injected intravenously 10 minutes post-impact. This model was then used to study the effects of dexamethasone on traumatic brain swelling, 1 mg. intravenously 1 hour post-impact and every 6 hours thereafter. All treated animals were alive at 72 hours post-impact. They were sacrificed at this time and the brain exhibited a significantly less amount of cerebral edema, as evidenced by microscopic examination as well as by Evans Blue staining.

e. Conclusion: Dexamethasone does have a beneficial effect in the treatment of severe closed head injury by its ability to prevent the magnitude of swelling.

f. Recommendation: This experimental model will be used to further study the pathophysiology of head injury and more precisely define the limits of steroid therapy.

8. Effect of In Vivo Hepatic Exclusion on Renal Function

a. Statement of problem: Normal liver function was the most frequent complication after sepsis in the wounded patient with post-traumatic renal insufficiency.

b. Background: The interaction of renal and hepatic mechanisms is poorly understood. The study was undertaken to elucidate the effects of hepatic inflow occlusion on renal function during a saline load. A saline load was used in the study to determine if the liver has a direct influence on renal sodium excretion.

c. Approach to the problem: The project was undertaken in 8 sham and 8 experimental dogs with side-side superior mesenteric-caval shunts. After a 1200 ml saline load, the 8 sham and 8 experimental dogs had a 50 minute control renal clearance collection. Next, the hepatic artery and portal vein were clamped in the experimental group and manipulated in the sham group. Twenty minutes later, clearance

studies were performed for 80 minutes. At the end of this time, the dogs had received 2800 cc of saline.

d. Results: During the control period, the results were similar for the sham and experimental groups. During the study period, the sham dogs had a significant increase in urine flow rate and sodium excretion, while the experimental dogs had no change in urine flow rate and a statistically significant decrease in sodium excretion. When the change in filtered sodium was correlated with the change in urinary sodium excretion from the control to study periods, there was no correlation in the sham group but a significant direct correlation between them in the experimental group. When the difference in response was analyzed in the sham and experimental dogs, there was a significant difference for urine flow rate and for total and fractional sodium excretion.

e. Conclusions: The increased sodium excretion in the sham dogs during the study period was related to changes in tubular reabsorption since there was no correlation with filtered sodium. However, the changes in sodium excretion in the dogs with hepatic inflow occlusion were directly related to the filtered load of sodium. The present experiment has not given conclusive evidence regarding the role of the liver in renal sodium excretion since two interpretations of the results can be offered. A natriuretic factor may have been operational in the sham dogs which was removed by hepatic inflow occlusion, thus enabling filtered sodium excretion. On the other hand, hepatic inflow occlusion may have had a direct but varying effect on both the intrarenal circulation and the filtered load of sodium which was superimposed on a natriuretic factor which was still present. Since natriuresis, though diminished in 6 of the 8 sham dogs, continued after hepatic inflow occlusion, additional factors regulating natriuresis exist which operate independent of the liver.

f. Recommendation: Further study of the relation of the liver to renal function should include (1) distribution of body sodium in the absence of any ascites, (2) the effect of liver damage on denervated kidneys, and (3) the operation of neurohumoral agents and blocking agents on the kidneys of dogs with experimental liver damage.

9. Effect of Liver Damage on Organ Water and Electrolytic Content and Oxygen Consumption

a. Statement of problem: Jaundice and liver damage are frequent accompaniments of sepsis following trauma in the wounded soldier.

b. Background: The protocol was undertaken to determine the effects of liver damage on the distribution of sodium and water in the body and to see if there was any metabolic depression of organs prior to ascites formation.

c. Approach to the problem: Non-ascitic rats with common bile duct division or sham surgery were studied.

d. Results: Increases in organ sodium and water were found in the liver, skin, stomach and jejunum following liver damage produced by common bile duct division. The portal pressure was moderately elevated at this time and total bilirubin averaged 10.0 mg%. In vitro tissue respiration was depressed for only liver and stomach.

e. Conclusions: If the changes in skin can be attributed to loss of fat, the remaining observations can be explained by congestion of the liver and portal system following the common bile duct ligation. The renal concentrating mechanism was also studied in the rats and found to be impaired.

10. Effect of Liver Damage on Respiratory Function

a. Statement of problem: Jaundice and liver damage have been recognized as an extremely frequent accompaniment of pulmonary insufficiency with sepsis in the wounded soldier. It has previously been recognized that the human with chronic liver disease has multiple abnormalities of respiration.

b. Background: The protocol was undertaken to see if respiratory abnormalities were associated with liver damage produced by common bile duct division in the rat.

c. Approach to the problem: Non-ascitic rats with common bile duct division or sham surgery were used in the study.

d. Results: Liver damage was associated with increased portal vein pressure and splenomegaly, hyperventilation respiratory alkalosis, increased arterial ammonia, decreased arterial and venous partial pressure oxygen, decreased oxygen consumption with normal arterial-venous oxygen content difference, decreased arterial partial pressure of oxygen on 100% oxygen, lacticacidemia, decreased stomach luminal pO_2 and a macrocytic anemia that was corrected by folic acid and unaffected by splenectomy. Liver damage had no effect on arterial percent saturation oxyhemoglobin, arterial pO_2 on 12% oxygen, in vitro lung compliance, and jugular or femoral vein ammonia. The lung had thickening of the alvolear septal walls.

e. Conclusions: Arterial hypoxia appears related to intra-pulmonary shunting of blood. Hyperventilation may be due to arterial ammonia and hypoxia.

11. Effect of Liver Damage, Nephrectomy and Metabolic Acidosis on Stress Ulcers Induced by Restraint

a. Statement of problem: Liver damage and jaundice as well as septic acute renal insufficiency are all associated with a high incidence of stress ulcers in the wounded soldier.

b. Background: The protocol was undertaken to determine if the incidence of restraint-induced gastric ulcers could be influenced by liver damage or by acute renal failure or metabolic acidosis.

c. Approach to the problem: Rats underwent either sham operation or nephrectomy, common bile duct division or ammonium chloride administration.

d. Results: Compared to sham operated rats, the rats with nephrectomy when studied at 12, 24, 30 and 48 hours post-nephrectomy had no increase in the incidence of gastric lesions. This occurred despite the presence of a metabolic acidosis and a marked increase in gastric acid production in the nephrectomized rats. When metabolic acidosis was produced by ammonium chloride administration for five days, there was a marked increased incidence of gastric erosions but not ulcers. Nephrectomy had no influence on gastric luminal pO_2 or arterial pO_2. Nephrectomized rats were studied in restraint with blood urea nitrogen between 70 and 220 mg% and plasma potassium of 6.0 to 11.0 mEq/L. Rats with common bile duct division demonstrated a sharp increase in gastric erosions and ulcers. Stomach in vitro respiration was depressed with succinate solution, and the gastric luminal pO_2 was depressed and tissue water was increased.

e. Conclusions: Acute renal failure above appears to have no influence on stress ulcer production in the rat. However, since there was a near significant increase in gastric erosions with nephrectomy, it is possible that nephrectomy might be potentiated by other variables following trauma and in this manner contribute to stress ulcer formation. However, liver damage appears to contribute to the development of stress ulcers in this model. This appears related to congestion of the stomach and impaired oxygen utilization.

12. Repair of Anterior Cruciate Ligament

a. Statement of problem: Rupture of the anterior cruciate ligament in the dog is the most common clinical condition of the stifle joint. It is a common problem of sentry and scout dogs. Repair has been largely unsuccessful in spite of a variety of surgical procedures which have been tried which work well in the human.

b. Background: The ligament may rupture due to trauma or unusual stress but often is the product of degenerative changes in the joint. Except for the size of the ligament and the intra-articular position of the tendon of the extensor digitorum longus muscle, the anatomy of the stifle joint of the dog and the knee of the man are essentially the same. The cruciate ligaments are critically important to the stability of the canine stifle joint. Due to normal angulation, the stifle joint in the dog, even in standing position, requires the support of the cruciate ligaments to prevent anterior-posterior movements of the demur on the tibia. This support is even more crucial upon motion of the joint. The situation is somewhat different in man as the normal angle of the knee is 170 degrees in standing position, and quadriceps muscles, if strengthened by exercises, may give sufficient support to the involved joint. Thus in some cases surgical correction may not be necessary.

c. Approach to the problem: There are several ways of repairing the cruciate ligament. All of the methods reviewed claimed some rate of success, but none of the techniques have been experimentally evaluated and statistically compared. Confusion remains as to the advisability of using any procedures. The purpose of our project was to select five of the most promising surgical techniques and compare them under controlled experimental conditions and statistically determine the most successful procedure.

Experimental subjects are 50 adult beagles. The anterior cruciate ligament is surgically severed in either hind leg and the dogs are separated into five experimental groups. In 7-10 days the cut ligament is repaired using one of the five selected procedures. All dogs are evaluated physically and radiographically during the three months following surgery.

Study is continuing.

Project 3A062110A821 COMBAT SURGERY

Task 00, Combat Surgery

Work Unit 121, Responses to trauma

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6468	71 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
70 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	3A062110A821	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CDC(G 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)
PRECEDING FISCAL YEAR 71	3	25
CURRENT FISCAL YEAR 72	4	100

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede Each with Security Classification Code) (U) Oxygen Toxicity; (U) Tissue Oxygenation; (U) Pulmonary Insufficiency; (U) Intravascular Microaggregation; (U) Stored Blood

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

23 (U) To examine in depth several factors that contribute to or result from defective tissue oxygenation and blood supply following combat injury and shock, such as (a) pulmonary insufficiency and oxygen toxicity, (b) alterations in the oxygen transport characteristics of stored blood, (c) the microaggregates of leukocytes and platelets that develop in stored blood and in vivo following shock and trauma, and (d) the alteration in energy metabolism of the gastric mucosa.

24 (U) Experimental models have been developed to study: (a) pulmonary oxygen toxicity with a double respirator that allows exposure of one lung to pure oxygen and the other to room air; (b) tissue oxygen transport in the isolated hind limb with a membrane oxygenator; (c) intravascular microaggregation with a particle counter which measures the size distribution of particles in blood during storage, shock, hypoxia, cardiopulmonary bypass and related conditions; (d) gastric mucosal aerobic and anerobic metabolism following shock and stress.

25 (U) 70 07 - 71 06 Oxygen has been shown to have a direct toxic effect on the lung not related to its systemic actions. The development of the aggregates in stored blood has been studied and an evaluation of various blood filters has found that Dacron wool is the most efficient filter. Studies in dogs and rats have shown that gastric mucosal oxygen tension falls during shock and lactate production increases. Studies have been initiated to relate pulmonary insufficiency following massive transfusion to the amount of particulate debris infused into baboons and to compare the oxygen transport characteristics of stored and fresh blood in the isolated hind limb of the dog. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

(Available to contractors upon originator's approval)

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 Jack A. Zeller, MC*; MAJ Phillip M. Levin, MC;
MAJ Robert T. Solis, MC; COL Robert T. Cutting, MC

1. The Effect of High Inspired Oxygen Tension and Its Relationship to Oxygen Toxicity.

a. Statement of the problem: High inspired oxygen tension produces pulmonary oxygen toxicity.

b. Background: The consequences of continuous exposure to oxygen at 1 atmosphere include decreases in oxygen uptake, infection, atelectasis, and destruction of pulmonary parenchyma.[1,2] The role of the respirator in the pathogenesis of the pulmonary lesion has been difficult to evaluate.[3]

c. Approach to the problem: Using 5 control and 10 experimental dogs 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, and pH and oxygen consumption determinations were made every six hours. These animals were then maintained on Acepromazine 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 lung. The oxygen-treated lungs were heavy and boggy and microscopically showed interstitial edema, the presence of hyaline membranes, destruction of capillary endothelium and alveolar epithelial cells. There were no lesions on the air-ventilated side.

e. Conclusion: Pulmonary oxygen toxicity is a local phenomenon caused by the direct effect of O_2 upon the alveoli and is independent of the level of hyperoxemia.

f. Recommendation: Patients should be given oxygen sufficient to maintain physiologic levels only of arterial oxygen tension. Mechanisms of oxygen toxicity await biochemical and enzymatic study.

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*Division of Experimental Pathology, WRAIR

2. Relationship of Oxyhemoglobin Dissociation Curve and Oxygen Uptake

a. Statement of the problem: The position of the oxyhemoglobin dissociation curve is to a great extent controlled by the interaction of organic phosphates (2,3 DPG) within the red cell, with hemoglobin. A shift to the left of the oxyhemoglobin dissociation curve is usually accompanied by a fall in 2,3 DPG level, thus increasing hemoglobin-oxygen affinity and theoretically decreasing the efficiency of oxygen delivery to the tissues. Storage of RBC for greater than one week has been shown to cause a decrease of red cell 2,3 DPG and an increased affinity of hemoglobin for oxygen.

b. Approach to the problem: Under anesthesia 500 cc of blood were withdrawn from each of 10 dogs and stored in ACD solution under normal blood banking conditions. On the 21st day each animal was anesthetized and another 500 cc of blood were withdrawn. The circulation of one hind limb was isolated from the rest of the animal and the iliac artery and vein were cannulated and ligated. The extremity was perfused alternately with 21-day old banked blood and with the fresh blood by means of a roller pump with an in-line membrane oxygenator.

c. Results and Discussion of the Results: Banked dog's blood 21 days old has a P_{50} value of 17-18 mm Hg while fresh ACD blood has a P_{50} of 24 mm Hg. Fresh heparinized blood has a P_{50} of 29 mm Hg. There is an increase in oxygen uptake noted in 2 of 3 perfusion studies carried out thus far.

The study is continuing.

3. Microaggregation in Whole Blood

a. Statement of the problem: The pulmonary insufficiency after trauma has been attributed to embolization of the microaggregates in stored blood.

b. Background: Previous methods of quantitating these microaggregates consisted of measuring the screen filtration pressure of blood or weighing the debris retained by the standard transfusion filter. However, in order to study the effects of these microaggregates, a new method of measuring them had to be developed.

c. Approach to the problem: The size distribution of the microaggregates in blood has been measured by utilizing the Model T Coulter Counter, previously used in industry but not in medical investigation. It counts the number of particles at 15 different volume sizes (from 8 to 164 u in equivalent spherical diameter) simultaneously.

d. Results: Initial studies quantitated the rate of development of the microaggregates in blood stored in ACD under standard blood bank conditions. An evaluation of various blood filters was then performed which demonstrated the ineffectiveness of the standard Fenwal transfusion

filter (170 μ pore) as compared to a small pore (40 μ) or Dacron wool filter. Subsequently the effects of centrifugation, anticoagulants, temperature and other variables on the physical characteristics of these microaggregates have been studied.

e. Conclusion: A system that quantitates the size distribution of the microaggregates in blood has been developed which can be further used to study their physiologic effects.

f. Recommendation: A more effective clinical blood transfusion filter must be developed. Assessment of new filters should include a physiological correlate system as well as current methods described above.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 122, Anesthesia and pulmonary complications of combat surgery

Literature Cited.

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Publication

Yhap, E. O., Zeller, J. A., Levin, P. M., Solis, R. T.: The Effects of alveolar hyperoxia and mechanical ventilation in the development of pulmonary oxygen toxicity. Clin. Res. XIX, No. 2: 523, Apr. 1971.

PROJECT 3A062110A822
MILITARY INTERNAL MEDICINE

Task 00
Military Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6469	71 07 01	DD-DR&E-AR(6)6

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
70 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. [redacted]	CDOG 1412(2)			

11. TITLE (Precede 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 71	20	365
FISCAL YEAR CURRENT 72	20	365

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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NAME: Wartofsky, MAJ, L. DA
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PII Redacted

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) - 70 07 - 71 06. Investigation of alterations in metabolic-hormone-energy-fuel relationship induced by trauma, immobilization, infection, and drugs were conducted. These special studies have included use of radioimmunoassay techniques, whole body radioactive counting, metabolic balance studies, and a dual isotope procedure for assessing rates of degradation and release of thyroid hormone. Studies evaluating osteoporosis and hypercalcemia which result in renal stones and prolonged convalescence in patients immobilized with war wounds and fractures have revealed that serum ionized calcium is frequently high while total serum calcium is usually normal. Alkali and thiazide reduce the hypercalciuria but not the ionized calcium. Ionized calcium has been an excellent diagnostic tool for hyperparathyroidism. Dual isotope studies of thyroid function during malaria infection reveal decreased thyroxine release. Evidence from in vitro studies indicates that no significant de nova purine biosynthesis occurs in malaria parasites and that preformed purines are utilized via salvage pathways. The steroid laboratory is providing support in a study to evaluate the effect of antimalarial drugs on endocrine function. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 June 71.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE AND 1498-1 1 MAR 68 FOR ARMY USE ARE OBSOLETE

Project 3A062110AA22 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Investigators.
Principal: LTC Jerry M. Earll
Associates: Marcus Schaaf, M.D.; MAJ Leonard Wartofsky, MC; MAJ William J. Howard, MC; MAJ Richard C. Dimond, MC; Joseph Bruton, Ph.D.

Description.

This work unit is concerned with investigations into basic mechanisms of disease 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, four endocrine fellows, diagnosis and treatment of endocrine patients, and technical laboratory support to other departments. The unit maintains the capability of mounting field studies.

Progress

1. Calcium Metabolism.

Immobilization and bed rest resulting from any injury or illness are characterized by negative nitrogen and calcium balance. Many war injuries result in prolonged inactivity and the longer the period of immobilization, the greater the increase of complications and slow rehabilitation. Hypercalciuria and negative calcium balance are almost a universal phenomenon in young men at bed rest.

Studies of the endocrine metabolic changes occurring in 10 immobilized orthopedic patients and 5 normal males ages 20-24 year. Negative calcium balance will be a major concern during prolonged space travel. Abnormal elevations of serum ioned calcium [Ca^{++}] (Orion electrode) were found in 24 of 38 determinations in the orthopedic patients (1.23 - 1.60 mM/l, mean 1.34) compared to values in 397 normal subjects (1.22 $\pm$ 0.10 mM/l = mean $\pm$ 2 S.D.). Corresponding total serum calcium values (TCa) were normal in 25 of 38

determinations (<10.2 mg/100 ml). Normal males were studied on a metabolic ward during 8 days of normal ambulation (NA), 12 days of bed rest (BR), and 4 days of reambulation (RA). Daily measurements of [Ca^{++}], TCa, and urinary calcium (UCa) were made. By the sixth day of BR [Ca^{++}] rose to abnormal levels (1.40 - 1.48 mM/1) and remained elevated until RA. Only 4 of 59 TCa values were abnormal during BR (10.3 - 10.5 mg/100 ml). UCa increased the first day of BR and progressively increased until RA. Alkali treatment of 2 additional normal subjects with 20 to 25 Gms equimolar sodium and potassium bicarbonate/day prevented the increase in UCa but did not alter the increase in [Ca^{++}]. Urinary Cyclic 3'5' AMP (cAMP) fell slightly during BR, and serum parathormone (PTH) was normal. In view of [Ca^{++}] values in range seen with hyperparathyroidism, it is unlikely that PTH is responsible, however, its detection in face of such high [Ca^{++}] could still leave it in a permissive role. The fall in cAMP makes increased sensitivity to TPH less likely, unless the increased sensitivity were selectively affecting bone. Urinary aldosterone and corticosteroids fell slightly during BR. (1,2).

Studies have been conducted to determine the value of thiazides as a test in differentiating idiopathic hypercalciuria and mild hyperparathyroidism. Chronic renal lithiasis due to idiopathic hypercalciuria is difficult to distinguish from mild hyperparathyroidism. Thiazide drugs are effective drugs in idiopathic hypercalciuria, but are contraindicated in hyperparathyroidism because they aggravate hypercalciuria. The response of serum total and ionized calcium to a brief thiazide challenge was explored as a diagnostic test of normocalcemic hyperparathyroidism. Hydrochlorthiazide, 50 mg q. 12 hrs, has been administered to 10 patients with normocalcemic or mildly hypercalcemic hyperparathyroidism, to 6 patients after surgical correction of primary hyperparathyroidism, and to 8 patients with idiopathic hypercalciuria; all patients were studied on a constant calcium-phosphorus intake. In all subjects urinary calcium decreased 20 to 60%, while urinary magnesium, phosphate sodium and potassium rose. Mean rise in total serum calcium was $\pm$ 1 mg% to frankly hypercalcemic levels in hyperparathyroid patients while in subjects whose hyperparathyroidism was corrected by surgery and idiopathic hypercalciuria, the rise in total serum calcium was less than 0.5 mg%. In response to thiazides ionized calcium fell slightly in post-operative hyperparathyroid patients, and accounted for 0 to 34% (mean 17%) of the total serum calcium rise in hyperparathyroid

patients and for 7 to 54% (mean 22%) in patients with idiopathic hypercalciuria. Thiazide challenge seems a helpful adjunct to total and ionized serum calcium determinations in the diagnosis of normocalcemic hyperparathyroidism.

Serum ionized calcium [Ca^{++}] and total calcium (Ca) were measured in 13 patients with proven diagnosis and 2 with presumptive diagnosis of primary hyperparathyroidism (PHP) to compare diagnostic accuracy of these two parameters. [Ca^{++}] was measured by a flow-through calcium ion-sensitive electrode and (Ca) by atomic absorption spectroscopy. [Ca^{++}] in the PHP patients ranged from 1.40 - 1.98 mM (5.60 - 7.92 mg%), well outside the 3 S.D. of the mean value of 1.22 $\pm$ 0.15 mM (4.88 $\pm$ 0.60 mg%) in 397 normal subjects. In contrast, 5 out of 15 (Ca) values, ranging from 2.49 - 3.67 mM (9.96 - 13.88 mg%) fell within the 3 S.D. of the mean value of 2.29 $\pm$ 0.36 mM (9.2 $\pm$ 1.4 mg%) in 231 normal subjects. Parathyroid adenomata (10) or adenomatous hyperplasin (3) were identified in 13 patients. Post-operative followup 2-11 months showed normal [Ca^{++}] and (Ca) in all. In the one negative exploration, [Ca^{++}] and (Ca) became and remained normal 5 months post-operatively. One patient awaits surgery. In 4 patients followed intra-operatively, an increase of 0.04 - 0.10 mM [Ca^{++}] above baseline values was observed within 2 hours of removal of adenoma, followed by a sharp drop to normal in 3 patients within 6 hrs. In contrast, (Ca) in 3 of these patients did not vary outside of the normal range during this period. [Ca^{++}] can be measured rapidly, simply and reproducibly. Its consistent elevation when (Ca) fluctuates in the normal range offers additional confidence in the diagnosis of normocalcemic PHP. (3)

2. Thyroid Metabolism.

A study examining a method to assess the latency, potency and duration of action of the various antithyroid drugs, which was presented at the 6th International Thyroid Congress (Vienna, June 1970) has just been published in "Further Advances in Thyroid Research" 1971. The method involves an i.v. 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*), 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 patio, 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 blockade (30 mg q 6 hrs).

The conclusions of this study were that 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.

A second study is currently nearing completion, which uses the general principles outlined above in that ^{131}I-T_4 is injected and the ratios of $U^{131}I/PB^{131}I$ were 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).

This has been a collaborative study with Dr Sidney H. Ingbar of Boston, and of the total of 14 subjects studied to date approximately two-thirds are better controlled by divided dose management. This contrasts with reports by Monte Greer that patients achieved satisfactory clinical control almost always with single dose therapy.

Another study nearing completion has examined the effect of acute malarial infection on thyroxine kinetics and hormonal secretion and 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 have now been completed on twelve

normal volunteers and indicate that acute malaria infection retards the peripheral disposal of thyroid hormone, and that there is an initial inhibition of thyroidal hormone release from the gland which is then followed by a compensatory rebound in release which continues through a period of convalescence. We plan to present this data at the meetings of the American Thyroid Association in Birmingham, Alabama during October 1971.

A fourth study involving an examination of thyroidal release mechanisms using the double-isotope technique described has been completed, also in collaboration with Dr Ingbar, and is soon to be published (J. Clinical Endocrinology & Metabolism 31: Sept 1971, in press).

Other work in progress includes an examination of the effect of drugs such as salicylates, adrenergic blockers, and phenobarbital on thyroidal function and thyroxine disposal utilizing the double isotope technique described in the malaria study above, and is designed to examine the effects of customarily used therapeutic doses of these agents 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 problems 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. In some 20 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 system.

Other animal studies done with the same *in vitro* (liver homogenate) T_4 deiodinating system, are in progress examining the effects of experimental malaria on thyroxine degradative rates.

3. Malaria.

Studies have been conducted to determine the source of purines in malaria parasites. Conclusive evidence has been accumulated that there is no significant de novo purine biosynthesis and that preformed purines are utilized via salvage pathways. Currently studies are under way to determine which purine bases, nucleosides or nucleotides are used preferentially. The mechanism of the salvage pathway and its kinetics are being investigated. Future studies are planned to look at various inhibitors of purine metabolism in malaria parasites and to determine their possible use as an imalarial agents.

4. Carbohydrate Metabolism.

Isolated fat cells have been prepared and are currently being employed to standardize a bioassay for insulin. Using this assay it is planned to study the biological activity of insulin on cultured cells under a variety of conditions. For example, the effects of aging, glucagon, the concentration K^+ and other hormones will be assessed in terms of their effects on insulin activity.

Skin fibroblasts from normals, diabetics and various other endocrine conditions will then be grown in culture and also studied by the above techniques to determine the biological effects of insulin in these conditions.

A human insulinoma has been successfully cultured by this laboratory and is currently proliferating in cell culture and continues to make insulin. Simple studies are currently underway to determine optimum conditions for cultures and growth of these cells and to maintain and support insulin secretion. Studies are currently being done on the effects of various concentrations of glucose in the culture medium on insulin secretion. When sufficient numbers of cells are obtained, extensive studies on mechanism of insulin secretion and synthesis are planned. These biochemical studies will be correlated with cell structure determined by electron microscopy. This is the only insulinoma which has been grown in culture with the continued production of insulin and as such offers an exciting and very valuable system for the study of insulin synthesis and secretion by human β cells.

Other endocrine tumors will be cultured as they become available. Presently, a pheochromocytoma is in the very early states of tissue

culture and a parathyroid adenoma will be cultured in the very near future.

5. Polypeptide Hormone.

The efficacy of medroxyprogesterone in the treatment of acromegaly has now been evaluated in six patients for periods of one week to six months. The effect of this drug on the growth hormone dynamics and the signs and symptoms of this disease have been thoroughly evaluated. Although serum growth hormone is reduced in some, glucose metabolism commonly deteriorates. The data is currently being analyzed and prepared for publication.

New immunoassay techniques for TSH, FSN and LH are being developed. A successful assay for insulin in urine has already been developed. As might be expected, urine insulin is higher during the day than during the night fasting period.

A new protocol to investigate the role of the B-adrenergic receptor and the adenyl cyclase system in mediating insulin secretion in man, and in particular in patients with reactive hypoglycemia.

An immunoassay for cyclic 3'5' AMP in urine and blood will be introduced during the next quarter.

Three members (father and two sons) of a family with vasopressin-responsive diabetes insipidus were studied in detail. All three had abnormal responses to hypertonic saline infusion, as well as to fluid deprivation. One member was able to concentrate his urine after i.v. nicotine. All of them responded to chlorpropamide treatment. The results of the study together with a review of the literature of hereditary vasopressin-responsive insipidus were submitted to the Annals of Internal Medicine for publication.

6. Steroid Metabolism.

Further study of adrenal function during acute experimental malaria has not suggested a significant role of the adrenal gland in some of symptoms seen during clinical illnesses. A collaboration study is under way with the Nutrition Laboratory at Fitzsimmons to evaluate any effects of common antimalarial drugs upon adrenal function.

Project 3A06211CA822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury.

Literature Cited.

References.

1. Heath, H., Earll, J.M., Schaaf, M., Piechocki, J.T., Li, T-K., and Baker, G.I. Abnormal serum ionized calcium with immobilization and bed rest. Abstract. Clin. Res., Vol. XIX, No. 2, p. 477, April 1971.

2. Heath, H., Low, J.C., Piechocki, J.T., Li, T-K., and Schaaf, M. Elevations of serum ionized calcium during immobilization and bed rest. Submitted for publication.

3. Low, J.C., Schaaf, M., Earll, J.M., Piechocki, J.T., and Li, T-K., The value of serum ionized calcium in the diagnosis of normocalcemic and hypercalcemic primary hyperparathyroidism. Presented at the National Meetings of the American Federation for Clinical Research, Atlantic City, N.J., 1 May 1971.

4. Low, J.C., Schaaf, M., Earll, J.M., Piechocki, J.T., and Li, T-K. Ionic calcium determination in primary hyperparathyroidism. Its value in borderline or normocalcemic states. Submitted for publication.

5. Dunn, M.J. Magnesium depletion in the Rhesus monkey: Induction of magnesium dependent hypocalcemia. Submitted for publication.

6. Danforth, E., Jr. Hormonal interrelationships in response to carbohydrate-free isocaloric diets in normal man. Submitted for publication.

7. Wartofsky, L., and Ingbar, S.H. Estimation of the rate of release of non-thyroxine iodine from the thyroid glands of normal subjects and patients with thyrotoxicosis. J. Clin. Endocrinol. & Metab., 31: Sept 1971, in press.

8. Low, J.C., Pedersen, P.R., Schaaf, M., and Earll, J.M. Hereditary vasopressin-responsive diabetes insipidus. Submitted for publication.

9. Heath, H., Low, J.C., Piechocki, J.T., Li, T-K., and Schaaf, M. Elevations of serum ionized calcium during immobilization and bed rest. Submitted for publication.

Publications.

1. Heath, H., Earll, J.M., Schaaf, M., Piechocki, J.T., Li, T-K., and Baker, G.I. Abnormal serum ionized calcium with immobilization and bed rest. Abstract. Clin. Res., Vol. XIX, No. 2, p. 477, April 1971.

2. Wartofsky, L., and Ingbar, S.H. A method for assessing latency, potency, and duration of action of antithyroid agents in man. In Further Advances in Thyroid Research, Proc. 6th International Thyroid Conf., Vienna. K. Fellinger and R. Hofer, Eds., Verlag der Wiener Medizinischen Akademie, Vienna. 1971.

3. Wartofsky, L., Martin, D., Graham, B.S., and Earll, J.M. Thyroid Function in Malaria. Abstract. Clin. Res., 19: 384, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6445	71 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 INSTR'N	8a. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	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

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 71	4	115
	CURRENT 72	4	115

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

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) 70 07 - 71 06 One S. dysenteriae 1 attenuated oral vaccine strain which was considered safe by laboratory criteria caused a reaction in 1 of 81 volunteers who received the strain per os. A second candidate strain is now being evaluated. Two further strains of penetrating E. coli - (O-143 and O-144) have been tested for pathogenicity in volunteers. These produced a disease indistinguishable from bacillary dysentery when 1 x 10^8 cells were ingested. Doses of 1 x 10^6 organisms failed to evoke symptoms. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon originator's approval

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PII Redacted

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 N. LaBrec, Ph.D.

Description.

The pathogenesis of enteric disease is studied to elucicate the mechanisms by which enteric pathogenes produce symptoms. By understanding the disease process improved procedures for prevention and treatment of diarrheal diseases will become evident.

Progress.

1. Work has continued on the pathogenesis of "non-specific" diarrhea in adults. Last year we demonstrated that two enterotoxin-producing strains of Escherichia coli isolated in Viet Nam were capable of causing disease in volunteers, but one dysentery-like strain of E. Coli (1272) failed to produce diarrhea even when fed in doses of 1×10^{10} cells. Two additional penetrating dysentery-like E. coli strains (1624 and 4608) have now been tested. A total of 26 men have ingested 1×10^{8} cells of these strains. Twelve of these men experienced severe disease which is defined as having a minimum of 10 diarrheal stools in a 48-hour period. The syndrome differed from that caused by the enterotoxin-producing strains and included chills, fever, abdominal cramps and bloody diarrhea. It is not presently known why strain 1272 failed to cause disease in man because it acted in laboratory models in a manner identical to strains 1624 and 4608. Strains 1624 and 4608, while causing disease when 1×10^{8} cells were administered, failed to evoke diarrhea when fed at a level of 1×10^{6} cells to a total of 14 men. However, when sodium bicarbonate was fed prior to the ingestion of 1×10^{6} cells of E. coli strain 1624, 2 of 3 men became ill. The disease pattern of one of these men is interesting in that it is an example of the wide spectrum of signs of disease which these organisms can produce. Within 12 hours of feeding, the individual had a non-febrile course of diarrhea for 8 days during which the organism was not isolated from the stool, and the colonic mucosa appeared normal at sigmoidoscopy. The disease appeared to be subsiding by the eighth day, when the stools became bloody and mucoid. Frank dysentery persisted for an additional 4 days.

Further information concerning the activity of toxigenic strains of E. coli has been accumulated in volunteers. In one study, the likely site of multiplication of the two pathogenic strains isolated in Viet Nam has been shown by intubation techniques to be the small intestine. In another, an enterotoxin-producing strain, isolated from piglets, failed to cause disease when fed in doses as high as 1×10^{10} cells.

2. Since 1945, S. dysenteriae 1 (S. shiga) has not caused a significant amount of bacillary dysentery and very little research on this organism has been conducted. However, during the past three years, a large epidemic with 20,000 deaths has occurred in Central America involving Guatemala, Honduras, and El Salvador. Because of the resurgance of Shiga dysentery, attempts are being made to prepare a vaccine. Shiga's bacillus differs from all other members of the genus Shigella in that it produces an exotoxin which has classically been called a neurotoxin. Recently, an exotoxin of Shiga has been described with enterotoxin activity, but it is not known whether the neurotoxin and the enterotoxin are the same substance with two different activities or are two different products of the bacterial cell. The elaboration of these toxins by the cell could complicate the development of a living attenuated oral vaccine. Our attempts to understand the genetic basis for toxin production have so far failed, and we are continuing our efforts in this area. We have found that mutant strains of S. dysenteriae 1 which are incapable of penetrating the bowel wall, but which still retain the capacity to produce exotoxin, fail to cause disease when fed in high doses (1×10^{11} cells) to monkeys. A non-penetrating toxin-producing mutant of S. dysenteriae 1 was mated with a male E. coli and a hybrid was isolated which had incorporated the xylose-rhamnose region of the E. coli chromosome. In this regard the hybrid resembled the S. flexneri 2a hybrid vaccine strain which so far has proved to be safe in man. This mutant-hybrid Shiga strain failed to cause disease in monkeys and protected these animals against experimental oral challenge with virulent Shiga bacilla. It was administered to volunteers in doses of up to 1×10^{11} cells. None of fifty men who received doses of either 1×10^{6} or 1×10^{8} living cells experienced a reaction and evidence of vaccine excretion was obtained on 48 of the men. Doses ranging from 1×10^{9} to 1×10^{11} cells were then fed to a total of 31 men. One man who received 2×10^{10} cells became ill 7 days following the administration of the vaccine and organisms which has reverted to the virulent form were isolated from his stool. Even though six other men, who were fed higher doses (1×10^{11} cells) did not experience reactions, this strain was not considered safe to use.

A second candidate hybrid vaccine strain has been prepared which has the purine E region as well as the xylose-rhamnose portion of the E. coli chromosome in its genome. We have previously identified the purine E region as one site which controls the ability of shigellae to penetrate the intestinal wall. This hybrid strain is safe when fed to monkeys, and is now being tested for safety in volunteers.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 0G, Military Internal Medicine

Work Unit 121, Pathogenesis of enteric diseases

Literature Cited.

Publications.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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70 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
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19. RESPONSIBLE DOD ORGANIZATION

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(U) Microbial Genetics; (U) Vaccine; (U) Enteric Bacteria; (U) Antigens; (U) Virulence; (U) Salmonella; (U) Drug Resistance

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

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) 70-07-71-06. In our continuing investigation of the genetic and molecular aspects of intergeneric bacterial hybridizations, we have gained additional knowledge of the behavior of Escherichia coli DNA when genetically transferred to Salmonella typhosa. We have found that S. typhosa is capable of recombining E. coli DNA, but that such recombination occurs in no more than 60% of hybrids selected for receipt of a particular E. coli gene, and is often as low as 20 to 30%. Generally, those hybrids showing a low recombination percentage display a high incidence of conservation of the E. coli DNA in partial diploid condition. Our molecular investigations of the physical nature of diploid E. coli DNA in S. typhosa hybrids indicate that it is maintained in the form of covalently closed, circular, supercoiled molecules. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 122, Microbial Genetics and taxonomy

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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. An Escherichia coli Hfr strain with negative chromosomal markers was employed in crosses with a Salmonella typhosa recipient to study the incidence of genetic recombination, as distinguished from unstable partial diploidy, in this mating system.

2. The behavior of coliphage λ in Shigella flexneri 2a, Salmonella typhosa and Salmonella typhumurium has been studied to develop new intergeneric transductional hybrids and to provide information on what effects host bacteria have on "alien" viruses.

3. Molecular investigations of the physical nature of diploid E. coli DNA in S. typhosa hybrids indicate that it is maintained in the form of covalently closed, supercoiled, circular molecules.

Progress.

1. Inefficiency of genetic recombination in hybrids between Escherichia coli and Salmonella typhosa

We have, in other studies, devoted considerable effort to examining the heterozygous, partial diploid hybrids formed when E. coli Hfr strains are mated with S. typhosa recipients.

However, the occurrence of genetic recombination in this mating system, and the consequent generation of haploid S. typhosa hybrids, has previously received little attention. The major problem in studying recombination in the E. coli X S. typhosa cross has been the difficulty invovled in differentiating true recombinants from stable partial diploid hybrids. When the E. coli gene inherited by the S. typhosa hybrid determines a positive phenotype character (as it must to be employed as a selective marker), its diploid nature may be discovered by its loss (by segregation) from the hybrid. However, the absence of detectable segregation, although suggestive of the haploid state of an E. coli gene in an S. typhosa hybrid, does not establish it.

It is possible to demonstrate the haploid state of an E. coli gene in an S. typhosa hybrid when that gene determines a recessive, or negative, phenotype for which the corresponding Salmonella allele is a dominant or positive determinant. Although negative donor alleles cannot be used as selective markers in a cross, their inheritance as unselected markers can be examined as an indicator of genetic recombination and, consequently, haploidy of the hybrid. We therefore employed, in matings with S. typhosa, an E. coli Hfr strain having negative chromosomal markers located within one minute of the positive markers used for selection. This Hfr strain, designated WR2009, has the negative markers leu$^-$, arg$^-$ and mtl$^-$ situated adjacent to the positive markers ara$^+$, rha$^+$ and xyl$^+$, respectively. In crosses between this donor and the S. typhosa recipient, WR4200, we selected hybrids which had received either the ara$^+$, rha$^+$ or xyl$^+$ E. coli markers and examined them for the presence of unselected donor genes. The mating media always contained, in addition to the cystine and tryptophan required by S. typhosa WR4200, leucine and arginine, so that hybrids undergoing recombination for the leu$^-$ or arg$^-$ donor alleles would not be contraselected.

Hybrids of S. typhosa WR4200, selected for receipt of the most distal (latest transferred) Hfr marker, xyl$^+$, displayed an extensive inheritance of the earlier transferred (proximal) E. coli genes tna$^+$, rha$^+$ and ara$^+$. Of 101 xyl$^+$ selected hybrids examined, 25 were of the xyl$^+$, tna$^+$ class, 24 were of the xyl$^+$ tna$^+$ rha$^+$ class, and 19 were of the xyl$^+$ tna$^+$ rha$^+$ ara$^+$ class. However, these hybrids proved to be unstable, partial diploids which segregated the inherited E. coli markers. Eleven hybrids exhibited only the xyl$^+$ marker, but instability was observed in only 3 of these. Only one hybrid expressed the negative donor marker leu$^-$ and the only positive E. coli character stably retained by this hybrid was the associated ara$^+$ marker. Similarly, one hybrid expressed the donor arg$^-$ marker, and it retained only the

associated rha$^+$ marker. Significant inheritance of a negative donor allele was noted only in the case of the mtl$^-$ gene. Twenty of the hybrids expressed this gene, 8 of them being of the xyl$^+$ mtl$^-$ type and 12 exhibiting the tna$^+$ marker (xyl$^+$ mtl$^-$ tna$^+$).

The xyl to mtl segment measures one minute on the standard time-length map of E. coli, and, since all of the mtl$^-$ expressing hybrids appeared stable for the selected marker, we might assume that this length of E. coli DNA might be haploid in these hybrids. The stability of the tna$^+$ marker in the 12 xyl$^+$ mtl$^-$ tna$^+$ hybrids might indicate the presence of haploid segments of 3 to 4 min in these hybrids, although it is, perhaps, unwise to attempt to draw any conclusions from stable, positive alleles. In any case, the situation in this cross does appear to be one in which relatively short E. coli chromosomal segments undergo recombination, whereas rather extensive segments may be conserved in the diploid state.

A similar situation was observed among S. typhosa WR4200 hybrids selected for receipt of the E. coli WR2009 rha$^+$ marker. Eleven of 56 rha$^+$ hybrids examined expressed the closely associated arg$^-$ allele. These hybrids appeared stable for the selected marker, but retained no other positive E. coli alleles. Only two hybrids exhibited the leu$^-$ character of the donor, and these also retained the associated ara$^+$ donor marker, but were unstable with regard to the selected rha$^+$ gene. Those rha$^+$ hybrids which also exhibited the xyl$^+$, tna$^+$, and/or ara$^+$ E. coli markers, as well as most of those containing only rha$^+$, were unstable diploids. Thus, analysis of both the rha$^+$ and xyl$^+$ selected hybrids indicated about a 20% incidence of recombination at the site of the selected marker, and about a 70% incidence of partial diploidy, with the remaining 10% of undetermined status. Significant recombination of the negative donor alleles was observed only at the site of the selected marker.

Hybrids of S. typhosa WR4200 selected for receipt of the proximally transferred ara$^+$ marker of E. coli WR2009 generally did not inherit the more distally transferred rha$^+$ and xyl$^+$ donor markers. Of 84 ara$^+$ hybrids examined, only 15 expressed distally transferred E. coli genes, and these hybrids proved to be unstable partial diploids. The leu$^-$ marker was expressed in 44 hybrids, all of which appeared stable for the associated ara$^+$ marker. The remaining 25 hybrids expressed only the ara$^+$ E. coli marker and 19 of these were observed to segregate this gene. Inasmuch as only 6 hybrids were thus undetermined as to their haploid or diploid status, the 52% incidence of leu$^-$

haploidy is probably close to being an accurate measure of recombination at the site of the *ara*$^+$ marker.

Our previous studies have placed considerable emphasis on the finding that the majority of hybrids resulting from the *E. coli* X *S. typhosa* cross behave as unstable partial diploids. The present findings indicate that a substantial percentage of recombination of *E. coli* deoxyribonucleic acid (DNA) can take place in *S. typhosa*, as evidenced by the 52% incidence of *leu*$^-$ haploidy among the *ara*$^+$ selected hybrids. Nevertheless, *S. typhosa* obviously is not capable of recombining *E. coli* DNA with the efficiency characteristic of its recombination, for example, of the DNA of *Salmonella* species. Moreover, selection of *S. typhosa* hybrids receiving the later transferred *E. coli* Hfr markers resulted in a lower recombination frequency (20%) and a higher incidence of unstable partial diploid hybrids than resulted from proximal marker selection. The amount of *E. coli* DNA which can be retained in the haploid state by *S. typhosa* hybrids appears to be defined by chromosomal segments which do not exceed 3 to 4 min in lengths; however, very extensive *E. coli* DNA segment (30 min or more), as we have previously seen, may be conserved in the diploid state. At the present time, little is known about the recombination enzymes of *S. typhosa*, thus making it difficult to assess their possible role in the recombination of *E. coli* DNA. We would expect, however, that the dissimilarities of the nucleotide sequences of *E. coli* and *S. typhosa* DNA's as previously demonstrated, is a major factor in the difficulty exhibited by *S. typhosa* in conserving *E. coli* genes.

2. Behavior of coliphages on other genera classified in the *Enterobactereaceae*

Unlike bacterial conjugation, which usually results in extensive transfer of chromosome from one cell to another, bacterial viruses, by the phenomenon of transduction, offer a system for exchanging a limited, discrete number of genes between bacteria. Studies from this department have previously established the feasibility of intergeneric conjugation between *E. coli* and recipients of other genera, such as *Salmonella*, *Shigella*, *Proteus*, *Serratia* and *Vibrio*. Under appropriate conditions, such intergeneric matings results in transfer of chromosomal segments of carrying size, yielding hybrids altered in a number of alleles.

We are now attempting to broaden such studies on intergeneric hybridization by developing intergeneric transduction procedures which allow for viral transfer of defined small segments of host DNA between different genera. Such studies in addition to elucidating

the behavior of viruses in alien hosts (an area of investigation still lacking in knowledge), would also enable one to prepare hybrids of different enterics which are solely altered in genes controlling virulence factors, LPS determinants and other medically important bacterial properties.

a. By means of phage P1, it has been possible to transduce genes of S. flexneri responsibilities for epithelial cell penetration, a primary step in the pathogenesis of bacillary dysentery, from one genus to another. This locus, termed kcpA phage P1 propagated on S. flexneri, has been employed to prepare intergeneric E. coli hybrids harboring the kcpA$^+$ genes of S. flexneri. Likewise, S. flexneri strains, which have received the kcpA$^-$ allele of E. coli via P1 transduction, have also been prepared and found to be unable to penetrate epithelial cells.

The intergeneric transduction of phage P1 of the E. coli surface receptor for coliphage λ penetration to S. flexneri has facilitated studies on the behavior of λ phage on S. flexneri. Such studies have revealed the following results:

In addition to the necessity for the E. coli surface receptor to be expressed by S. flexneri, the adsorption of coliphage λ is affected by the smooth somatic O antigen determinants of S. flexneri. This smooth LPS layer appears to block or interfer with the adsorption process. Rough mutants of S. flexneri hybrids expressing the λ surface receptor have been shown by adsorption and efficiency of plating experiments to be more efficiently infected by coliphage λ than smooth derivatives of the same strain.

Upon infection of S. flexneri, coliphage λ appears to replicate normally and is capable of lysogenizing near the galactose genes of the S. flexneri chromosome. Such λ lysogenic S. flexneri can be induced to yield both functional infectious particles (λ) and defective (λdg) particles able to transfer intergenerically S. flexneri gal$^+$ genes.

Restriction-modification studies on the intergeneric level have shown that S. flexneri does not restrict (i. e., destroy the DNA) λ phages propagated on E. coli strains K-12, B and C. S. flexneri, however, is able to modify λ DNA, since λ phage grown on S. flexneri is restricted by E. coli K-12 and B.

b. Salmonella typhosa strains normally are unaffected by λ, a lysogenic bacterial virus whose natural host is Escherichia coli K-12. By hybridization using E. coli K-12 as the donor of genetic material to S. typhosa recipients, we have been able to

characterize certain discrete regions of the K-12 chromosome as being required for the propagation of coliphage λ.

Notwithstanding the presence of 30% of the K-12 genome including the λ receptor locus, the S. typhosa hybrid strain WR4255, which we have characterized earlier, remains unable to replicate λ to produce free phage particles. This hybrid, nevertheless can maintain both λ prophage and the defective phage element λdg following transduction of the K-12 galactose genes carried by λdg in high frequency transducing preparations composed of λ and λdg.

E. coli-S. typhosa hybrids recently isolated in an Hfr strain of S. typhosa which have the continuous chromosomal segment including the pro$^+$, ara$^+$, rha$^+$, xyl$^+$ selective markers were shown to plate coliphage λ. In contrast, the λ insensitive S. typhosa hybrid WR4255 does not contain the xyl$^+$ region of K-12. These results seemed to implicate the genetic region near the xyl$^+$ selective markers as being necessary for λ replication. That this supposition was indeed the case was established by transferring to an S. typhosa hybrid containing only the λ receptor locus the xyl$^+$ region from two K-12 strains, one of which, WR2010 transfers its markers in the order 0-tna$^+$ ... xyl$^+$ ... str$^+$ while the second Hfr strain, WR2020 transfers its markers in the reverse order 0 ... str ... xyl$^+$ ... tna$^+$ ... This mapping procedure effectively limits the region required to the position of the K-12 genome circumscribed by the origins of the two K-12 Hfr strains employed. Most of the hybrids isolated were diploids for the tna$^+$ ... xyl$^+$ region and were susceptible to λ. Segregants of these S. typhosa diploids which lost the tna$^+$ ... xyl$^+$ region also lost their λ susceptibility. The region of the K-12 chromosome implicated in the replication of λ by these experiments encompasses a 10 min segment (min 64 to min 74) of the K-12 genome representing 1/9 of the chromosome.

The necessary region providing WR4255 with the ability to replicate λ was further defined by a K-12 episome F-xyl$^+$ which does not possess the 71 to 74 position of the K-12 chromosome. Transfer of this F-xyl$^+$ episome resulted in the isolation of WR4255 F-xyl$^+$ hybrids susceptible to λ. These experiments decreased the chromosomal region to a 5 min segment from min 66 to min 70 or less than 6% of the K-12 chromosome.

To define further other gene regions necessary for λ replication which were already present in S. typhosa hybrid WR4255, the previously hybridized strain of S. typhosa with the λ receptor locus (λ rcp$^+$) only was employed as a recipient. S. typhosa λ rcp$^+$ hybrids are not susceptible to wild-type λ

or the λ sx mutants, although mutants of λ imm434sx can be isolated on them at high frequency. Addition of the xyl$^+$ region by transfer of the F-xyl$^+$ episome to the S. typhosa λ rcp$^+$ hybrid, however, did not permit this hybrid to plate λ as was the case with WR4255 F-xyl$^+$ containing hybrids.

The inability of the S. typhosa λ rcp$^+$ hybrid containing the F-xyl$^+$ episome to plate λ indicates an additional requirement for a K-12 genetic locus within the 74 to 64 min region of the E. coli chromosome.

At the present time, we are unable to define the specific nature of the gene products which are controlled by the chromosomal regions involved. However, the use of hybrids containing either none, one or both of the required regions should make it possible to analyze the specific functions of these K-12 gene regions.

The availability of hybrid strains of S. typhosa which plate λ permitted the testing of possible restriction of K-12 grown λ by S. typhosa as well as the possible restriction of S. typhosa grown λ by E. coli K-12. The results established that λ previously grown on K-12 was not restricted by S. typhosa, whereas λ propagated on S. typhosa was now restricted when plated on E. coli K-12. This can be explained by a modification of the DNA of λ by S. typhosa causing it to be degraded by K-12. These results were confirmed in transduction experiments which showed that the λdg element carrying the galactose genes of S. typhosa was restricted by K-12 while the K-12 λdg was not restricted by S. typhosa hybrid recipients.

c. The information derived from a study of coliphage λ on S. typhosa has facilitated the development of a system for investigating genetic recombination between two unrelated, evolutionary divergent bacteriophages; E. coli phage λ and Salmonella phage P22. Since S. typhosa appears refractory to phage P22, a P22 sensitive derivative of S. typhimurium was chosen for this study. Employing mutation techniques and intergeneric recombination with E. coli K-12 Hfr donors, a hybrid derivative of S. typhimurium SC19 has been constructed which has inherited a number of E. coli properties essential for sensitivity to coliphage λ. Originally derived by mating donor strain E. coli K-12 Hfr P4X6 with recipient strain S. typhimurium LT7 SC19 and selecting for the inheritance of the donor xyl$^+$ and thr$^+$ alleles, the S. typhimurium hybrid was subsequently found to have inherited also the E. coli mal$^+$-λ rcp locus needed for λ adsorption, one or more loci concerned with the replication of wild-type λ in S. typhimurium, the matB negative donor allele, as well as the E. coli genes for type I pili. Since this λ sensitive hybrid strain

was defective also in its rough A lipopolysaccharide locus thus preventing efficient P22 adsorption, the defect was repaired by conjugation with a smooth S. typhimurium donor, yielding a S. typhimurium hybrid which expressed sensitivity to both the E. coli phage λ and the S. typhimurium phage P22. Preliminary results from studies on this unique hybrid have revealed that:

(1) The strain can be lysogenized individually with each phage or simultaneously with both, and express the expected immunity patterns.

(2) Induction experiments have shown that the phages are capable of replication in this host.

(3) By infecting a λ lysogenic derivative of this hybrid with phage P22 and allowing the phage growth, presumed λ-P22 hybrid phages have been recovered. Preliminary characterization of 3 such phage hybrids indicate that the hybrid phage express the host range of coliphage λ. The immunity-repressor characterizations of existing phage hybrids are presently being performed. In addition, other classes of hybrid phages will be sought.

3. Examination of circular DNA isolated from Salmonella typhosa hybrids obtained from matings with Escherichia coli

a. Examination of S. typhosa hybrids derived from matings with E. coli Hfr WR2010. The first group of hybrids of S. typhosa WR4200 which we examined resulted from a mating with E. coli Hfr WR2010. Hybrids were selected for receipt of the xyl^+ (utilization of xylose) marker of the donor, and about 30% of them inherited also the donor tna^+ (production of tryptophanase) character. A total of nine hybrids (5 containing the xyl^+ tna^+ markers and 4 containing only the xyl^+ marker) were examined by the dye-buoyant density method, and 4 of these (all containing both xyl^+ and tna^+ E. coli markers) were found to contain circular DNA. Fractionation of the DNA from these hybrids yields a large peak consisting of highly labeled Salmonella chromosomal DNA and a smaller more dense peak containing the supercoiled, covalently closed circular E. coli DNA. The ratios of counts in the small peak compared to the total counts indicates that the supercoiled, circular DNA comprises 1-2% of the total DNA from the cell. This is probably a low estimate of the true value because some of the supercoiled circles are undoubtedly converted to open circles or linear molecules because of breakage of one or more strands during the extraction of the DNA.

The supercoiled circular DNA was analyzed in a neutral sucrose gradient. The sucrose gradient provided additional

evidence that the DNA fraction represented by the small peak in the CsCl gradient with ethidium bromide was in fact, supercoiled, circular DNA; it provided as well a method for estimating the molecular weight according to the relationship described by Bazaral and Helinski.

The supercoiled circular DNA from the 4 *xyl*$^+$ *tna*$^+$ hybrids was also examined by electron microscopy. The electron micrographs provided a means of more accurately measuring the size of the circular DNA molecules as well as a confirmation of the circularity of these molecules. The results of these analyses are shown in the upper part of the table. Three of these hybrids exhibited circular DNA molecules with a molecular weight of 66 X 10^6 daltons and one showed a molecular weight of 160 X 10^6 daltons. In this group of hybrids, it is not possible to make any correlation between the size range of the circular DNA molecules and the detectable *E. coli* genetic markers, because no markers were available to measure the inheritance of *E. coli* genes in the region immediately distal to the selected *xyl*$^+$ marker. We suspect that the hybrid containing the circular DNA of 160 million molecular weight contains *E. coli* DNA from that region in addition to the DNA indicated by the presence of the *xyl*$^+$ and *tna*$^+$ markers.

Since segregants from these *xyl*$^+$ *tna*$^+$ hybrids could be isolated which had lost the inherited *E. coli* genetic markers, we decided to examine one of these, designated WR4220, to determine whether this loss was accompanied by loss of the circular DNA molecules. The segregant, WR4200, was analyzed by the dye-buoyant density method. The only peak observed was the chromosomal DNA peak. There was no peak corresponding to circular DNA in the segregant. In order to determine whether remating this segregant would again result in hybrids which contained circular DNA molecules, we mated *S. typhosa* WR4200 with *E. coli* Hfr WR2010. The *xyl*$^+$ selected hybrids from this cross appeared similar in all respects to those obtained in the earlier mating with *S. typhosa* WR4200. Six *xyl*$^+$ *tna*$^+$ hybrids of *S. typhosa* WR4200 were examined by the CsCl-EtBr method and 5 of them, as shown in the table were found to contain circular DNA. Circular DNA molecules from two of these hybrids, as determined by electron microscopy were found to have molecular weights of 84 X 10^6 and 160 X 10^6 daltons. These molecules are thus within the size range previously determined for the *xyl*$^+$ selected hybrids of *S. typhosa* WR4200.

We have not, in the past, observed any transfer of *E. coli* diploid material from *S. typhosa* hybrids except when that material was contained in F$^-$ or Hfr strains. Nevertheless, we did examine the *xyl*$^+$ *tna*$^+$ hybrids which exhibited circular DNA for possible

transfer of the *xyl*$^+$ marker to *E. coli* WR3051. None of these hybrids showed any evidence of transfer of the *xyl*$^+$ marker. The possibility was considered also that the present hybrids might contain a terminal marker episome derived from *E. coli* WR2010 (F-*ilv*). However, none of them showed any transfer of the *ilv* (isoleucine-valine production) marker to the *ilv*$^-$ *E. coli* recipient WR3026.

We examined also the ability of the circular DNA-containing *xyl*$^+$ *tna*$^+$ hybrids to propagate the male specific phage R-17. Inasmuch as this phage does not form plaques on male derivations of *S. typhosa* WR4200, the testing was accomplished by the titer increase method. *S. typhosa* WR4200 containing F-*lac*, when tested by this method, showed a 1000-fold increase in titer, but no increase was observed with any of the *xyl*$^+$ *tna*$^+$ hybrids.

b. Examination of S. typhosa hybrids derived from matings with *E. coli* Hfr WR2015 and *E. coli* Hfr WR2009. We were interested in obtaining hybrids in which the genetically predicted extent of the *E. coli* diploid material (from unselected marker inheritance) could be correlated with the molecular size as determined by electron microscopy. Therefore, we mated *S. typhosa* WR4200 with *E. coli* Hfr WR2015 and selected those *S. typhosa* hybrids which received either the *rha*$^+$ (utilization of rhamnose) or the *xyl*$^+$ *E. coli* markers. Hybrids selected for receipt of the *rha*$^+$ gene generally received only that marker; approximately 15% of these *rha*$^+$ selected hybrids also received the distal *tna*$^+$ marker, but none received the *xyl*$^+$ gene. Hybrids selected for receipt of the *xyl*$^+$ marker frequently received both proximal markers *tna*$^+$ and *rha*$^+$, although some were obtained in which only the *xyl*$^+$ and *tna*$^+$ markers were conserved. We were able to isolate circular DNA from representatives of three of these hybrid classes, the *rha*$^+$ class, the *xyl*$^+$ class, and the *xyl*$^+$ *tna*$^+$ *rha*$^+$ class. We were not able to isolate circular DNA from those hybrids of the *rha*$^+$ *tna*$^+$ class which we examined. We suspect that our inability to demonstrate circular DNA among these *rha*$^+$ *tna*$^+$ hybrids was due to the fact that they were very unstable with regard to their conservation of the inherited *E. coli* alleles. The problem of instability was encountered also with some of the previously examined diploid hybrids derived from mating with *E. coli* WR2010, in which we were not able to demonstrate circular DNA. It would be expected that in unstable diploid populations, in which many segregant cells are observed to have lost the *E. coli* DNA, the number of cells containing diploid material is too small to provide enough circular DNA for detection.

Those hybrids derived from the E. coli WR2015 X S. typhosa WR4200 crosses in which we demonstrated circular DNA are shown in the table. As anticipated, the smallest circular DNA molecules measured (40 and 42 million daltons) were those isolated from the two hybrids containing only the rha^+ marker. By comparison, the xyl^+ tna^+ rha^+ hybrid contained a circular DNA molecule with a molecular weight of 120 million. An intermediate size of circular DNA (63 million) was isolated from the xyl^+ tna^+ hybrid. Thus, the measurements of the circular molecules obtained from rha^+ hybrids, as compared with the hybrid containing the tna^+ and xyl^+ markers in addition to rha^+, are consistent with the notion that the size of the circular DNA is directly related to the size of the inherited E. coli chromosomal segment.

We also employed S. typhosa WR4220 in a mating with a different E. coli Hfr, WR2009, in an attempt to obtain circular DNA molecules of larger size than those which we had already isolated. The selection of xyl^+ hybrids from this mating generally results in hybrids which conserve rather extensive E. coli genetic segments in the diploid state because of the position of the selected marker with relation to the Hfr origin. Thus, xyl^+ selected hybrids expressing both tna^+ and rha^+ markers, but lacking the more proximally transferred ara^+ (utilization of arabinose) marker might be expected to contain greater amounts of E. coli DNA than did the xyl^+ tna^+ rha^+ S. typhosa hybrids derived from E. coli Hfr WR2015. We examined 7 xyl^+ tna^+ rha^+ hybrids and one xyl^+ tna^+ hybrid derived from the E. coli WR2009 X S. typhosa WR4220 mating for the presence of circular DNA. Only the xyl^+ tna^+ hybrid yielded a positive result. The circular DNA extracted from this hybrid, also included in the table, had a molecular weight of 97 million daltons.

Summary and Conclusions.

1. An Escherichia coli Hfr strain in which three negative chromosomal alleles (leu^-, arg^- and mtl^-) were closely linked to three positive alleles (ara^+, rha^+ and xyl^+, respectively) was employed in crosses with a Salmonella typhosa recipient to study the incidence of genetic recombination in this mating system. The detected expression of the negative E. coli alleles in S. typhosa hybrids was used to determine the occurrence of haploid recombinants as distinguished from partial diploid hybrids. Recombination measured at the sites of the distally transferred Hfr markers xyl^+ and rha^+ was about 20%, the majority of these hybrids being unstable partial diploids. Selection of a proximally transferred Hfr marker (ara^+) resulted in a 52% incidence of recombination as determined by the expression of leu^- haploidy. The lengths of

CHARACTERIZATION OF HYBRIDS FROM MATING OF

E. COLI Hfr WITH *S. TYPHOSA* STRAINS

Mating Pair	*S. typhosa* Hybrid	Inherited *E. coli* Genetic Markers	Circular DNA	Molecular Weight of Circular DNA in Daltons	
				Sucrose Density Gradient	Electron Microscopy
WR2010 X WR4200	Xyl 4	*xyl*$^+$ *tna*$^+$	+	58 X 10^6	66 X 10^6
	Xyl 6	*xyl*$^+$ *tna*$^+$	+	60 X 10^6	66 X 10^6
	Xyl 8	*xyl*$^+$ *tna*$^+$	+	60 X 10^6	66 X 10^6
	Xyl 9	*xyl*$^+$ *tna*$^+$	+	150 X 10^6	160 X 10^6
WR2010 X WR4220	Xyl 5	*xyl*$^+$ *tna*$^+$	+	-	-
	Xyl 6	*xyl*$^+$ *tna*$^+$	+	-	-
	Xyl 10	*xyl*$^+$ *tna*$^+$	+	81 X 10^6	84 X 10^6
	Xyl 16	*xyl*$^+$ *tna*$^+$	+	-	160 X 10^6
	Xyl 17	*xyl*$^+$ *tna*$^+$	+	-	90 X 10^6
WR2015 X WR4200	Rha 9	*rha*$^+$	+	41.5 X 10^6	40 X 10^6
	Xyl 10	*xyl*$^+$ *tna*$^+$ *rha*$^+$	+	110 X 10^6	120 X 10^6
	Xyl 12	*xyl*$^+$ *tna*$^+$	+	59 X 10^6	63 X 10^6
	Rha 13	*rha*$^+$	+	-	42 X 10^6
WR2009 X WR4220	Xyl 17	*xyl*$^+$ *tna*$^+$	+	97 X 10^6	-

E. coli chromosomal segments undergoing recombination in these S. typhosa hybrids appeared rather short (3 to 4 min or less) whereas quite extensive E. coli gene segments were conserved in the diploid state.

2. Studies on the behavior of coliphage λ on the other genera classified in the Enterobactereaceae have led to the following conclusions.

In a S. flexneri hybrid recipient, coliphage λ behaves, for the most part, as it normally does in its natural host, E. coli, being able to replicate efficiently and to lysogenize near the galactose genes of the S. flexneri chromosome. Upon induction of a λ lysogenic S. flexneri, both functional infectious particles and defective phage particles (λdg) are produced. Smooth S. flexneri 2a hybrids were found to be poor in adsorbing λ. In contrast, when the smooth LPS antigen layer is altered to a rough state (either by mutation or hybridization) λ was found to adsorb efficiently.

Unlike S. flexneri, S. typhosa hybrids were found to exert a natural block on the lytic production of λ, although they efficiently adsorbed this phage. Subsequent studies revealed that this natural block can be obviated by the addition of at least two further E. coli genes, one located between the malA$^+$ and xyl chromosomal markers, the other between the xyl-ilv loci. Such hybrids, fully sensitive to λ, are being analyzed to determine the specific functions of these genes.

Similar studies with a P22 sensitive S. typhimurium have shown it possible to prepare S. typhimurium hybrids fully sensitive to coliphage λ and Salmonella phage P22. As with S. typhosa, the natural block to λ replication was obviated by the addition of appropriate E. coli genes from E. coli Hfr donor strains. Such S. typhimurium hybrids can be infected with both phages to yield hybrid phages between λ and P22 C$^+$. These are being studied further.

3. Heterozygous, partial diploid S. typhosa hybrids obtained from matings with E. coli K-12 Hfr strains were observed to contain supercoiled, circular DNA when examined by the dye-buoyant density method. Molecular weight determinations of circular DNA molecules isolated from a number of S. typhosa partial diploid hybrids were made by sucrose density gradient ultracentrifugation and electron microscopy. These studies revealed a range of molecular sizes consistent with the presence in the hybrids of various lengths of transferred E. coli chromosomal segments. It was concluded that the E. coli Hfr genetic segments transferred to these S. typhosa

hybrids were conserved, in the diploid state, in the form of supercoiled, circular DNA molecules.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 122, Microbial Genetics and taxonomy

Literature Cited.

Publications.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6446	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRAD NO	8a. DISPN INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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. [redacted]	CDOG 1412A(2)			

11. TITLE (Procede 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)
PRECEDING FISCAL YEAR 71	6	100
CURRENT FISCAL YEAR 72	6	100

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede 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. Procede 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 tritiated thymidine and histochemical investigations

25.(U) 70 07-71 06The delineation of the starvation model of duodenal stress ulcers in guinea pigs as studied by gross anatomic, histopathological, histochemistry and radioautography is nearing completion. Studies on experimental cholera in the rabbit have resulted in a manuscript "Localization of Cholera Toxin in Rabbit Intestine: An Immunohistochemical Study: which has been submitted to Lab. Invest. and another "Experimental Cholera: Localization of Immune Response in Ligated Loops of Rabbit Ileum" was presented to the FASEB in Chicago 1971. An abstract,"Cholera Toxin: Response to Intramural Inoculation" has been published in Sastroentrol. Studies in progress on the role of staphylococcal enterotoxin in diarrheagenesis have demonstrated in the monkey that this toxin is also capable of producing positive loop responses in the jejunum but not in the ileum. It also does not cause increased skin permeability. The effects of this toxin in the intestinal localization of radioactively labelled compounds are in progress. A manuscript, "Pathogenesis of E. Coli Diarrhea" has been accepted for publication by the N.Eng.J.Med. Studies on spirochetal components of intestinal microflora have been presented."Gastric Spirilla in the Rhesus Monkey"FASEB and "Intestinal Spirochetosis in Monkey and Man" International Academy of Pathology. A chapter "Current Aspects of Bacterial Enterotoxins" for Current Topics in Pathology, Springer Verlag is in preparation. For technical reports see Walter Reed Army Institute of Research Annual Progress Report,1 Jul 70-30 Jun 71.

*Available to contractors upon originator's approval

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

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123, Histopathologic manifestations of diarrheal diseases

Investigators.
Principal: LTC Daniel G. Sheahan, MC
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MAJ V. Kao, MC; MAJ R. Nagle, MC; MAJ J. Ballo, MC;
MAJ J. Mooney, MC

Problem

To better understand the pathogenetic mechanisms of diarrheal diseases with specific emphasis on those of bacterial origin which have military significance.

Background

This department has since its inception been concerned with investigators on diarrheal disease. Those mediated by bacterial enterotoxins in addition to those caused by invasion of enteric pathogens have been continuously studied.

Approach

The greatest emphasis has been placed on the study of morphological alterations in diarrheal disease states using light and electron-microscopy, mucin and enzyme histochemistry autoradiography and immunological tracer methods.

Results

The sequential changes in epithelial mucosubstances in response to staphylococcal enterotoxin were described (Sheahan et al 1970). These alterations occurred at the same time as the morphological and enzyme histochemical changes previously described from this department indicating the diffuse effect of this toxin. A result of this study was the decision to investigate the normal pattern of epithelial mucosubstance distribution in the gastrointestinal tract of the commonly used laboratory animals. This is currently in progress.

Studies on cholera enterotoxin showed that inoculation of the toxin into the mesenchymal tissues of the intestinal wall was not associated with fluid exsorption in contrast to that normally seen with intraluminal instillation (Sheahan and Sprinz 1971). This observation provided further evidence for the prerequisite of cholera enterotoxin/epithelial surface interaction to produce fluid exsorption in the gut.

Collaborative studies with the Department of Applied Immunology and the Department of Infectious Diseases, Baltimore City Hospital, have described at least 2 different pathogenetic mechanisms by which E. Coli organisms may produce diarrheal disease (Dupont et al 1971). Some strains mediate their pathogenicity by their capability to invade the gastrointestinal mucosa and others by elaborating an enterotoxin. This significant observation has far-reaching implications because the currently used routine bacteriological methods do not differentiate between the invasive and enterotoxin producing strains of E. Coli. A chapter describing the recent developments in toxin mediated bacterial enteric diseases is currently in press (Sheahan 1971).

Studies on the immunological aspects of experimental cholera have indicated that parenteral immunization of rabbits with cholera toxin produced a certain degree of immunity which could be overcome with massive infection. IgG was localized in the crypt epithelium and was discharged into the intestinal lumen with specific enterotoxin challenge. These observations were presented at the annual FASEB meetings in April 1971 (Kao et al 1971). A manuscript, "Localization of cholera toxin in the rabbit intestine," has been submitted to Laboratory Investigation in revised form.

The relationship of constituents of the normal bowel microbial flora to the intestinal mucosa has been studied morphologically. Light and electronmicroscopic observations have indicated a symbiotic-like relationship of intestinal spirochetes and intestinal epithelium in monkey and men (Takeuchi et al 1971) and also between gastric spirilla and gastric mucosa in monkey (Takeuchi 1971). A series of monographs describing the invasive effects of various enteropathogens have been published (see references).

Investigation of the ingestion of salmonella and shigella organisms by mouse and monkey macrophages revealed no difference in the uptake of these two organisms. Studies of the phagocytosis of synthetic liposomes, into which known quantities of antigen may be incorporated, by mouse macrophages in vitro have been undertaken to further understand the roles of complement and antibody in phagocytosis.

Attempts to find a simple direct method of immunizing animals to narcotic agents to detect specific antibodies are also being made with the use of these synthetic liposomes.

The delineation of the starved guinea pig model of duodenal stress ulceration is completed and a manuscript is in preparation.

Studies on the effects of neutron gamma radiation on the intestinal mucosa of conventional and germfree mice has been published (Jervis et al 1971).

Other investigations

1. Ultrastructural studies comparing the morphology of canine Heidenhain pouch and normal gastric mucosa insitu have commenced and changes induced by urea on the gastric mucosa are being studied morphologically and physiologically.

2. In collaboration with the Department of Surgery, WRAIR, morphological studies have indicated that high alveolar oxygen tension is the primary cause of oxygen toxicity in the lung (Yhap et al manuscript submitted).

3. Studies using staphylococcal enterotoxin have indicated that this toxin can also produce intestinal fluid exsorption and increased vascular permeability but only with doses largely in excess of those which are normally enteropathogenic for monkeys.

Support role

This department continues to give support to other divisions and services in their investigations. Histochemical studies on both human and experimental animal biopsy material from the Walter Reed General Hospital Organ Transplant Service have been performed and are included in a presentation to be given to the Electron Microscopy Society of North America (August 1971).

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 MILITARY INTERNAL MEDICINE

Work Unit 123 Histopathologic manifestations of diarrheal diseases.

Publications:

1. Sheahan, D. G., Jervis, H. R., Takeuchi, A., and Sprinz, H.: The effect of staphylococcal enterotoxin on the epithelial mucosubstances of the small intestine of Rhesus monkeys. Am. J. Path. 60: 1-18, 1970.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6485	71 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. ORG'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Provide 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 71	2	45
FISCAL YEAR CURRENT 72	2	45

19. RESPONSIBLE DOD ORGANIZATION

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NAME:

NAME: DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide KEY, 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, precede 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 availability of these nutrients for absorption from these foodstuffs in normal subjects and in populations where nutritional deficiencies and chronic infections are commonplace.

25. (U) 70 07 - 71 06 Standard methods for the measurement of serum iron concentration in serum specimens and for standardizing these methods were developed in collaboration with ICSH and WHO. Similar studies for standardization of measurements of serum transferrin concentration are in progress. Various factors affecting the absorption of iron were studied and biochemical explanations for varying absorption of iron from different foodstuffs was provided. The effect of protein deficiency upon iron absorption was quantified in animal experiments. For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 70 - 30 Jun 71.

*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 125, Hematology . nutritional deficiencies of military importance

Investigators.
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Associate: LTC George M. Bernier, MC, MAJ Phillip P. Toskes, MC, Mr. Harold L. Williams

Description.

Standard methods for the measurement of serum iron concentration in serum specimens and for standardizing these methods were developed in collaboration with ICSH and WHO. Similar studies for standardization of measurements of serum transferrin concentration are in progress. Various factors affecting the absorption of iron were studied and biochemical explanations for varying absorption of iron from different foodstuffs were provided. The effect of protein deficiency upon iron absorption was quantified in animal experiments.

Progress and Results.

During the last four years, this laboratory has made and distributed serum specimens to nine international laboratories participating in a collaborative undertaking to establish standards and standard referee methods for measurement of iron and iron binding protein in serum specimens. A method has been developed which permits the reproducible measurement of iron in serum specimens in the various collaborating laboratories. The method and means of standardization have been forwarded through the International Committee for Standardization in Hematology to both the International Society of Hematology and World Health Organization for acceptance. Similar salutory results have not been forthcoming in the standardization of methods for measurement of transferrin in serum specimens. This measurement is important because it differentiates hypoferremia secondary to iron deficiency from that observed in patients with chronic inflammatory diseases and infection. Immunologic methods have not been highly reliable and reproducible because of variation in transferrin in different serum specimens; this can be demonstrated by electrophoresis. Further, estimates of the molecular weight of transferrin which vary from 68,000 to 90,000 in different studies make it impossible to calculate the amount of iron which should be bound to protein from immunologic measurements. Chemical methods of measurement of iron bound to serum proteins have been more successful. Improvements in methods have produced

reproducibility within laboratories during measurements in batches of materials. However, temporal variation within single laboratories and by comparison of results between laboratories remains far greater than can be tolerated for standard referee methodology. A number of factors have been identified in our laboratory during the past year which we believe will markedly diminish errors in the procedure. These include: identification of a buffer which does not bind iron, proper control of pH at all stages of the procedure by the appropriate use of buffers, identification of factors which prevent complete binding of transferrin with iron and methods to decrease the formation of macromolecular iron which is unbound to transferrin from solutions and prevent falsely elevated values. Further, an independent method using titration has been developed which permits standardization.

Iron deficiency is widespread among populations consuming a protein-deficient diet, and it is found with greatest frequency in women during the childbearing years and in children during periods of maximal growth. Among protein starved humans the most important nutritional factors causing iron deficiency are the low iron content of most protein-deficient diets and a lack of readily absorbed heme in the diet. Other factors which diminish iron absorption in protein deprived populations are histologic alterations of the small intestinal mucosa, a diminished corporeal stimulus to absorb iron, a decreased concentration of the amino acids in the diet which facilitate iron absorption from the intestinal lumen, chronic diarrhea with increased intestinal motility and chronic infection. Studies of starvation and protein depletion in experimental animals showed ferrokinetic abnormalities and diminished iron absorption. This was not caused by a direct intraluminal effect of starch or sucrose diets on iron absorption. The abnormality was attributed to a retarded growth rate and diminished hemoglobin synthesis in these animals. Protein deprived animals seemed to attempt to re-establish and maintain a normal body iron concentration by decreased absorption and increased excretion of iron. Animals receiving an iron replete, protein-deficient diet for prolonged periods developed anemia which was normocytic and normochromic. A microcytic hypochromic anemia occurred only when iron was not added to the protein-deficient diet. The decreased absorption of iron resulting from protein depletion was not accompanied by increased iron content or concentration in the duodenal mucosa. However, in starved animals increased amounts of dialyzable iron were shown to be incorporated into the duodenum from body stores and may act to diminish the uptake of iron from the lumen of the gut into mucosal cells.

The effect of cobalt upon iron absorption was studied because these metals seem to share a common intestinal absorptive pathway, and it was believed that studies of competition and of the similarities and dissimilarities in absorption would provide information of value about the absorption of each metal which could not be obtained otherwise.

Cobalt decreases iron absorption as effectively as equimolar amounts of carrier iron. Factors which influence the absorption of one metal significantly affect the absorption of the other. Cobalt does not decrease iron absorption by a nonspecific toxic effect upon the gut or by competing for intraluminal compounds which enhance iron absorption. The lack of incorporation of iron into ferritin indicates that ferritin is not involved in any absorptive pathway common to both cobalt and iron. The capability of ferritin to hold absorbed iron in intestinal cells and prevent the transfer of unneeded iron into the body could diminish iron but not cobalt absorption. This provides a possible explanation for the observation that cobalt absorption is not always decreased by conditions which diminish iron absorption.

Studies were performed in animals to determine the site and mechanisms involved in the suppression of iron absorption by doses of cobalt. Significantly less iron was observed in both the carcass and small intestine of animals fed cobalt with test doses of radioiron than in animals receiving iron alone. This suggested that cobalt diminished the mucosal uptake of iron from the intestinal lumen. Iron absorption studies in animals receiving various doses of iron with and without added cobalt suggested that cobalt was capable of saturating a common pathway in intestinal absorptive cells for the absorption of both metals. Studies of ferritin synthesis showed that iron administered orally or parenterally enhanced the production of this iron bearing protein in intestinal cells. On the contrary, doses of cobalt had no effect upon ferritin synthesis showing that this metal did not exert its effect upon iron absorption through this mechanism. The finding that parenteral iron induced significant synthesis of ferritin in the distal small intestine where iron is poorly absorbed suggested that ferritin functions chiefly as a barrier to excess iron absorption and an important mechanism for iron excretion.

Conclusions and Recommendations.

An acceptable and reproducible method has been developed for the measurement of iron in serum specimens in our laboratory which has been recommended for use as a referee method by WHO and the International Society of Hematology. A similar requirement for standardization of measurements of iron binding protein is needed and is under study. A better understanding of the relative availability of iron in various foodstuffs is needed in order to make recommendations regarding the appropriate diet for people in various parts of the world where iron deficiency is an important nutritional problem. An understanding of the mechanism by which various constituents of the diet and secretions of the gut affect iron absorption is important for the achievement of this goal.

Project 3A062110A822, MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 125, Hematology of nutritional deficiencies of military importance

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PROJECT. 3A062110A823
MILITARY PSYCHIATRY

Task 00
Military Psychiatry

1000

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6470	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISPN INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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. CONTRIBUTING	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 71	8	220
FISCAL YEAR CURRENT 72	8	220

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Stress Performance; (U) Human Volunteer; (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, psychosomatic disease, or drug addiction, 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) 70 07 - 71 06 A survey of drug users was completed at Ft. Meade establishing incidence and prevalence figures. This is being followed up with interviews of individual users to establish contingencies of drug use. Work commenced investigating relevant variables in problems of race relations, and difficulties in readjustment of Vietnam returnees. A longitudinal study of Officer Candidates at Ft. Benning, Ga., was carried out during the 4th and 22nd week of training. Performance, social, psychological and endocrine variables are being correlated, and a follow-up of career performance is underway. Aggressive behavior of men in prison and early in their adolescence was studied and the latter found to correlate with testosterone. A study of endocrine responses to hospitalization in different groups of psychiatric patients admitted to WRGH was started. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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 68 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00, Military P. ch' itry

Work Unit 030, Military Psychiatry

Investigators.
Principal: Robert M. Rose, M.D.
Associate: LTC Avrom C. Segal, MC; MAJ Donald R. Bardill, MSC;
MAJ Jonathan F. Borus, MC; MAJ James T. Grosshans, MC;
MAJ Leo E. Kreuz, MC; MAJ Jay P. Mohr, MC;
MAJ Gene R. Moss, MC; MAJ Edgar P. Nace, MC;
CPT John W. Holaday, MSC; CPT Larry H. Ingraham, MSC;
Gloria J. Balcom, Ph.D.; and David H. Marlowe, Ph.D.

I. INTRODUCTION

Three major areas continue to be emphasized within the Department of Psychiatry. Studies have continued of effects of social and psychological stress on various hormones using animal and human studies. The application of behavioral principles to specific clinical problems, both neurologic and psychiatric, continues to be studied. Finally, increasing emphasis has been placed upon studies of military mental health principles and the delivery of services with a major focus on drug abuse problems.

II. HORMONES AND BEHAVIOR

Dr. Rose and MAJ Kreuz with CPT Jennings of the Department of Experimental Psychophysiology have studied the effects of stress on hormone production among officer candidates. A company of three platoons of approximately twenty men each was studied in Oct 1970 and March 1971 at Officer Candidate School (OCS), The Infantry School, Fort Benning, Georgia. These men were first studied during the fourth week of training (Oct 1970), generally rated as one of the most stressful periods, and during the twenty-second week, just prior to graduation. The latter time was used for comparison as men at this time were relaxed and confident of graduating. Numerous measurements were made, including the study of urine and blood samples. CPT Jennings administered a questionnaire designed to assess coping ability during the first period of study to see if ability to graduate could be predicted. All men were also interviewed by the three investigators for approximately forty-five minutes each during this first study period. An analysis of endocrine, behavioral, and performance data is in process and should be completed by January, 1972. It is already apparent that the stress of OCS provoked a significant decrease in plasma testosterone levels in men studied to date. This is the first direct documentation of this relationship in the medical literature. Plans have been made

to follow members of this company to assess their performance on their first duty assignment in order to compare this performance with leadership ratings during OCS as well as to test the possible predictive value of various psychological and endocrine measures made during training.

In collaboration with CPT Robert Lenox, a resident in Psychiatry at Walter Reed General Hospital, a pilot study of behavioral-endocrine correlates of psychiatric patients was begun. Plasma samples on a number of patients with different diagnoses were drawn over a two to six week period, and the relationship between testosterone and cortical response to hospital admission will be studied along with endocrine correlates of changes in clinical state.

MAJ Kreuz and Dr. Rose completed a study of 21 adult men institutionalized at the Patuxent Institution for repetitive anti-social behavior. Aggressive and anti-social behavior were assessed in terms of (a) fighting in prison; (b) scores on psychological tests designed to measure aggressiveness; and (c) history of past criminal behavior. Plasma testosterone was drawn on each man six times during a two week period. Levels of testosterone did not correlate with either frequency of fighting in prison nor with psychological tests. However, they did correlate with age of onset of more violent, anti-personal crimes. Men who had earlier histories of rape, murder, assault, etc., tended to have higher plasma testosterone levels in adulthood. These findings suggest that in individuals who are susceptible to anti-social or criminal behavior by virtue of social and familial variables, the rise of testosterone associated with normal onset of puberty may serve to stimulate such aggressive behavior.

III. CLINICAL STUDIES

MAJ Mohr has undertaken the study "Traumatic Brain Injury: A Combined Behavioral and Neurophysiologic Study" in the Neurosurgical Ward, Walter Reed General Hospital. MAJ Peter Williamson, Department of Experimental Psychophysiology, is co-investigator on this project. Patients with traumatic aphasia and language disorders from other etiologies and control subjects are being studied in daily sessions. Patients studied include those who have suffered gunshot wounds to the left, right and bilateral sides of the head, multiple fragment wounds from booby traps, head injury from auto accidents, and a single case of Herpes Simplex encephalitis. He has also undertaken work at the University of Maryland and has studied two patients at that institution. One was a person with aphasia whose case was presented at the annual meeting, American Academy of Neurology in April 1971. Another patient was found to have neuropathologic findings which appeared to be unique among cases of hypotension.

MAJ Moss has been collaborating with John J. Boren, Ph.D., Professor of Psychology at The American University, for the past two and one-half years studying the application of operant technology to psychiatric treatment using video tape. This study has resulted in: (1) programs for specifying psychiatric problems and treatment goals in operational terms; (2) a functional approach to psychiatric classification; and (3) specific operant treatment procedures for certain psychological disorders. He has also been involved in the development of bio-feedback apparatus for conditioning of blood pressure in hypertensive patients. This apparatus has been designed and constructed. However, due to problems in the pick-up mechanism, it has not proven of sufficient reliability to begin studying patients. Evaluation is underway on a new low-frequency microphone that will provide sufficient reliability of pick-up to begin a formal medical study.

IV. FIELD AND PREVENTIVE STUDIES

An effort was made to establish a relationship between the Department of Psychiatry and MEDDAC, Fort Meade, Maryland for the purpose of developing a comprehensive community mental health center in a military environment which could serve as the focal point of research into factors associated with prevention of behavioral disturbances, the treatment of behavioral casualty, and the assessment of delivery of mental health services. LTC Segal, MAJ Bardill, CPT Ingraham and Dr. Marlowe primarily were responsible for developing this program. Data were being gathered about the functioning of the clinic and certain clinical programs, especially in the area of drug and alcohol abuse, and the establishment of inpatient services at Kimbrough Army Hospital were proceeding well when the project was abruptly ended in January 1971.

The major focus of field studies then switched to that of programs related to drug abuse. MAJ Grosshans, an epidemiologist from the Division of Preventive Medicine had become chief of an interdisciplinary team within the Department of Psychiatry whose task it was to develop and carry out field research on the problem of drug abuse. They were to determine aspects of the problem which required further research, and were to develop a set of research protocols which would provide an integrated and comprehensive clinical research program. This basic research was, in part, to provide the basis of information necessary to the organization of an intervention program for drug abuse initially scheduled to begin in FY 1973. MAJ Grosshans, MAJ Nace, and CPT Ingraham have developed a series of proposals to study individual and small group aspects of drug use as well as several epidemiologic surveys.

MAJ Nace has undertaken a study to analyze drug-taking patterns of active duty personnel in their pre-service civilian lives and in their military careers, and the relationships between drug-taking behaviors and other behavioral variables. A semi-structured interview was constructed for use with drug abusing patients who presented themselves for help at mental hygiene units and fifty-six multi-drug using patients were interviewed extensively, both at Fort Meade, Maryland and Fort Carson, Colorado. This group, in addition, received and completed several standardized, non-projective psychological tests. Preliminary analysis of the data is being undertaken. To prepare for the study of factors which influence the delivery of mental health services at a large Army post, MAJ Nace also analyzed two hundred medical charts at Mental Hygiene Consultation Service, Fort Meade.

MAJ Bardill has completed a preliminary study relating to factors influencing the delivery of mental health services. The results of this preliminary study revealed three areas of importance in the delivery of mental health services: the existing Army regulations specific to MHCS's and their impact on MHCs work, the training and supervision of enlisted specialists, and the tasks and services performed by enlisted specialists. Based on preliminary findings, a more comprehensive investigation of delivery of mental health services has been designed and is being implemented.

MAJ Borus is continuing his work in studying returnees from the Republic of Viet Nam. He is studying a prospective sample of 1010 men during their first six months at a unit at Fort Meade. Approximately 75% of these men had returned from Viet Nam within a short time prior to their selection for the study. He has completed data collection and is analyzing the results of interviews with seventy Viet Nam returnees from the above listed sample. He is also continuing work on his Racial Perceptions Inventory (RPI). He composed, pilot tested, revised and retested the RPI at Fort Benning, Georgia in Nov 1970 and April 1971 and presented the preliminary results to a Department of Army Race Relations Conference at Fort Monroe, Virginia in Nov 1970. He also has provided preliminary data to the Air Force University at Maxwell AFB, Alabama for their race relations course to senior officers. He is presently scaling the items in the inventory, working out details to use the RPI further at the request of Schofield Barracks, Hawaii, Fort Lewis, Washington, and Walter Reed General Hospital.

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00, Military Psychiatry

Work Unit 030, Military Psychiatry

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6456	71 07 01	SD-DRAR-R(A)S

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISSEM INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	3A062110A823	00	031
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(3)			

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 00940C Man-Machine Relat 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 07	CONT	DAI	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) 71	4.5	135
FISCAL YEAR (CURRENT) 72	5	150

19. RESPONSIBLE DOD ORGANIZATION
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PII Redacted

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) Stressful environments, physiological conditions 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 and learning methods, computer processing, and electrophysiologic techniques behavioral and psychologic events are isolated and correlated. Sensory and informational inputs are correlated with behavioral and neurophysiologic events; physiologic rhythms and imposed work-rest schedules are studied under specified normal and stressful conditions to characterize altered patterns of performance.

25. (U) 70 07 - 71 06 Progress includes the development of cortical evoked potential techniques for clinical use with traumatically brain injured patients. The isolation of physiological and performance power spectra characterized the sleep deprived and moderately tranquilized subject. Electroencephalographic and visual studies have related brain activity to general intelligence and some specific relations have been found between evoked cortical potentials and signal detection and processing. Developmental changes in the brain's electrical activity are also under study. A study of perceived stress, methods of coping and performance in Officer Candidate School is nearing completion. In preliminary work, brief changes in stress-related autonomic functions have been found to relate specifically to different types of information processing. Both autonomic reactions and processing may be related to degree of obesity. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 70 - 30 JUN 71.

DD FORM 1498

Project 3A062110A823, MILITARY PSYCHIATRY

Task 00, Military Psychiatry

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

Investigators.
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Description.

The elucidation of the biological substrates of stress and performance decrements is important to both military psychiatry and to the performance of normal military missions. The basic research strategy of this work unit is psychophysiological in nature, i.e. concurrent measures of psychological processes and physiological activity are made. Within this approach applicable techniques range from those of clinical medicine to those of mathematics and statistics. Specific areas of current research include: 1) vigilance performance decrement and environmental-physiological decoupling due to sleep deprivation and different work-rest cycles; 2) stress-related reactions of the autonomic nervous system and of the central nervous system to different information processing demands; 3) the relation of evoked cortical potentials and performance in traumatically brain-injured patients; 4) studies of brain-behavior relationships during problem solving as a function of age, mental deficiency, and normal development; 5) brain wave control and performance change; and 6) various comparative studies of visual information processing and microelectrode recording techniques.

Progress.

1. Spectral analysis of physiological and behavioral responses to stress.

The development of power spectrum analysis as a useful technique in detecting the more subtle effects of stress on physiology and performance has continued. Work has been completed on a project designed to show the techniques's validity in evaluating drug effects. The main hypothesis under study here is that a drug effect and its time course can be determined by evaluating the effect of periodic (at regular intervals) administrations on the power spectrum of the measure of interest. The periodic drug administrations have the effect of inducing a rhythm which can be detected by the spectrum analysis at a

frequency specified by the interval between administrations. This technique, it is felt, will have many advantages, not the least of which is that the location of the drug effect on the spectrum can be specified by the investigator. The data analysis has begun on this project, but is as yet incomplete.

Another project has been completed using the same analytic technique to delineate the endogenous rhythms which may be present in heart rate and spontaneous galvanic skin responses under conditions of bed rest. There is little if any work in the literature concerning the presence of ultradian (less than 24 hours) biological rhythms in these response systems. Another important aspect of this study is the determination of the degree of coupling between these systems. The data analysis has revealed strong evidence for the existence of a 90 minute periodicity, possibly related to the REM cycle of sleep which also has an approximate 90 minute rhythm.

A recently completed study examined the effects of signal frequency in multiple channels on vigilance performance. Signal presentation was designed to parallel operant reinforcement schedules. The results indicate that the classic operant approach to data analysis can be greatly extended and refined by the use of spectrum analysis of response rates. Briefly, different response rates on a fixed interval schedule of reinforcement were accurately reflected by the presence or absence of harmonics in the spectral analysis. Regarding a most controversial issue in operant conditioning, this study revealed, through the use of coherence analysis, that there is coupling between schedules in a concurrent schedule paradigm. In terms of vigilance performance on the three signal displays, these results indicate that individuals involved in similar tasks are greatly influenced by the schedule or rate of signal occurrences on a single channel. This operates on his observing behavior by controlling the rate of observing the other channels being monitored. Such control would lead to detection errors on the two subsidiary or slow-rate channels.

2. Autonomic correlates of information processing.

The reactions of autonomic nervous system have been related classically to stress. Recent evidence (Lacey, 1967) has indicated, however, that relatively non-stressful information processing may cause autonomic reactions. These non-stress reactions may either exacerbate or lessen classical autonomic stress reactions. Of equal importance, certain autonomic reactions may facilitate information processing. Thus, change in autonomic function due to stress may serve to impair information processing by interfering with the autonomic aspects of this cognitive functioning.

Research on autonomic correlates of information processing was designed initially to identify processing tasks which were associated with large autonomic reactions. Reactions that were different for different tasks were sought. Preliminary data suggested that three types of function -- input, storage, and manipulation -- were associated with distinctive autonomic reactions. Furthermore, these differences were influenced by constitutional factors -- specifically, overweight persons reacted differently from underweight persons (see Jennings & Orr, 1970, Schachter, 1970). The preliminary results are now being verified. Experiments are being planned manipulating autonomic and stress states.

3. Evoked brain responses to meaningful stimuli as indicants of neural processing.

The Averaged Evoked Potential (AEP) is extracted by computer techniques from the bio-electrical activity recorded from the scalp of human subjects. Since they originate in the brain, they may be influenced by a wide variety of parameters including the physical characteristics of the stimulus and the psychological characteristics of the stimulus, e.g. the history of the individual and the meaning of the stimuli to him. The data coming from our laboratory has supported both kinds of influences on the AEP. This work has several ramifications: (a) the AEP represents one of the few techniques now available for looking at brain functioning that may be related to psychological processes in man and (b) it also represents one of the few techniques for examining sensory systems of the brain of man.

One of our lines of investigation has been to fractionate pre- and post-stimulus effects. In considering the effects of the physical parameters of stimuli, it has been found necessary in work at all levels from single-cell recording to psychophysical judgements to deal not just with the discrete stimuli but also to be concerned with the recent history of the system. For example, in the visual system, not only the characteristics of the light flash influence the response, but also the adaptation level which has resulted from the recent light history. This has been sometimes categorized as the state of the system (light- or dark-adapted state, neural or photochemical adaptation, etc.).

In a similar way the psychological state of the organism is important to the psychological or behavioral response to stimulation. In considering brain processing of stimuli, what the brain does with stimulus information depends on the task that the brain is performing at that time, i.e. on the relevance of this information to the task. If differences in brain processes are found in two situations, is the difference related more funamentally to post-stimulus processes or to the state existing prior to the stimulus presentation?

From the standpoint of studying neural processing, it is advantageous to be able to control what processing takes place and when it takes place. If we unleash the processing by a discrete stimulus which requires the subject to perform some particular task, then we are able to study neural processing. AEP experiments often appear to have those two characteristics, but unfortunately, both the what and the when in many of these experiments have lacked precision. What processing has often been "controlled" by presenting "meaningful" stimuli, e.g. pictures, words, etc. by asking the subject to pay attention, to count the stimuli, to guess the next stimulus, etc. When the processing takes place has presumably been controlled by presenting a discrete stimulus. The time of stimulation and averaging may be precise, but the time of processing often is doubtful. Because the AEP depends on averaging, the same stimulus has often been repeated throughout a run. This procedure makes it unnecessary to even "perceive" the stimulus. If the stimuli are presented rhythmically, a counting task can successfully tolerate large gaps of not sensing. Presenting "meaningful" stimuli without designating a task, does not seem like a good way to control what processing, if any, is resulting from the stimulus. Nor does asking the subject to guess which stimulus will appear next exercise much control over what the brain does when the stimulus does appear, since the guess is occurring prior to the stimulus. What an adequate experimental design ought to do is: (a) assure that each and every stimulus is processed in a defined way; (b) separate effects due to general states of the organism, such as activation level; (c) separate effects due to recent history from post-stimulus effects; and (d) have adequate controls for the physical stimulus reaching the receptors.

In the experimental design that we have used, the particular stimuli were changed in a random fashion with each presentation. These stimuli were incorporated in a task that required that the relevant stimuli be perceived and processed in a particular way and the subject's performance at the designated tasks was measured. The stimuli were presented as brief flashes, so no second looks and no AEP effects could be produced by retinal scan from eye movements. The control responses were obtained to stimuli mixed in the same trials (less than 1 sec. apart) so that any effects may not be attributed to general states that may change over a slower time course. In addition, a measure of the general brain state was taken in the form of the alpha electroencephalograph (EEG) score. By presenting two relevant and two irrelevant stimuli on each trial, our analysis yielded two post-perceptual processes and three expectancy states due to sequence biases. These distinctions are important because they relate to the general question of the specificity of AEP effects, "Can the AEP tell us about the particular post-stimulus brain processing or only about the excitability of the brain in being prepared or ready for the stimulus?" It is the possibility of pre-stimulus differences that has clouded interpretations attempting to relate AEP differences to

post-stimulus processes. A variety of response measures at various brain sites showed that both pre- and post-stimulus effects occur and that relatively subtle task effects may be detected by these techniques. Measurements have been made in terms of response area and amplitudes at fixed latencies, e.g. 105 and 225 msec. The differences obtained with these measures indicate that more elaborate multivariate techniques might be useful for extracting the information available in these complex waveforms. It is concluded that AEPs may be used to study brain processes that are of a higher order than simple attention, uncertainty (information theory), or stimulus gating.

4. Control of alpha EEG.

Previous work in this and other laboratories has demonstrated that various EEG activities, e.g. alpha EEG, are modulated by various mental and problem solving tasks. Our work in the past has focused on changes in the EEG that are controlled by the subject's tasks, i.e. does task processing cause a change in brain activity? More recently, there has been a great deal of interest in the possibility of running the causality chain in the other direction, i.e. can a person's internal state (and thus his task processing) be controlled by controlling brain activity. Since alpha EEG is associated with an awake, relaxed state, inducing alpha may serve to decrease stress states or to increase activation from sleepy states. By applying learning and association techniques (Peper, 1970) it may be possible for subjects to learn to produce, at will, high or low amounts of alpha activity and perhaps, thereby, to correspondingly alter their internal state and subsequently their task performance. These hypotheses are being tested by experiments in progress.

One of the important considerations in the study of brain waves is the role of the visual system and eye activity, since the classical way of producing alpha EEG is to close the eyes or extinguish the room illumination. Recent work had suggested that much of the increase in alpha EEG activity following eye closure in the classic "eyes open - eyes closed" test is due to the accompanying tendency for the eyes to turn upward. This hypothesis was tested by measuring the amount of alpha activity (using an electronic analog scorer previously developed in this laboratory) present with eye positions "ahead" and "up" in the light and in the dark and comparing these results with changes in alpha activity with eyes "opened" and "closed" in the light. Data were obtained from a preliminary group of 13 subjects and a main group of 22 subjects, where an electrooculogram measure of eye position and visual targets in the light conditions were added. The data were evaluated by analyses of variance of both groups as well as on the repeated measures of each of the 35 subjects individually. The dark condition was especially critical for testing the hypothesis since it removed the possibility of differential visual input which might be associated with ocular orientation. The data consistently showed that

the position of the eye was not a primary factor in controlling alpha EEG, while eye closure and darkness dramatically increased alpha activity. Differential eye input appears to be a major factor.

Several reports (Lippold and Novotny, 1970; Lippold, 1970) have cast doubt on the cerebral origin of two prominent features of the EEG, alpha and kappa activity, which are hypothesized to be artifacts related to eye activity. Although various reports have implicated eye activity, their specific hypotheses have varied, including emphases on the corneoretinal potential of the eye, tremor of the extraocular muscles, eye position, accommodation, and eyelid flutter. If their interpretations are correct, it is necessary to reconsider the meaning of the EEG as a measure of cerebral function. Hypotheses that alpha and kappa EEG activity are due to eye activity go considerably beyond the well-recognized fact that eye activity sometimes may intrude on EEG records. It has previously been shown that kappa EEG activity is independent of eye or lid flutter (Armington and Chapman, 1959).

In a recently published study (Chapman, Ernest, & Cavonius, 1971), data were presented from subjects with either one eye or both eyes absent. Evidence of normal EEG activity from these subjects further argues against the eye artifact hypotheses noted above. Following the hypothesis that "the source of current for generating the waveform of alpha rhythm is the standing potential across the eye," we recorded the EEG of a man without ocular globes. Although this subject had relatively little alpha or kappa activity, both brain activities were modulated by the tasks in the same way shown by the majority of normal subjects in our previous work with normals. Since both eyes were missing, these data demonstrate that neither alpha nor kappa EEG activity must originate from the standing potential across the eye; nor must they depend on eye position, accommodation, or eye movement. It has been suggested that the extraocular muscles that remain after enucleations may be the source of alpha activity. To study this question we investigated bilateral EEG activity from two subjects who had undergone complete exenterations of one orbit. Neither subject showed amplitude differences from the two sides as great as predicted by the ocular artifact hypotheses. These data, together with those previously reported, lead to the conclusion that alpha and kappa activity are not directly dependent on the corneoretinal potential of the eyeball, tremor of the extraocular muscles, eye position, accommodation, or eyelid flutter.

Research has begun using what is sometimes known as "biofeedback," in this case feeding back to the subject information as to when he does or does not have alpha brain waves. The major experimental question is whether the subject can utilize this information to alter the amount of alpha EEG he exhibits and, if so, whether this changes his reported states and task performance. Previous work in this area

has not come to grips with the experimental design problems, so considerable attention has been devoted to the design of the necessary controls. In order to monitor the possible changes that might take place with repetition, these controls are a regular part of the training sequence during each session. Because of the possible interaction of visual stimuli with the alpha system, auditory stimuli are being employed as carriers of the feedback. More data needs to be gathered before a reasonable conclusion can be offered to the question posed above.

5. Developmental studies of the clinical uses of electroencephalography.

The study of sensory evoked EEG potentials in normal children and in children with sensory and neurological disorders has continued. The laboratory has also performed EEG sleep and evoked potential studies of patients with Down's syndrome and in patients with dystonia musculorum deformans being studied for the possible therapeutic benefits of L-Dopa administration.

Several investigations of the characteristics of click and flash evoked potentials in normal children have been completed (Barnet, Ohlrich, and Shanks, 1970, 1971; Barnet, 1971 (a)). Statistically significant age-related changes have been demonstrated. Latencies of several components decrease with age and amplitude increases. Response complexity increases with age.

A study comparing the effects of repetitive auditory stimulation on normal and 21-Trisomic, 1-year and under children was completed. Mongoloid infants do not show response decrements with repeated stimulation as do normal infants of the same age. Mongoloid responses do not show well-developed late components (P_3) by 6 months of age as do normal infants. The hypothesis that abnormal sensory processes in the mongoloid may be related to their cognitive defect was advanced.

A report describing serial changes in visual evoked responses (VER) of patients with sudden onset of cortical blindness was published (Barnet, Manson, Wilmer, 1970).

A report on EEG audiometry in 100 abnormal and 141 normal-hearing infants and children was completed (Barnet, 1971(a)). Test-retest data are included. Our experience indicates that EEG evoked response audiometry is a valuable clinical hearing test.

A double-blind study of the effects of administered 5-Hydroxytryptophan on the development of children with Down's syndrome is in progress.

A preliminary report was made on a mentally retarded child with chronic high levels of blood 5 HT (serotonin). This child had abnormal EEG sleep patterns. Para-chloropheonylalamine (pCPA) was administered to this patient and the effects on EEG sleep and biochemical measures of 5 HT metabolism were studied (Coleman and Barnet, 1970).

6. Intelligence quotient and auditory evoked potentials.

The Army faces complex personality, cultural, and military problems in determining the performance capabilities of its personnel, both during normal duty assignments and following injuries, especially neurological injuries. The extremes of ethnic and economic backgrounds make any single test a prejudicial procedure for some individuals or groups (Montague et al., 1957; Williams et al., 1959). Reports of a new EEG correlate of intelligence seemed to open possibilities for a culture-free assessment of I.Q. and performance capability (Whittaker, 1967; Ertl, 1969). The simplicity of the new assessment was one of its most inviting features. It involved flashing light stimuli and recording of the evoked electrical response from the brain by electrodes attached to the scalp by a clay paste. There was no task involved and this prevents the falsification of intelligence level by the subject. Furthermore, the electronics needed could be reduced in size and easily made portable for checking decrements of capacity under drugs or other stresses. The values of this testing system, particularly to military testing, is quite clear.

In order to assess the military feasibility of this testing system, enlisted men in a holdover status were tested. The men volunteered to participate. The use of enlisted men added a secondary advantage in time and economy because of the availability of a test performance record in the form of their Army General Classification Test score to correlate with the evoked response data. Ideally, the electronic I.Q. determination could be made in a few minutes but our procedure used a three-determinations format to permit statistical evaluation. In the first stage of the experiment we processed 60 subjects. We were unable to replicate the Whittaker results (Whittaker, 1967) which had been the model for our procedures, so we analyzed the evoked response data in a variety of ways, eventually using a total of 130 correlates in the computer analysis. Thereafter, a new experimental approach was initiated based on our few positive correlates and also on new reports from other laboratories entering this field of investigation (Conners, 1969; Chalke & Ertl, 1965; Callaway, III, 1968; Weinberg, 1969; Plum, 1968; Rhodes et al., 1969; Stone, 1968; and Straumanis, et al., 1965). We ended up with a multi-faceted experiment requiring more than an hour of evoked response gathering and, of course, a much greater time commitment for data measurement and computer programming. Among the parameters added to the experiment were right and left cortex evoked responses, pattern vs. diffused light stimuli, and filtered vs.

unfiltered evoked responses. In addition, the subjects were given a greater number of light flashes. Sixty-seven additional subjects were processed. The data have only been partially analyzed. The important correlates of the first part of the experiment were confirmed in absolute value but a difference in direction of the relationship has occurred which has not yet been resolved or explained. The added features of the experiment have not been completely analyzed due to the quantity of data involved as well as computer programming problems. The data analyzed indicate that both the hemispheric recording sites and intra-session temporal sequences are implicated in the correlation between intelligence quotient and evoked potential.

7. Electrophysiological studies in brain damaged subjects.

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 likelihood of survival following brain injury. One major consequence has been the estimated four-fold increase in cases of total disability following wounds in Vietnam as 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. The 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 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.

This project is progressing well. To date, approximately eight brain-injured subjects and ten controls have been studied with visual, auditory and somatosensory evoked potentials. Some anticipated results have not been realized. Specifically, we have not observed the enhancement of the visual evoked response with meaningful or significant stimuli. The reason for this is not clear. Modifications of experimental design are presently being done in hopes of rectifying this problem.

We have observed some unexpected results in several of the patients studied. These results are being carefully examined in order to determine their possible significance.

It is anticipated that sufficient data from control subjects and patients will have been obtained within the next three months to allow the initial phase of this project to be completed.

8. Microelectrode dye techniques developed in the frog diencephalon.

The goal of this project is the development of 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.

Completion of this project has been delayed by our lack of success in obtaining anatomical confirmation of the recording sites. We have been successful in developing a microelectrode dye marking technique but for reasons that are not entirely clear, we have been unable to obtain satisfactory recordings using the dye-filled electrodes. Work is currently under way attempting to solve this problem. Hopefully, this study can be completed within the next month.

9. Studies on a primitive photoreceptor in Limulus.

A great deal of our present knowledge about the visual process has been derived from the work of Hartline and co-workers who studied the functional organization of the compound lateral eyes in horse-shoe crab (Ratliff, 1965). The studies have been extremely rewarding in answering questions concerning how the single visual receptors respond in photic stimuli, and how receptors are organized in functional units. Interaction among various receptor units was also studied. These interactions quantitatively describe lateral inhibition as the physiological basis for the age-old phenomenon of Mach bands observed in humans. Thus, the compound lateral eye of the horseshoe crab has provided us with a convenient experimental preparation for studying complicated visual mechanisms in a simpler system than man. In recent years, we have investigated the median dorsal ocellus in Limulus with respect to its photic properties. The ocelli are simple eyes and are presumably evolutionarily more primitive than the compound eyes. They contain two spectral mechanisms presumably housed in two receptor types, near ultraviolet and visible (Chapman and Lall, 1967). It is hoped that the ocellus would provide an experimental preparation where the interaction between two receptor types would be studied on a cellular level.

Last year we concentrated on studying the dark-adaptation in the median dorsal ocellus for these two types of receptors. The method employed was to record the EEG elicited by illumination from the intact ocellus. A dark-adapted median ocellus was light-adapted for short duration times (30, 60 and 120 sec.) with either near ultraviolet light (300-420 nm) or visible light (>450 nm). The log threshold of a 360 nm or 530 nm test light (for a criterion response of 50μV, - constant response method) as a function of time in the dark after chromatic light adaptation was plotted. The overall dark-adaptation curves for "UV" and "visible" spectral systems (i.e. receptors) did not differ significantly from each other. The threshold changed over 4 to 5 log units. The UV receptor system appeared to dark-adapt slightly faster than the visible one. With short-duration light adaptation flashes, both "UV" and "visible" receptors were dark-adapted in about 40 - 50 minutes.

10. Electronic instrumentation.

Four advances in electronic instrumentation relevant to this work unit occurred. The first of these is a simulator of normal EEG sleep during sleeping and waking. This instrument assists in the calibration and testing of measurement devices. The device uses integrated circuit amplifiers to filter high frequencies from a standard white noise source. Different stages in the filtering simulate the waking and sleeping EEG.

The second device is a random pulse generator to control the sequencing of experimental events. Constructed of readily available semi-conductor devices, the pulse generator summates several semi-dependent uni-junction oscillators to produce an output pulse that is random within an adjustable window.

The third device is a calibrator and tester for differential laboratory amplifiers used to transform biological signals. This is a highly flexible unit which allows the user to manipulate seven important parameters of input test signals for the biological amplifier.

The fourth is a simple device to convert triangle waves to sine waves. By using a differential amplifier in its amplitude limiting region, high harmonics of the triangle wave fundamental are removed and a high-purity sine wave is produced. The simplicity and compactness of this device recommend it for a wide variety of bio-instrumentation applications.

11. Opthalmologic hypoxia studies.

This project assessed the effects of hypoxia on certain aspects of vision. A common concomitant of performance in special and thus, stressful environments, e.g. high-altitude, is a decrease in environmental oxygen leading to hypoxia. Decreases in visual

sensitivity associated with hypoxia were reported in last year's progress report. This project is now completed and the research has been published (Ernest, 1971; Ernest, 1971; Ernest & Clamann, 1971; Ernest & Potts, 1971; Rosenberger & Ernest, 1971).

Project 3A062110A823, MILITARY PSYCHIATRY

Task 00, Military Psychiatry

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

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PROJECT 3A062110A824
IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00
Ionizing Radiation Injury, Prevention and Treatment

1024

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6471	71 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. ORG'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	621110A	3A062110A824	00	055
b. CONTRIBUTING				
c. [illegible]	CDOG 1212B(21)			

11. TITLE (Procode 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 71	6	150
	CURRENT 72	6	150

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Rothe, W. E. COL DA

NAME:

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procode 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. Procode 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 a 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) 70 07 - 71 06 The synthesis of new aminoalkylaminoalkylphosphorothioates is continuing. Adamantane and amidino derivatives, and symmetrical and unsymmetrical aminoalkyl disulfides continue to show promise as radioprotective drugs. Emphasis is being placed on orally effective agents. Dogs were protected by a combination of WR 2822, MEA and PAPP. Monkeys were protected by WR 77913. Thiazolidines which are protective in mice have failed to protect dogs or monkeys. The clinical protocols for WR 2529 and WR 2721 have been revised, and these drugs are scheduled for clinical evaluation. Animals have tolerated chronic intravenous administration of WR 2823 at 100 mg/Kg/day, and an IND has been prepared for clinical studies. Compound WR 149024 is undergoing preclinical evaluation. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

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
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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. At the present time one compound is being studied in the clinic and three others have received final approval to begin clinical studies.

Chemistry

II. Chemistry - Contract Synthesis Program

As of the end of FY-71 there are five active synthesis contracts and one contract for the synthesis of potential antiradiation agents on a preparative (pilot plant) scale. The contract with Parke-Davis was terminated in August, while the New England Nuclear Corporation contract for the synthesis of radiolabled compounds is now operating on an individual compound basis.

During FY-71 there were 141 potential antiradiation agents submitted for testing, the vast majority of which were target compounds. Thirteen compounds were re-synthesized on a larger-than-laboratory scale under the preparative contract.

Emphasis has been placed on the evaluation and exploitation of current leads. Interest continues in compounds possessing the

adamantane moiety as well as those having an aminoalkylaminoethyl or propyl backbone. The difficulties associated with the synthesis and assay of the important phosphorothioate functional group have been investigated.

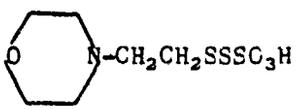
All in all, the vigorous offensive to develop potent antiradiation agents is being carried forward unabated on all fronts.

III. Chemistry - Organic Synthesis Section (WRAIR)

The investigation of reactions leading to novel and improved antiradiation agents has continued to yield interesting findings in sulfur chemistry. In the study of the conversion of aminoalkanethiosulfuric acids to their corresponding disulfides, it was found that the action of hydrogen sulfide in aqueous or aqueous-alcoholic solution readily accomplished this end according to the following equation if the amino group was mono- or disubstituted:

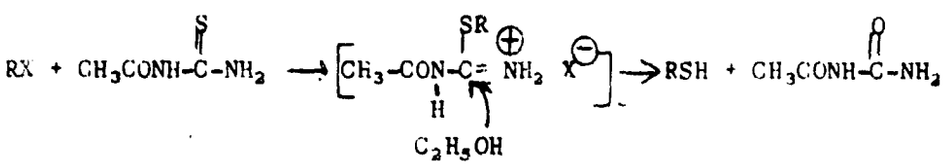
$$2\ RNHCH_2CH_2SSO_3H + 3H_2S \longrightarrow (RNHCH_2CH_2S-)_2 \cdot H_2S_2O_3 + 2H_2O + 3S^{o}$$

The amino-disulfides were obtained as their stable thiosulfuric acid salts, some of which have already shown excellent antiradiation activity. Of the tertiary-aminothiosulfuric acids studied, only the N,N-dimethyl derivative behaved toward hydrogen sulfide as indicated in the above equation. Other tertiary-aminoalkanethiosulfuric acids led to tetrasulfides, while diheptylaminoethanethiosulfuric acid gave a tetrasulfide coordinated with six molecules of hydrogen sulfide. This highly unusual brilliant yellow compound is surprising stable at room temperature. 2-Morpholinoethanethiosulfuric acid, on hydrogen sulfide treatment, yielded a mixture of the corresponding trisulfide and sulfenylthiosulfuric acid:



There are only one or two other examples of 2-sulfenylthiosulfates in the chemical literature.

A new synthesis of thiols is now being developed on the basis of our earlier observation that S-methyl derivatives of acylthioureas react with hydroxylic compounds. The best conditions for the generation of a thiol involve heating under refulx an alkyhalide with 1-acetyl-2-thiourea in ethanol (cf. equation).



Primary halides give about an 80% yield of thiol after 24 hours, however, the reaction with secondary halides is slower. The distinct advantage of this thiol synthesis over the usual method which utilizes thiourea is that basic conditions, known to accelerate the oxidation of thiols, are not needed.

IV. Rodent Testing Program

Chemical compounds synthesized under government contract for the Army's Antiradiation Drug Development Program were evaluated for radioprotective activity by the Rodent Testing Section of the Division of Medicinal Chemistry. Additional compounds were screened for radioprotective activity in mice by the Woodard Research Corporation, Herndon, Virginia under the direction of Dr. Henry Horn. The Woodard Laboratory primarily screened off-the-shelf compounds. Active agents were further evaluated at Walter Reed. The Woodard contract has been terminated, and all screening in mice is now done in-house. Evaluation of radioprotective activity is based upon the survival of treated mice to 30 days after exposure to lethal whole-body radiation. Both treated and control mice are exposed simultaneously to either 1000 R of Cobalt-60 gamma radiation or 825 R of 250 KVP x-radiation All irradiated control mice die within 30 days. Initially the compounds are administered 15 or 30 minutes before irradiation at two dose levels - the maximum tolerated dose and one-half of that dose. Female ICR mice 8 to 9 weeks old, from the WRAIR colony are used in radiation studies. Preliminary toxicity studies are performed using male mice. Woodard Laboratories used ICR "Astro" mice obtained from Charles River Mouse Farms, Inc.

After screening, active radioprotective compounds are studied to determine the duration of action, radiation dose reduction factor and optimum route of administration. As much data as possible regarding acute toxic and pharmacologic effects of each compound is recorded at the time of the primary test.

During the period of this report two factors curtailed the screening program: 1. Shut down of the Cobalt-60 irradiator during December and January and 2. the presence of intercurrent infection in mice obtained during part of this report period. Prior to and following the reloading of the cobalt source, the mice were found to harbor bacteria which enhanced their radiosensitivity.

Since February 1971, the new source has been calibrated, and LD_{50} determinations on the mice have been performed. The dose rate of the cobalt irradiator is now 232 rads/min., about 7 times greater than before. LD_{50} evaluations are listed below:

Mouse Strain	Age & Sex (wks)	Dose Rate	LD_{50} ± 2SE
ICR/FG	8-9 F	35 R/min	800 ± 31
ICR/FG	9-10 F	35 R/min	810 ± 20
ICR/FG	11-12 F	35 R/min	820 ± 19
ICR/FG	10-11 F	234 R/min	840 ± 17
ICR/FG	10-11 F	234 R/min	800 ± 25

The testing activities of the WRAIR and Woodard Laboratories during

the past fiscal year are summarized in the table below. Primary, confirmatory and secondary tests are included in the total number. The number of active compounds reflects re-testing of compounds previously known to have activity as well as new compounds with activity.

Summary of Antiradiation Testing

Rodent Testing Section, WRAIR

Test	Number of Tests	Good Activity [1]	Slight Activity [2]
Primary - Intraperitoneal	84	33	19
Primary - Oral	49	15	17
Other	3	2	0
Total	136	50	36

Woodard Laboratories

Primary - Intraperitoneal	1077	199	
Total	1213	249	36

[1] Greater than 50% survival after lethal irradiation.

[2] Less than 50% survival after lethal irradiation.

V. Radiation Protection in Dogs

Selected compounds which have shown promising radioprotective activity in mice were selected for further evaluation in dogs. Prior to radiation test, preliminary acute toxicity studies were performed. Radioprotection studies were generally performed with the maximum tolerated drug dose.

Healthy, mature beagle dogs weighing 9 to 12 Kg were exposed to a lethal dose of 650 rads of gamma radiation in the wood-lined exposure room of the Triga Mark IV Nuclear Reactor at the Harry Diamond Ordnance Laboratory Facility. During exposure, the dogs were confined in lucite cages arranged in a four cage array parallel to the gamma isodose curve produced by the reactor flux. The mid-line of each of the cages was 130 centimeters distant from the tank wall. With the reactor operating at 250 kilowatts of power at the steady state, the gamma dose rate at the mid-line was between 100 and 108 R per minute as measured by a tissue equivalent ionization chamber. By maintaining a thickness of 70 centimeters of tank water between the core of the reactor and the exposure room, the neutron contribution to the total dose was selectively reduced to less than 2% of the gamma dose. Neutron energies were predominantly thermal.

The $LD_{50}/30$ days was 432 R under these conditions (Table 1). Forty-seven control dogs were irradiated during this fiscal year and none survived. The mean survival time of controls was 12.0 days (Table II).

The results of all drug experiments in dogs conducted during the past year are summarized in Table III. A comparison with the results in mice is indicated. One drug combination study was performed in 1971. The results are shown in Table IV.

VI. Radiation Protection in Monkeys

Healthy, Macaca mulatta monkeys weighing 1.5 to 3.5 Kg were exposed to a lethal radiation dose of 850 rads of gamma radiation at the Harry Diamond Nuclear Reactor Facility. With this experimental configuration at 250 kilowatts of steady state power, the dose rate was 95 R/min. The animals were restrained upon plexiglass boards with leather and "Velcrow" harnesses. These harnesses permit the experiment to be conducted with a minimum of animal handling and allow greater ease in drug administration. Some problems were encountered with exposure times during fiscal 1971 resulting in delivery of doses of radiation either greater or less than desired. These problems appear to have been solved at this time. A comparison of the results obtained in monkeys with those obtained in mice is shown in Table V.

RADIATION LETHALITY STUDY
DORF REACTOR-GAMMA
DOGS
Table I

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	47	47	100

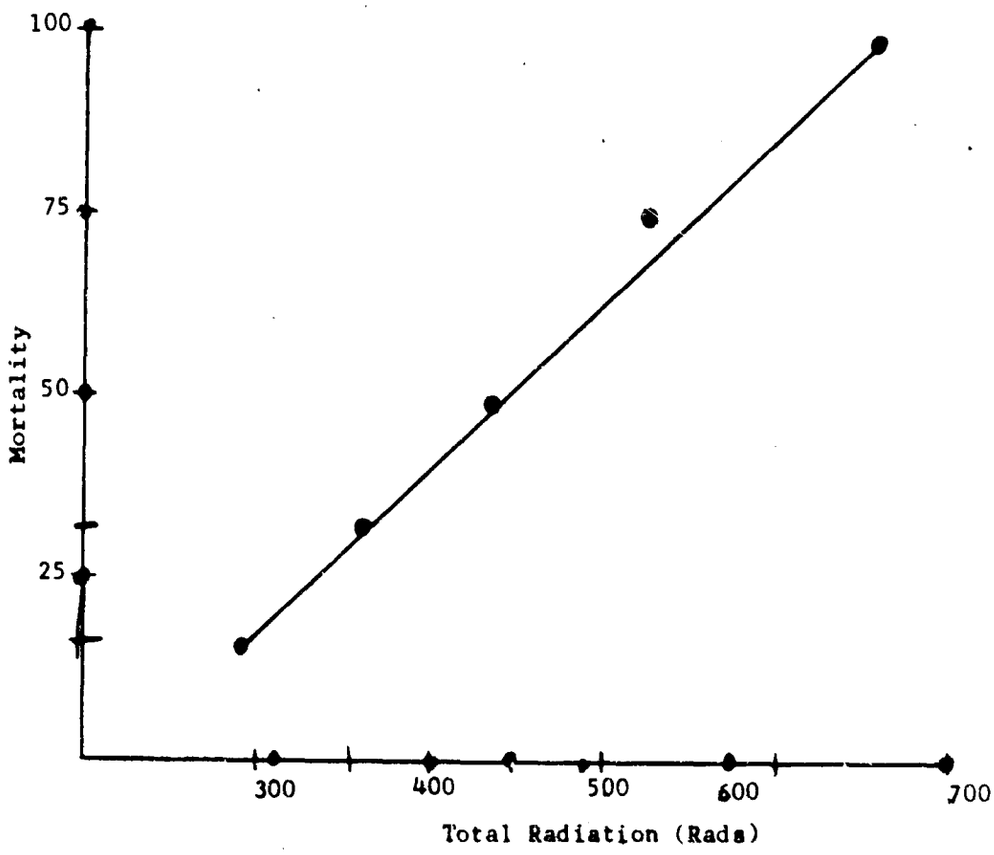


Table II
Control Mortality
Dogs
650 R Gamma Radiation

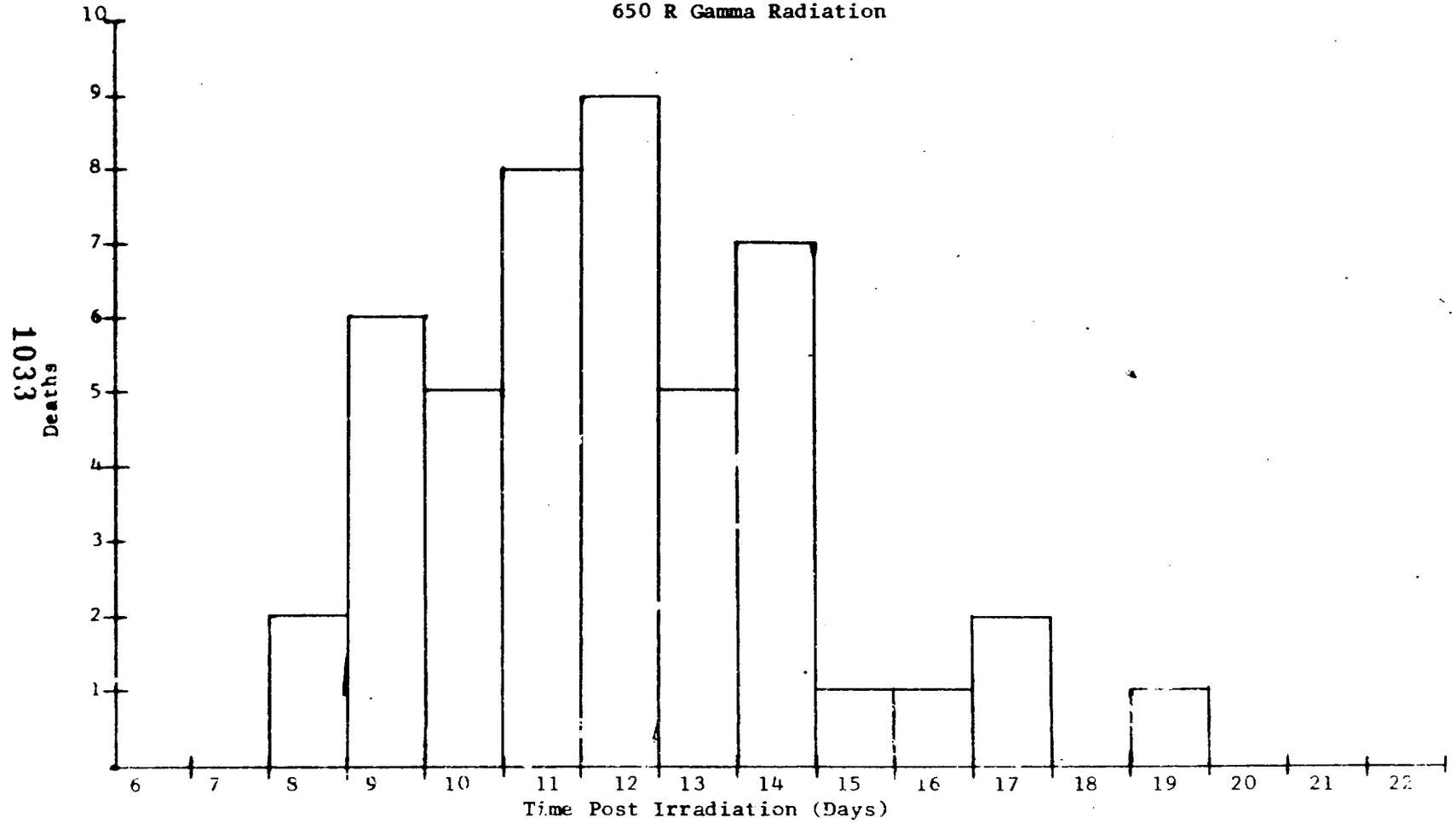


Table III

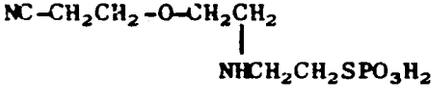
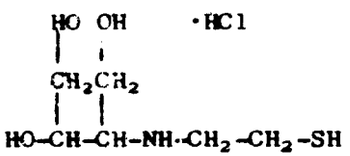
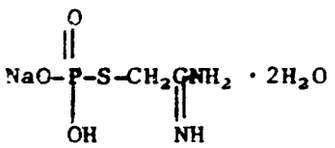
Structure	WR No.	Test Animal	Test Dose	Route	Minutes-Pre Irradiation	No. of Animals	% Survival
	46359 AB	Dog	150	IV	30	7	0
	(AV00693)	Dog	200	IV	30	8	12.5
	46359 AA	Mouse	300	IP	30	15	7
			600	IP	30	15	60
			250	IP	15	15	20
			500	IP	15	15	27
			1000	PO	15	15	20
			1000	PO	30	15	13
	2347	Dog	350	IV	30	9	56
	2347 A	Mouse	600	IP	15	15	73
			1200	IP	15	15	100
	2347 H	Mouse	150	IP	30	15	0
			160	IP	30	15	0
			300	IP	30	15	7
	108,250 AE	Dog	15	IV	30	6	0
		Mouse	37.50	IP	30	15	93
			75.00	IP	30	15	53
			25.00	IP	30	15	20
			50.00	IP	30	15	67
			37.50	PO	30	15	73
			75.00	PO	30	15	80
			60.00	PO	30	15	67
			120.00	PO	30	15	93
			25.00	PO	15	15	0
			25.00	PO	30	15	7

Table III (Continued

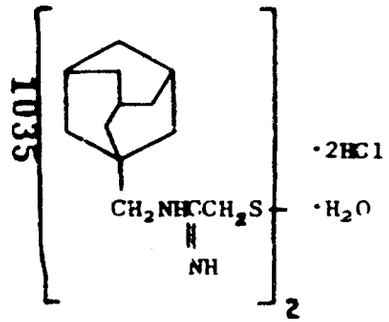
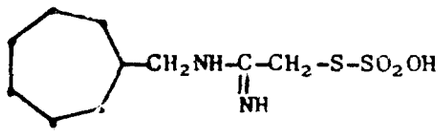
Structure	WR No.	Test Animal	Test Dose	Route	Minutes-Pre Irradiation	No. of Animals	% Survival
	108,250 AA	Mouse	90.00	IP	15	10	0
			45.00	IP	15	15	67
			22.50	IP	15	15	7
			12.25	IP	15	15	0
			350.00	PO	30	15	93
			350.00	PO	60	15	73
	155,419	Dog	12.50	IV	30	9	0
		Mouse	12.50	IP	15	15	0
			25.00	IP	15	15	7
			100.00	PO	15	15	33
			200.00	PO	15	15	0
			75.00	PO	15	15	13
			75.00	PO	30	15	33
			50.00	PO	15	15	13
			50.00	PO	30	15	80
			25.00	PO	15	15	13
			25.00	PO	30	15	47
			12.50	PO	15	15	0
			12.50	PO	30	15	0
	150,637	Dog	12.50	IV	30	9	0
	150,637 AB	Dog	12.50	IV	30	9	11
	150,637 AA	Mouse	7.50	IP	15	15	67
			15.00	IP	15	15	87
			3.75	IP	15	15	0
			7.50	IP	15	15	80
			75.00	PO	30	15	47

Table III (Continued

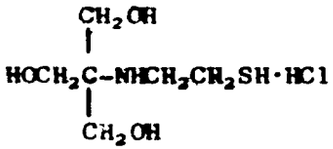
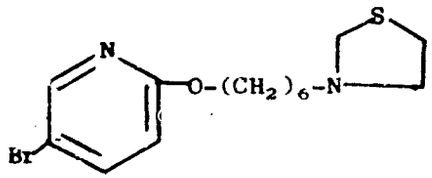
Structure	WR No.	Test Animal	Test Dose	Route	Minutes-Pre Irradiation	No. of Animals	% Survival
	150,637 AA	Mouse	75.00	PO	60	15	27
			50.00	PO	15	15	47
			50.00	PO	30	15	80
			12.50	PO	15	15	0
	1616	Dog	200	IV	30	9	33
	1616 A	Mouse	750	IP	15	20	95
			750	IP	15	20	100
	1616 B		500	IP	15	6	50
			1000	IP	15	6	50
	1616 C		400	IP	15	10	33
			800	IP	15	15	60
			500	IP	30	15	53
			1000	IP	30	15	33
			500	SC	30	12	0
			500	SC	60	15	0
			400	IP	15	15	40
			200	IP	15	15	0
	91496	Dog	35	IV	30	9	0
		Mice	150	IP	30	15	47
			150	IP	60	15	13
			100	IP	15	15	80
			50	IP	15	15	0
			300	PO	15	15	47
			300	PO	30	15	67

Table III (Continued)

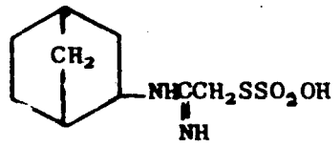
Structure	WR No.	Test Animal	Test Dose	Route	Minutes Pre-Irradiation	No. of Animals	% Survival
	91496	Mice	150	PO	15	15	27
			150	PO	30	15	60
			75	PO	15	15	20
			75	PO	30	15	27
	113191	Dogs	25	IV	30	6	0
		Mice	30	IP	30	15	80
			30	IP	60	15	67
			30	IP	90	15	33
			15	IP	30	15	0
	77913 AD	Dog	375	IV	30	15	60
$H_2NCH_2CH(OH)CH_2SPO_3HNa$		Mouse	750	PO	15	15	47
	77913 AC	Mouse	1500	PO	60	15	60
			1500	PO	90	15	67
			400.00	IP	30	15	100
			800.00	IP	30	15	100
			500.00	IP	30	13	100
			1000.00	IP	30	14	100

Table IV

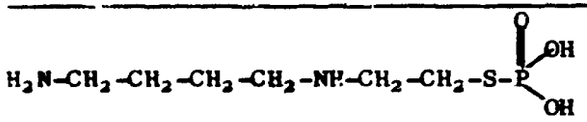
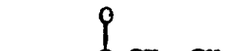
Structure	WR No. Comb.	Test Animal	Test Dose	Route	Minutes Pre Irradiation	No. of Animals	% Survival
	2822	Dog	100	IV	30	9	100
	347		50				
	302		5				

Table V

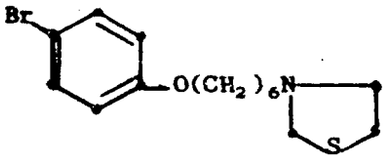
Structure	WR No.	Test Animal	Test Dose	Route	Minutes Pre-irradiation	No. of Animals	% Survival
	91496	Monkey	50.00	IV	30	6	50
			500.00	PO	45	6	0
	91496 AA	Mouse	50.00	IP	15	15	0
			100.00	IP	15	15	80
			300.00	PO	15	15	67
			300.00	PO	30	15	93
			300.00	PO	60	15	87
			300.00	PO	90	15	13
			100.00	IP	30	15	67
			100.00	IP	60	15	53
			150.00	PO	30	15	47
			150.00	PO	60	15	13
	91496 AB	Mouse	50.00	IP	15	15	7
			100.00	IP	15	16	75
			75.00	PO	15	15	0
			75.00	PO	30	15	0
			300.00	PO	15	15	60
			300.00	PO	30	15	67
	91496 AC		50.00	IP	15	15	27
			100.00	IP	15	15	47
			300.00	PO	15	15	47
			300.00	PO	30	15	67
			150.00	PO	15	15	27
			150.00	PO	30	15	60
			75.00	PO	15	15	20
			75.00	PO	30	15	27

Table V (Continued)

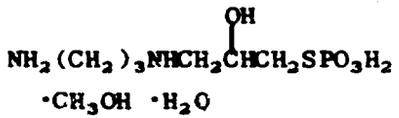
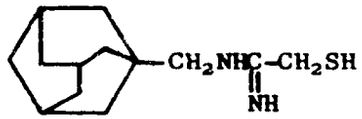
Structure	WR No.	Test Animal	Test Dose	Route	Minutes Pre-irradiation	No. of Animals	% Survival
	142,469 AA	Monkey	75.00	IV	30	6	17
		Mouse	200.00	IP	15	15	100
			400.00	IP	15	15	100
			50.00	IP	15	15	0
			100.00	IP	15	15	47
			300.00	PO	30	15	0
			600.00	PO	30	15	0
	109,342	Monkey	125.00	PO	?	6	0
			125.00	PO	60	6	17
	109,342 AA	Mouse	6.00	IP	15	15	67
			12.00	IP	15	15	93
			1.50	IP	15	15	0
			3.00	IP	15	15	33
			30.00	PO	15	15	93
			30.00	PO	30	15	93
			12.00	IP	30	15	67
			12.00	IP	30	15	80
			12.00	IP	30	15	73
			30.00	PO	60	15	67
			30.00	PO	90	15	33
	109,342 AB	Mouse	16.00	IP	30	6	83
			32.00	IP	30	6	17
			8.00	IP	30	6	100
			60.00	PO	15	15	80
			60.00	PO	30	15	80

Table V (Continued)

Structure	WR No.	Test Animal	Test Dose	Route	Minutes Pre-irradiation	No. of Animals	% Survival
	109,342 AB	Mouse	30.00	PO	15	15	80
			30.00	PO	30	15	80
			15.00	PO	15	15	40
			15.00	PO	30	15	73
			7.50	PO	15	15	7
			7.50	PO	30	15	0
			30.00	SC	180	15	67
			30.00	SC	240	15	13
			20.00	SC	15	15	53
			20.00	SC	30	15	93
			20.00	SC	60	15	67
			20.00	SC	120	15	20
			20.00	SC	180	13	31
			20.00	SC	240	15	33
			10.00	SC	120	15	0
			10.00	SC	180	15	0
			6.00	IP	15	15	80
			12.00	IP	15	15	93
			1.50	IP	15	15	0
			3.00	IP	15	15	7
			12.00	IP	90	15	27
			12.00	IP	120	15	0
			12.00	IP	30	15	27
			12.00	IP	60	15	60
			6.00	IP	90	15	0
			6.00	IP	120	15	0
			12.00	IP	30	13	100
			12.00	IP	60	13	69

Table V (Continued)

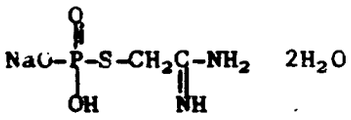
Structure	WR No.	Test Animal	Test Dose	Route	Minutes Pre-irradiation	No. of Animals	% Survival
	139,342 AB	Mouse	12.00	IP	60	15	73
			12.00	IP	90	15	20
$H_2NCH_2CHOHCH_2SPO_3HNa$	77913 AD	Monkey	700.00	IV	30	6	100
			1500.00	PO	30	6	17
			1500.00	PO	60	6	0
			700.00	IV	30	6	100
			1500.00	PO	60	5	[illegible]
			700.00	IV	30	1	100
		Mouse	750.00	PO	15	15	47
	77913 AC	Mouse	1500.00	PO	60	15	60
			1500.00	PO	90	15	67
			400.00	IP	30	15	100
			800.00	IP	30	15	100
			500.00	IP	30	13	100
			100.00	IP	30	14	100
	108,250 AB	Monkey	50.00	IV	30	6	50
		Mouse	37.50	IP	30	15	93
			75.00	IP	30	15	53
 $2H_2O$			25.00	IP	30	15	20
			50.00	IP	30	15	67
			37.50	PO	30	15	73
			75.00	PO	30	15	80
			60.00	PO	30	15	67

Table V (Continued)

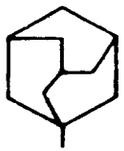
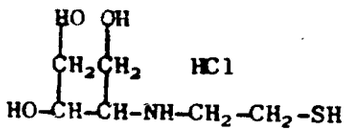
Structure	WR No.	Test Animal	Test Dose	Route	Minutes Pre-irradiation	No. of Animals	% Survival
	108,250 AB	Mouse	120.00	PO	30	15	93
			25.00	PO	15	15	0
			25.00	PO	30	15	7
	108,250 AA	Mouse	90.00	IP	15	10	0
			45.00	IP	15	15	67
			22.50	IP	15	15	7
			12.25	IP	15	15	0
			350.00	PO	30	15	93
			350.00	PO	60	15	73
	155,419	Monkey	50.00	IV	30	6	50
 $\cdot 2HCl$ $\cdot H_2O$		Mouse	12.50	IP	15	15	0
			25.00	IP	15	15	7
			100.00	PO	15	15	33
			200.00	PO	15	15	0
			75.00	PO	15	15	13
			75.00	PO	30	15	33
			50.00	PO	15	15	13
			50.00	PO	30	15	80
			25.00	PO	15	15	13
			25.00	PO	30	15	47
			12.50	PO	15	15	0
			12.50	PO	30	15	0
 $HO-CH-CH-NH-CH_2-CH_2-SH$ HCl	2347	Monkey	250.00	IV	30	6	0

Table V (Continued)

Structure	WR No.	Test Animal	Test Dose	Route	Minutes Pre-irradiation	No. of Animals	% Survival
	2347 A	Mouse	600.00	IP	15	15	73
			1200.00	IP	15	15	100
	2347 H	Mouse	150.00	IP	30	15	0
			160.00	IP	30	15	0
			300.00	IP	30	15	7

VII. Liver Perfusion Studies

It has been reported in the Annual Report for 1970 that it is possible to administer a supra-lethal dose of nitrogen mustard to the liver through the hepatic artery and effect survival of an experimental dog provided the portal circulation was perfused with a nitrogen mustard antagonist. The antagonist previously reported consisted of a mixture of D-L-threo-3 (2 mercaptoethyl) amino, 1, 2, 4 butanetriol hydrochloride (WR 2347) at a dose of 300 mg/Kg and cysteine hydrochloride (WR 348) at a dose of 400 mg/Kg.

During 1971, six control dogs were perfused with 1.5 mg/Kg of nitrogen mustard through the hepatic artery. No nitrogen mustard antagonist was administered through the portal system. Three of the six control animals survived. It had been shown previously that all dogs survive when a 1.0 mg/Kg dose of nitrogen mustard is administered through the hepatic artery. These experiments demonstrate that 1.0 mg/Kg of nitrogen mustard administered intra-hepatically is the maximum which can be administered safely. Since nitrogen mustard can be administered intra-hepatically in a dose of 2.0 mg/Kg when the portal circulation is also perfused with a nitrogen mustard antagonist, the amount of this drug which could otherwise be administered is approximately doubled. These results offer encouragement for improved drug therapy of hepatic neoplasia by permitting the administration of a dose level which is presently prohibitive due to toxicity.

VIII. Anti Allergic Properties of Radioprotectant Aminothiols

In Vivo

The test system being utilized for these studies (as described in last year's report and cited in the literature) tended toward inconsistent test results. Not only was difficulty encountered obtaining repeatable results with the drug treated monkeys, but the control animals also showed erratic responses.

Therefore, an attempt was made to standardize the *in vivo* test system by systematically varying the test procedures. The amount of serum, antigen and Evans Blue Dye were independently changed in several instances, yet no truly acceptable method was found. Time before and after challenging were varied. The mixing of antigen with the dye and serum with the dye was attempted without success. Changing the serum from the local intradermal location to an intravenous injection, and changing the antigen from intravenous to local intradermal injection was also attempted but to date has been unsuccessful.

Currently, several other modifications are being tried in an attempt to find a method that can be consistently utilized as a drug screen. These methods include aerosol sensitization with ragweed antigen;

serial injections of ragweed antigen IV; sensitization of the conjunctiva with ragweed antigen; and inhalation of ragweed pollen.

The compounds tested during this fiscal year were WR 377, WR 1963, WR 698, WR 638, WR 2721, WR 2977, WR 49808 and WR 4853. In the event that a suitable test procedure is found, these drugs will be retested for confirmation of anti-allergic activity.

Anti-allergic Properties of Radioprotectant Aminothiols In Vitro

Skepticism of the realiability of the in vitro test system has arisen because nearly all drugs tested gave a positive result (i.e. blocked allergic response) to some degree. We currently suspect that during incubation of the serum with the test drug in the in vitro portion of the test, the serum protein (antibody) is denatured or in some other way inactivated, and therefore does not respond to the antigen when tested in vivo. Since the in vitro test appears to have little value in predicting the in vivo antiallergic activity of test compound, the in vitro drug screen has been abandoned early in the fiscal year.

IX. Evaluation of Radiation Compounds as Possible Therapeutics for Wilson's Disease

Various compounds are being tested in order to obtain insight into the mechanism of binding of D-penicillamine to copper and to find a more effective chelator of copper for the treatment of Wilson's Disease (hepatolenticular degeneration).

Additional compounds tested were: N-(2-mercaptoethyl)-1, 3, propanediamine (WR 1065); B-mercaptoethyl-B phenylethylammonium chloride ·1/2 H_2O (WR 633); N-(2-mercaptoethyl) ethylenediamine .2HCl (WR 884); 2-(2-mercaptoethyl amino)-2- hydroxymethyl-1,3 propanediol .HCl (WR 1616); N-(2-mercaptoethyl) 1, 5 pentanediamine .HCl (WR 1729); and 2-(2-mercaptoethyl) amino 1,3 propanediol .HCl (WR 2753). These compounds were tested for their ability to chelate neonatal hepatic mitochrondrocuprein of rats ranging from 12-48 hours of age. Atomic absorption spectrophotometry was utilized to determine the amount of hepatic mitochrondrocuprein chelated. As noted by H. Porter, the principal subcellular site of hepatocuprein in Wilson's Disease is the mitochondrial fraction. This is distinctly similar to the distribution of copper and serum ceruloplasm in the newborn. Porter found 35% of the total copper located in the mitochondrial fraction. Experimentally, our results yielded a total of 33%. Evans, etal have also found the hepatic mitochondrocuprein distribution and serum mitochondrocuprein distribution and serum ceruloplasm in rat neonates to coincide with that of Wilson's Disease.

Procedures have been developed in our laboratory to determine the amount of copper present in each centrifugal fraction (homogenate, nuclei, combined supernates, microsomal and mitochondrial) thus

Table I

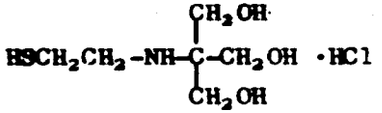
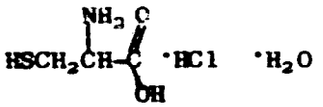
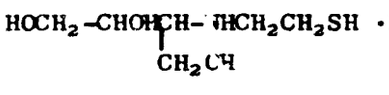
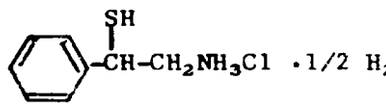
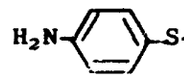
WR No.			% Chelated Copper
WR 1605	$HSCH_2CH_2NHCH_2CH_2CH_2NH_2$	N-(2-Mercaptoethyl)-1,3 propanediamine	75%
WR 377		D-penicillamine	39%
WR 2346	$HSCH_2CH_2NHCH_2CH_2COOH$	N-(B-Mercaptoethyl)- B alanine	39%
WR 1616		2-(2-Mercaptoethylamino)-2-hydroxy-methyl-1,3 propanediol ·HCl	38%
WR 348		L-cysteine ·HCl Hydrate	32%
WR 347	$HSCH_2CH_2NH_2 \cdot HCl$	2 Mercaptoethylamine ·HCl	10%
WR 1729	$H_2N(CH_2)_5NH-CH_2CH_2SH \cdot 2HCl$	N-(2-mercaptoethyl)-1,5 Pentanediamine ·HCl	7%
WR 2347		dl-Threo-3-(2-Mercaptoethyl) Amino-1,2,4-Butanetriol ·HCl	0%
WR 2753	$HOCH_2CH(CH_2OH)NHCH_2CH_2SH \cdot HCl$	2-(2 Mercaptoethyl) amino -1,3 propanediol ·HCl	0%
WR 633		B-Mercapto-B phenylethyl ammonium chloride ·1/2 H_2O	0%

Table I (Continued

WR No.			% Chelated Copper
WR 884	$H_2N-CH_2CH_2-NH-CH_2-CH_2-SH \cdot 2HCl$	N-(2-Mercaptoethyl) ethylenediamine $\cdot 2HCl$	0%
WR 15206		Para-aminothiophenoxy acetic acid	0%

allowing for determination of the total amount of copper present prior to chelation. After freezing each of the fractions they are lyophilized for 24 hours, ashed at 500°C for 24 hours, diluted with 0.5 N HNO_3 and analyzed for total copper through atomic absorption spectrophotometry. This procedure was used on the compounds recently tested and also on those compounds reported in last year"s Annual Report. See Table 1 for the total percentage of chelated copper by selected compounds.

N-(2-mercaptoethyl)-1,3 propanediamine (WR 1065) is approximately twice as effective in copper chelation as D-penicillamine. Toxicity testing of WR 1065 has been completed. Toxicity studies of several other agents are in progress. In one experiment 10 mg/Kg of WR 1065 was administered IV daily for 20 days to a Rhesus monkey. No toxic side effects of the drug were noted at this dose level after 90 days of observation.

Presently, WR 1065 and several related chemical agents are being studied to investigate the possibility of utilizing this compound in the treatment of Wilson's Disease.

X. Snake Antivenin Studies

A. Relative Potency of Polyvalent Antivenin (Lots 143 E and 143 F) Against Naja Naja and B. Caeruleus Snake Venoms

Assays of 3 lots of polyvalent antivenin were performed for the Department of Pharmacology to confirm the data published by the supplier, Haffrine Institute, Bombay, India. Naja naja (Indian) dried venom was obtained from USAMRL, and the Miami Serpentarium, Miami, Florida (Lot # NS6TV). B. caeruleus lyophilized venom (Lot 108A15) was obtained from the Miami Serpentarium. The lyophilized venoms were reconstituted in physiologic saline prior to use. LD_{50} estimations of these venoms were determined by administering graded doses in volumes of .2 ml into the tail veins of 8-9 week old female albino mice. The lyophilized polyvalent antivenins (Lots 143 E and 143 F) were reconstituted with 10 ml sterile distilled water prior to use and administered IV immediately after administration of a lethal dose of venom. The volume of antivenom injected did not exceed .1 ml. Mortality was recorded over a 24 or 48 hour period.

Results

The survival of mice given Naja venoms are shown in Tables 1 and 2. The approximate LD_{50} value of Indian Naja venom (Lot USAMRD) was 8 µg/mouse (.32 mg/Kg) while the LD_{50} of Lot NS6TV was 6 µg/mouse (.24 mg/Kg). A dose of 10 µg (.4 mg/Kg) produced 100 per cent mortality. The LD_{50} of B. caeruleus venom (Lot 1-8A15) (Table 3) was 2.35 µg/mouse (.09 mg/Kg). At 5 µg (.20 mg/Kg) 100 per cent lethality was observed.

Table 1

Mortality and Survival Times of Mice Given Graded Doses of Naja naja Venom (USAMRD Lot) Intravenously.

Assay Date	No. of Mice	µg Venom*	ml Venom	Percent Mortality	Survival Time
20 June 1970	10	.625	.2	0	--
	10	1.250	.2	0	--
	10	2.500	.2	0	--
	10	5.000	.2	0	--
	10	7.500	.2	40	Over Night
	10	10.000	.2	100	33-103 Min.

Table 2

Mortality and Survival Times of Mice Given Graded Doses of Naja naja Venom (Lot NS6TV) Intravenously.

Assay Date	No. of Mice	µg Venom*	ml Venom	Percent Mortality	Survival Time
19 Aug 1970	10	2.5	.2	0	--
	10	5.0	.2	40	86-120 Min. &Over Night
	10	7.5	.2	100	31-120 Min. &Over Night
	10	10.0	.2	100	29-63 Min.

Table 3

Mortality and Survival Times of Mice Given Graded Doses of B. caeruleus Venom Intravenously.

Assay Date	No. of Mice	µg Venom*	ml Venom	Percent Mortality	Survival Time
21 Jun 1970	10	1.25	.2	0	--
	10	2.50	.2	60	240-360 Min.
	10	5.00	.2	100	109-240 Min.
	10	10.00	.2	100	60-107 Min.
	10	25.00	.2	100	48-60 Min.

* Per 25 gm mouse

Table 4

Efficacy of the Polyvalent Antivenin Lot 143 F in Preventing Lethality in Mice After IV Administration of Naja naja (Indian-USMARL) Venom.

Assay Date	No. of Mice	µg Venom*	ml Venom	ml Anti-venin	Percent Mortality	Survival Time
20 Jun 1970	10	25	.2	.1	10	22 Min.
	10	50	.2	.1	40	13-65 Min. & Over Night
	10	75	.2	.1	70	16-49 Min.
	10	100	.2	.1	100	6 Min.

Table 5

Efficacy of the Polyvalent Antivenin Lot 143 F in Preventing Lethality in Mice after IV Administration of B. caeruleus (108A1S) Venom

Assay Date	No. of Mice	µg Venom*	ml Venom	ml Anti-venin	Percent Mortality	Survival Time
21 Jun 1970	10	50	.2	.1	00	--
	10	75	.2	.1	40	87 Min. & Over Night
	10	100	.2	.1	80	85 Min. & Over Night

Table 6

Protective Effect of Antivenin 143-E Against Lethality Induced by Naja naja and B. caeruleus Snake Venoms in the Mouse

Assay Date	Venom	µg* Venom	ml Venom	ml Anti-venin	No. of Mice	Percent Mortality	Survival Time
19 Aug 1970	Naja	25	.2	.1	10	0	--
		50	.2	.1	10	54	35-180 Min.
		75	.2	.1	10	100	4-136 Min.
20 Aug	B. caeruleus	50	.2	.1	10	30	55-120 Min. & Over Night
		75	.2	.1	10	40	250 Min. & Over Night
		100	.2	.1	10	100	50 Min. & Over Night

*per 25 gm mouse

Table 7

Relative Potencies of Different Lots of Polyvalent Antivenin Against Naja and B. caeruleus Venoms

Venom	A.V. Lot #	Venom LD_{50} (µg/mouse)	LD_{50} of Antivenin Treated Mice (µg/mouse)	Dose Reduction Factor
Naja	143-F	8	60	7.5
	143-E	5.4	48	8.8
B. caeruleus	143-F	2.35	80	33.3
	143-E	2.35	74	30.8

For the determinations of relative potency of the anti-venins, the lowest dose selected for both venoms was 25 µg/mouse. Lot 143 F completely neutralized 25 µg of Indian naja venom in the mouse (Table 4) and raised the LD_{50} of venom injected mice by 7.5 times (Table 7). Against B. caeruleus venom, antivenin of Lot 143 F neutralized 50 µg venom (Table 5) and increased the LD_{50} of the venom in treated mice by over 30 times (Table 7). The potency of Lot 143 E was similar to Lot 143 F in reversing the lethality induced by both venoms (Tables 6 and 7).

Discussion

According to the brochure accompanying the antivenins, the potency (as assayed by the Haffkine Institute) is considerably higher against Naja than against B. Caeruleus venom. Their findings indicate that 1 ml of the polyvalent antivenin will neutralize .60 mg Naja naja venom. In our assays, 1 ml of the antivenin neutralized not more than .35 mg Naja venom in mice.

Haffkine Institute claims the antivenin will neutralize .45 mg of B. caeruleus venom. We have found comparable potency. In our assays, 1 ml neutralized at least .5 mg of B. caeruleus venom. We conclude that these polyvalent antivenins are truly extremely potent and effective antidotes against Naja naja and B. caeruleus venoms.

B. Evaluation of Potencies of Several Lots of a Monovalent Antivenin Against Agkistrodon rhodostoma venom

The purpose of these investigations was to assay the potency and specificity of 3 lots of a monovalent antivenin prepared and submitted by the Science Division, Thai Red Cross Society. The antivenin was specific for Malayan pit viper venom (Agkistrodon rhodostoma).

Materials and Methods

Lyophilized Agkistrodon rhodostoma venom (Lot # AR6TU) was obtained from the Miami Serpentarium, Miami, Florida. Prior to use, the venom was dissolved in physiologic saline. Freshly prepared solutions were used for each assay. The lyophilized antivenin samples were reconstituted with 10 ml sterile distilled water prior to use, and thoroughly mixed and shaken to dissolve all particulate matter. Both the venom solutions and antivenins were kept cold throughout the assays.

ICR female mice 10-11 weeks of age, obtained from the Walter Reed colony were used in all experiments. The weight of the mice was 25-27 grams.

The toxicity of the venom was first established in groups of ten mice by injecting graded doses into the tail veins. The volumes injected did not exceed 0.2 ml. The relative potency of the antivenin was

determined by injecting mice with a 100 per cent lethal dose of venom followed by the intravenous injection of 0.1 ml of the antivenin. Those mice alive at 48 hours were counted as survivors.

Results

The results of these studies are summarized in Tables 1 - 3. Lots 4 and 6 were truly antidotal, and had a greater neutralizing capacity for reversing lethality of the venom than Lot 5. One ml of these antivenins neutralized at least 1.5 µg of venom. Their relative potencies (the ratio of the LD_{50} of controls to venom treated mice) were 168% and 150%.

Lot 5 appeared to be the weakest in potency (Table 2). One ml of this lot of antivenin neutralized only 1 µg of venom. Its relative potency was 100%.

Discussion

In these studies, 100-150 µg of A. rhodostoma venom killed 40-88% of mice given a single intravenous injection. Time to death following injection decreases with an increase in the amount injected.

Data on the neutralizing capacity of this type of antivenin were not previously available. Our results demonstrate that this monovalent antivenin is effective in reversing the lethality induced by Malayan pit viper venom in the mouse. The neutralizing potency does vary, however, among lots of the same antivenin. The toxicity of the venom in antivenin treated mice was decreased by factors of 1.50-1.68 for Lots 4 and 6, and 1.0 for Lot 5.

C. Observations on the Toxicity of Agkistrodon Rhodostoma Venom in Male and Female Mice

The effects of A. rhodostoma venom on the survival were compared in male and female adult mice by two routes of administration. The females were more resistant than the males by the intravenous route, while by intraperitoneal injection, there was no difference. The LD_{50} value for females (350 mice) was 123 micrograms or 4.9 mg/Kg; for males (240 mice) the value was 61.3 micrograms or 1.86 mg/Kg.

By the intraperitoneal route, the LD_{50} values were: For females (90 mice) 105 µg or 4.2 mg/Kg, and for males (89 mice) 108 µg or 3.3 mg/Kg.

The regression coefficients of the dose response curves by both routes were comparable for both sexes.

The males showed a shorter survival time than females after IV injection, but after IP injection their survival time was longer than

that of the females. Possible hormonal factors involved in these sex differences in response to venom are being investigated.

XI. Additional Projects

A. The toxicity of a phenanthrene methanol (WR 122455) synthesized for the malaria program was studied in the mouse. The results have been included in the IND reports of the Department of Pharmacology.

B. A study of the radioprotective activity of a 5-carbon aminothiol salt submitted by the French Department of Defense was performed and a detailed report of our findings was submitted.

XII. Effect of WR 2823 on Renal Damage From Tumbling Trauma

Background

Several antiradiation chemicals have been reported to prevent mortality in mice from tumbling injury. The mechanism of death from this type of injury is obscure as is the mechanism by which these agents prevent death from this and other experimental stress. The only grossly observable damage seen in mice subjected to tumbling stress is a non-contusive, hemorrhagic necrosis of the renal medulla. Since renal failure accompanies so many types of disease and shock; an effect of any of these antiradiation chemicals to prevent renal damage following stress could have crucial implications for therapeutic exploitation.

Methods

One half of the mice were treated with WR 2823 (50 mg/kg IP) 15 minutes before tumbling for 10 minutes; simultaneously, the remainder received water injections before tumbling. The survivors were sacrificed serially for observation of renal pathology. These studies were conducted in cooperation with the Division of Experimental Pathology.

Results

As reported before, WR 2823 prevented immediate and delayed mortality from tumbling. The degree of renal pathology was also less in the animals pretreated with WR 2823.

Discussion

We have resisted the temptation to call WR 2823 the panacea of the nuclear battlefield, but the therapeutic implications for the application of this type of drug in mass casualty situations to prevent mortality from disease and injury are staggering. Clearly, the key to the prevention of renal failure is the prompt establishment of renal function as evidenced by the results using diuretics. Conse-

quently, the timely application of this non-toxic agent as a prophylaxis could conceivably prevent significant numbers of renal failure cases associated with trauma and disease. This is clearly an area of potential clinical investigation following the proposed Phase I human studies.

XIII. Absorption, Distribution and Excretion of WR 109,343

Background

WR 109,342, given orally or intraperitoneally, was reported to afford prolonged protection against the lethal effects of ionizing radiation in mice. ^{35}S labelled WR 109,342 was administered in mice via IP and PO routes to test the hypothesis that this prolonged action was due to delayed excretion of the chemical agent.

Methods

^{35}S labelled WR 109,342 was administered at 30 mg/Kg (orally) and 12 mg/Kg (IP). Tissues and excreta were examined at various times following administration.

Results

Absorption, distribution and excretion patterns were similar following both routes of administration. The levels reached maximum at about 90 minutes and remained high from 15 minutes through 4 hours (IP) or 6 hours (PO). Excretion of label is nearly quantitative at 48 hours (75-85%).

Discussion

These data are consistent with good oral absorption of this agent and prolonged plasma and tissue levels as a result of slow excretion of the chemical in mice.

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	71 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
70 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. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede 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 N/A

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 71	3	100
	CURRENT 72	3	

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: BUESCHER, COL E. L.

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)

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NAME: MOON, A. P. DA

NAME:

PII Redacted

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) 70 07 - 71 06 Anti-macrophage globulin caused a marked decrease in circulating phagocytes in Plasmodium berghei infected rats, but parasitemia did not change even in several different dosage patterns. Anti-macrophage globulin did not block hyperimmune serum from lowering parasitemia in early infections. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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: E. H. Sadun, Sc.D., Lib. Doc.
Associate: M. A. Dunn; MAJ S. H. Lourie, MC

1. Immune response in rodent malaria modified by anti-cell globulins.

Anti-lymphocyte globulin (ALG) given to rats before infection with P. berghei causes an increase in both the number of parasites and duration of the parasitemia. The immediate effect on the course of infection is a more rapid climb of the parasitemia during the first 48 hours. The later effects are a markedly increased parasitemia, more severe anemia and prolonged patency. Most of the increased mortality after ALG treatment is in the latter period. The effect of ALG could be reversed by the administration of hyperimmune rat serum at the time of infection. The hyperimmune serum blocked both the early and late effects of ALG; the infection in animals treated with both ALG and hyperimmune serum was not different from controls. The role of phagocytosis in this system was studied with anti-macrophage globulin (AMG). The AMG caused a marked fall in circulating phagocytic cells. However, there was no alteration in the pattern of parasitemia when it was given in several dosage patterns. Furthermore, the anti-macrophage globulin did not block the activity of the hyperimmune serum in lowering the circulating parasite level in the early stages and there were no delayed effects on the course of the disease. These studies support the concept that humoral factors play a crucial role in protection against P. berghei, especially in the early stage. Further, the humoral factors are effective in the presence of impaired delayed hypersensitivity of decreased circulating phagocytes.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 106, Antigenic fractionatio. . serology of malaria

Literature Cited.

Publications:

Sadun, E. H., Hickman, R. L. and Briggs, N. T.: Use of immune globulin in the prevention and therapy of malaria. Immunoglobulins: 282-292, Ed. Ezio Merler, National Academy of Science, Washington, D. C., 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6506	71 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
70 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:

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 71	11	215
FISCAL YEAR CURRENT 72	11	215

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Drug Effects

(U) Plasmodium berghei; (U) Enzymes (U) Drug Analysis; (U) Mechanisms; (U) Drug Action;

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

23. (U) Chemotherapeutic agents particularly antimalarial drugs will be examined for their metabolic interaction with the biological environment. The mechanism of action of each drug against the malarial parasite will be biochemically defined.

24. (U) Plasmodium species in various animal hosts will be used to test organisms to evaluate intermediary metabolism of the parasite, and the responses of the host to the antimalarial agent under study. Analytical definitions of the chemotherapeutic agent and its distribution in biological tissues are studied. Synthesis of important metabolites will be done.

25. (U) 70 07 - 71 06 Studies with the CO_2 fixing enzyme phosphoenopyruvate carboxylase isolated from Plasmodium berghei have demonstrated the inhibitory effect of quinine, chloroquine, iron, copper and mercury while magnesium, manganese and cobalt are required for the enzyme to function. The effect of iron on parsite growth and multiplication has been examined and the data suggest that chloroquine increases intracellular iron thus inhibiting CO_2 fixing enzymes in the parasite with the resultant denial of essential intermediates such as oxaloacetate, amino, keto and other organic acids to the parasite. Studies involving diaminodiphenylsulfone (DDS) have been carried out analytically to define impurities found in DDS preparations. Four major impurities have been characterized and are either analogs of DDS or substituted chloro and hydroxyl derivatives. "In vitro" studies have established that DDS is indeed a potentiator of MHb formation through the formation of an N-OH derivative. "In vivo" studies of the interaction of chloroquine and primaquine with alcohol dehydrogenase has demonstrated that under the usual conditions, CP tablets have no effect on the oxidation of alcohol. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 June 71.

1061

*Available to contractors upon originator's approval

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

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 108, Biochemical effects and mechanism of action of chemotherapeutic agents

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC; Betty J. Boone, Ph.D.; Larissa DeBarre, M.D.; Seymour Garson, Ph.D.; CPT Paul A. Kramer, MSC; LTC Ting-Kai Li, MC; Elvio A. Levri, M.S.; Robert T. Lofberg, Ph.D.; Benjamin Mehlman, M.S.; Joseph T. Piechocki, M.S.; Patrick M. L. Siu, Ph.D.

DESCRIPTION.

The technical objectives of this work unit are to examine chemical compounds found to be of value in the treatment of malaria from the analytical chemistry point of view; develop methodology for the compounds of interest in the biological environment; examine and quantify the enzymes required for the metabolism of the compound; isolate and chemically define metabolic intermediates; and to study the intermediary metabolism of the parasite along with its response to the chemical compounds of interest.

PROGRESS.

1. Analytical Chemistry of Antimalarials.

During the reporting period efforts in this area have been limited to the following compounds: diaminodiphenylsulfone (DDS); diformyldiaminodiphenylsulfone (DFD); 6-bromo-α-di-n-heptylaminomethyl-9-phenanthrene methanol hydrochloride (WR 33063); and 6,8-dichloro-2-(3',4'-dichlorophenyl)-α-(di-n-butylaminoethyl)-4-quinoline methanol hydrochloride (WR 30090). The two sulfones when examined by thin layer chromatography were shown to have small quantities of impurities that do not exceed 5% of the total compound. DDS contains a total of four impurities namely 4-amino-4'-chlorodiphenylsulfone; 4-aminodiphenylsulfone; 2,4-diaminodiphenyl-sulfone; and 4-amino-4'-hydroxydiphenylsulfone. These similar structural analogs were in the original material used by the manufacturer and were not introduced in the pilling process. DFD was shown to have two impurities that were defined as the monofarinylderivative and DDS. These results are consistent with the slow conversion of the diformyl dapsone to the monformyl dapsone and then to DDS. WR 33063 and WR 30090 were examined by gas liquid chromatography and have not given any indication of inherent impurities.

2. Methodology Development.

Microbore column chromatography employing an ethyl acetate gradient coupled with an ultraviolet detection system has been established for chemical definition of the above compounds. This technique has been successful in the separation of impurities allowing them to be prepared in quantities suitable for ultraviolet, infrared and nuclear magnetic spin resonance measurements. Gas chromatography has been employed as the method of choice for the detection of WR 33063 and WR 30090. Both the free base and the silyl derivatives of both compounds can be quantitatively determined with a yield of 90% by means of flame ionization. However, precision and the limit of detection can be materially improved by use of a Ni-63 electron capture detector. Synthesis efforts have included the preparation of each of the impurities found in DDS and DFD to allow for chemical definition and characterization.

In addition, four antimalarials containing α-(di-n-butyl-aminomethyl) side chain, and varying substituents in 3- and 6-positions were selected for synthetic work. The selection of these compounds was based upon previous test results in the hopes that the compounds would show a high degree of antimalarial activity, an acceptable level of side effects and/or phototoxicity.

The amounts of the compounds obtained at this time were sufficient to establish their structures by various analytical and physical methods. Attempts are being extended to improve the yield so that they may be tested for their biological activity.

Secondly, the following intermediates of the DDS family were synthesized for testing their biological activity as chemotherapeutic agents.

a. 4-amino-4'-hydroxylaminodiphenylsulfone.

b. 4-acetylamino-4'-(N-acetyl-O-acetylhydroxy-amino)-diphenylsulfone.

c. 4,4'-acetylaminodiphenylsulfone.

d. 4-hydroxy-4'-aminodiphenylsulfone.

e. 4-hydroxy-4'-chlorodiphenylsulfone.

f. 4-chlorophenyl, phenyldisulfone.

3. Metabolism of Chemotherapeutics in Malaria.

a. Effect of p-substituted diphenylsulfones on methemoglobin formation in vitro. Dapsone is an antimalarial drug which can produce abnormal amounts of MHb in vivo, apparently through the action of an

unidentified metabolite. In order to discern the chemical nature of such a compound, 13 p-substituted derivatives were examined for their ability to oxidize hemoglobin to methemoglobin in human erythrocytes in vitro. 4-amino, 4'-hydroxylaminodiphenylsulfone was found to be most effective, 1.9 x 10^{-5} M drug oxidizing as much as 60% of the hemoglobin available or about 100 heme equivalents/mole drug in two hours. Glucose and oxygen were required for this process. With dialyzed hemolysates and solutions of purified hemoglobin, the heme equivalents oxidized/mole drug become less than 10, but a ratio of 80-100 can be restored by adding reducing compounds such as GSH or NADPH. Apparently, 4-amino, 4'-hydroxylaminodiphenylsulfone produces MHb by coupled oxidation with hemoglobin and oxygen and can be regenerated by reducing compounds to yield the high heme oxidation ratio. Incubation of galactose-treated erythrocytes with 4-amino,4'-hydroxylaminodiphenylsulfone resulted in less NADPH production by the hexose monphosphate shunt than in glucose-treated cells and a decreased formation of methemoglobin and reduced GSH level was observed. Depletion of intracellular GSH by treating the erythrocytes with methylphenylazoformate also reduced the amount of methemoglobin produced by 4-amino,4'-hydroxylaminodiphenylsulfone. These observations implicate GSH and/or NADPH in the recycling of this drug in erythrocytes. Consistent with these interpretations, 4-amino, 4'-hydroxylaminodiphenylsulfone produced only 1/3 as much methemoglobin in erythrocytes from 2 individuals with glucose-6-phosphate dehydrogenase deficiency as it did in normal erythrocytes. The present observation that 4-amino,4'-hydroxylaminodiphenylsulfone is a potent producer of MHb reinforces the likelihood that DDS-NOH is indeed produced as a quantitatively minor metabolite of dapsone which nevertheless plays a significant role in the methemoglobinemia observed in persons using the drug.

b. Effect of malaria chemotherapeutic agents upon alcohol metabolism and alcohol dehydrogenase. Last year, it was reported that primaquine and a number of 8-amino-6-methoxyquinoline compounds inhibited horse liver alcohol dehydrogenase. This study was extended to human liver alcohol dehydrogenase. The enzyme was isolated from a single liver, obtained at autopsy and purified approximately 200-fold. Kinetic studies showed that primaquine also inhibited human liver alcohol dehydrogenase competitively with respect to the coenzyme, NAD. However, the inhibition constant was 30 μM as compared to 2 μM for the horse liver enzyme. Thus, the human enzyme was not as susceptible to inhibition by primaquine as is the horse enzyme. As with the horse liver enzyme, chloroquine did not inhibit at all.

These studies suggested that primaquine may interfere with alcohol metabolism in vivo. To examine whether or not primaquine, in doses used for malaria prophylaxis, actually causes a disturbance of ethanol metabolism, CP tablets (45 mg primaquine phosphate and 300 mg chloroquine phosphate) were administered to six healthy human subjects and alcohol tolerance tests performed. The rates of ethanol disappearance from blood after ingestion of the CP tablet was compared to that

performed without the CP tablet. No differences in rates were observed. These data thus indicate that when given in the dose and schedule employed for malaria prophylaxis to healthy individuals, primaquine does not detectably alter ethanol oxidation. However, since primaquine is an effective inhibitor of alcohol dehydrogenase, it is conceivable that its effects may become manifest physiologically when liver functions are compromised or when the degradation and excretion of primaquine is impaired.

c. Carboxylase activity in Plasmodium berghei. Phosphoenolpyruvate carboxylase was isolated from P. berghei and partially purified. The enzyme is shown to be inhibited by quinine, chloroquine, iron, copper and mercury. Magnesium, manganese, or cobalt is required in the reaction. The study of the effects of small molecules on the carboxylase shows that it is not affected by acetylCoA, fructose diphosphate and cyclic AMP-3',5'. Avidin also had no effect in concentrations of 30 µg and 60 µg per ml of the reaction mixture. A rapid loss of enzymic activity was observed, however, in the presence of added urea. The carboxylase is shown to be composed of subunits and the assembly of these subunits is affected by Mg^{++}. Chloroquine stimulates the uptake of iron into the blood cells of mice. The incorporation of radioactive iron from iron-59-citrate is approximately 33% greater in the presence of chloroquine than the saline control. In vivo studies with mice showed that the average parasitemia in infected mice treated with iron is 4 to 6 times less than that found in infected mice kept on a low iron diet. Mice treated with iron plus chloroquine show an even lower average parasitemia, i.e., 10 to 13 times less than mice kept on a low iron diet. The results were statistically significant with p values of less than 0.001.

d. The effect of iron on Anopheles stephensi. It has been shown that water containing iron is anopheline sterile and that iron soils are inhibitory to breeding. Larvae from normal A. stephensi were allowed to develop in different concentrations of ferric chloride solutions to adulthood. It was previously determined that second instar stage larvae of A. stephensi were killed within 18 hours when allowed to develop in iron solutions containing .01-1.0 mg/ml iron. Large numbers of eggs from normal A. stephensi were allowed to hatch and develop into adulthood in a solution of 2.5 µg ferric chloride per ml. The adults were fed on a rabbit and divided into two groups. One group (Group 1) of A. stephensi were allowed to oviposit in tap water and the second group (Group 2) in a solution containing 2.5 µg ferric chloride per ml. Two hundred fifty eggs were placed into pans with media of different ferric chloride concentrations and allowed to hatch and pupate. The results obtained are as follows:

Medium	Percent Pupation		
	Group 1	Group 2	Difference
Control (no iron)	40	35	5
2.5 µg Fe^{+++}/ml	49	30	19
1.25 µg Fe^{+++}/ml	77	22	55
0.625 µg Fe^{+++}/ml	57	17	40
0.313 µg Fe^{+++}/ml	63	56	7
0.156 µg Fe^{+++}/ml	73	50	23
0.078 µg Fe^{+++}/ml	25	15	10

The results thus far obtained are considered preliminary in that no quantitative conclusions can be made from the data. It appears from the data obtained that iron does indeed have an effect on the development of A. stephensi. Special note must be given to the fact that the percent of pupation from the eggs is consistently lower when the eggs were allowed to develop under constant iron pressure than when the eggs were allowed to develop in iron after oviposited in water.

4. Experimental Malaria in the Hamster.

a. Thyroid function and 2,3-diphosphoglycerate content of erythrocytes in experimental malaria. Preliminary observations indicate that a significant reduction in the size of the thyroid occurs in young adult male hamsters at 12 to 14 days after intraperitoneal inoculation with 2 x 10^7 parasitized (Plasmodium berghei) red blood cells. This was observed in 17 of 20 hamsters examined; parasitemias at this time were approximately 18%. Determinations of total circulating thyroxine (T-4) by a competitive binding procedure showed that T-4 values in 4 of 5 infected hamsters were decreased as much as 50% when compared to control values. Conclusions regarding the physiological and homeostatic implications of these observations will be delayed pending their corroboration. In this regard, a comprehensive study will be undertaken to include other parameters of thyrometabolic function. Other rodents infected with P. berghei will also be examined; subsequent studies, if warranted, could involve different malaria parasites.

It has been reported that elevated concentrations of erythrocyte 2,3-diphosphoglycerate (2,3-DPG) occur in hyperthyroid patients as well as in normal red cells incubated with T-3 (3,5,3-triiodi-L-thyronine) or T-4, with a subsequent increase in dissociation of oxygen from hemoglobin. Accordingly, it was thought that a reduced thyroid function might contribute to a state of tissue anoxia regarded by some

investigators to be the primary pathogenic manifestation of malaria infection; anemia, except under extreme conditions, would appear to be of minimal significancein this regard. Moreover, splenic erythrostasis, as might be encountered in malaria, with an associated fall in pH and subsequent activation of 2,3-DPGase would also lower the red cell content of 2,3-DPG. This is currently under investigation in P. berghei infected hamsters; however, preliminary results, contrary to expectations, show that 2,3-DPG levels are moderately elevated in infected red cells when compared to non-infected cells.

b. Serum lipids in experimental malaria. The evidence pertaining to a disordered lipid metabolism in malaria is still quite fragmentary. Fatty livers have been observed in rodent and similar malarias. However, investigations on the blood levels of various lipid components in malaria infections have yielded variable results. The interrelationships of drug hepatotoxicity and lipid metabolism, with some relevance to malaria, have been the subject of several reports. The present study was undertaken to determine if a broad investigation of lipid metabolism in experimental malaria is warranted. An examination was made for possible alterations in serum lipoprotein patterns and in the serum concentrations of various lipid fractions throughout the course of infection with P. berghei in the hamster.

Separate groups of fasted male hamsters were bled from the heart under light ether anesthesia at intervals of 4, 7, 12, 17, 21 and 26 days subsequent to intraperitoneal inoculation with 2×10^7 parasitized red blood cells. Non-infected controls were included at each interval. Parasitemia and hematocrit determinations were made prior to exsanguination on blood obtained from the orbital venous pleurus. Measurements in serum were made of: total lipids, total cholesterol, triglycerides, phospholipids and free fatty acids. Lipoprotein patterns were obtained by means of agarose gel electrophoresis.

This study is still in progress; however, the available data may be summarized briefly. Parasitemia progressed from a mean value of 2.0% on the 4th day of infection to a maximum of 38.7% on day 21, then declined on day 26 to 14.5%. The latter group represented survivors in an apparent state of recovery from the infection. Mean hematocrit values decreased from 55.6% on day 4 to 18.8% on day 21, then rose to 44.6% on day 26. The total serum lipid and cholesterol concentrations showed remarkably similar patterns throughout the course of infection. This involved a significant reduction on the 7th day of infection which persisted through day 12, followed by a progressive rise through day 21 (for total lipids) and day 26 (for total cholesterol). Comparable results have since been obtained in a subsequent experiment. These observations are especially interesting in light of previous reports by Krishnan et al., (1936) and Kehar (1937) dealing with P. knowlesi infections in monkeys. These authors noted a fall of total blood lipids and cholesterol 24 hours prior to the onset of hemoglobinuria, followed

by a sharp rise before death. In monkeys which did not develop hemoglobinuria, the fall in cholesterol continued uninterrupted. No satisfactory explanation has been offered for these changes. Elsewhere in the current reports we have noted the onset of an intense hemoglobinuria in hamsters during the second week of infection with P. berghei.

Triglyceride values showed a moderate, but progressive, rise through day 17, with little further change in days 21 and 26. The lipoprotein pattern which emerged in the course of infection was essentially one of hyperbeta-lipoproteinemia accompanied in the final intervals by a decrease in alpha-lipoproteins, the latter probably indicative of advancing liver disease. A further discussion of the results will be delayed pending completion of this study.

c. 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 investigations of malarial pathophysiology.

The course of P. berghei infection in fasted male hamsters inoculated with 2 x 10^7 parasitized red blood cells was studied sequentially in terms of parasitemia, hematology, blood chemistry, relative organ weights, tissue enzyme levels and selected urinary constituents.

Death from infection first occurred 14 days after incoluation; survivors on day 21 were extremely debilitated. Parasitemia progressed from a mean value of 1.9% on the 4th day of infection to a maximum of 36.2% on day 21 and was paralleled by a rise in reticulocytes from 0.6% to 47.0%. Mean hematocrit values during this period decreased from 55.4% to 15.3%, however, individual hematocrits as low as 9.0% were recorded. Splenomegaly was evident as early as the 4th day of infection.

The onset of an intense hemoglobinuria was often noted in the second week of infection. Concomitant or subsequent urinary changes included the occurrence of polyuria and aminoaciduria without obvious changes, however, in the pattern of free amino acid excretion or in blood amino acid excretion.

Hypoalbuminemia and hyperglobinemia were present beyong the 4th day of infection. Infected hamsters also showed progressive elevations in SGO-T, SGP-T, urea nitrogen, creatinine and lactic acid. Levels of serum glucose and uric acid markedly decreased. Glutamic-oxalacetic transaminase and glucose-6-phosphatase activities in infected liver and kidneys were moderately decreased in the course of infection.

d. Tissue culture growth of Plasmodium fallax. When subcultures of embryonic turkey brain inoculated with the exoerythrocytic or tissue stages of an avian malarial parasite P. fallax were incubated for 48 hours, parasitemias rose 50 to 85%. This was accompanied by alteration in free amino acid pool of parasitized cells. This observation may have some effect on the protein synthesis of parasites. This study was undertaken in collaboration with personnel of the Naval Medical Research Institute.

SUMMARY.

Chemotherapeutic agents particularly antimalarial drugs were examined for their metabolic interaction with the biological environment. The mechanism of action of each drug against the malarial parasite was biochemically defined. Plasmodium species in various animal hosts was used to test organisms to evaluate intermediary metabolism of the parasite, and the responsens of the host to the antimalarial agent under study. Analytical definitions of the chemotherapeutic agents and its distribution in biological tissues was studied. Synthesis of important metabolites was accomplished. Studies with the CO_2 fixing enzyme phosphoenopyruvate carboxylase isolated from P. berghei demonstrated the inhibitory effect of quinine, chloroquine, iron, copper and mercury while magnesium, manganese and cobalt are required for the enzyme to function. The effect of iron on parasite growth and multiplication was examined and the data suggest that chloroquine increases intracellular iron thus inhibiting CO_2 fixing enzymes in the parasite with the resultant denial of essential intermediates such as oxaloacetate, amino, keto and other organic acids to the parasite. Studies invovling diaminodiphenylsulfone (DDS) were carried out analytically to define impurities found in DDS preparations. Four major impurities were characterized and are either analogs of DDS or substituted chloro and hydroxyl derivatives. In vitro studies established that DDS is indeed a potentiator of methemoglobin formation through the formation of an N-OH derivative. In vivo studies of the interaction of chloroquine and primaquine with alcohol dehydrogenase demonstrated that under the usual conditions, CP tablets have no effect on the oxidation of alcohol.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 108, Biochemical effects and mechanism of action of chemotherapeutic agents

Literature Cited.

References:

1. Hjelm, M. and DeVerdier, C. J.: Biochemical Effects of Aromatic Amines - I. Methaemoglobinaneamia, Haemolysis and Heinz-Body Formation Induced by 4,4'-diaminodiphenylsulphone. Biochem. Pharmacol., 14: 1119 (1965).

2. Sandosham, A., in Malariology with Special Reference of Malaya, University of Malaya Press, Singapore, 1965.

Publications:

1. Kramer, P. A., Glader, B. E. and Li, T.-K.: The Effect of P-Substituted Diphenylsulfones on Methemoglobin Formation in vitro. Fed. Proc. 30: #3, 1199 (1971).

2. Siu, P. M. L.: Effect of Iron and Chloroquine on Malarial Infection in Mice. Fed. Proc. 30: 1200 (1971).

3. Forrester, L. J. and Siu, P. M. L.: P-enolpyruvate Carboxylase from Plasmodium berghei. Comp. Biochem. Physiol. 38B: 73-85 (1971).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6471	71 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
70 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				
~~c. SECONDARY~~	CDOC 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 71	3	177
FISCAL YEAR CURRENT 72	8	360

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Drug Resistant Malaria; (U) Chloroquine; (U) In vitro Models; (U) Tetracycline; (U) Immunity; (U) Cerebral Malaria

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 and to determine suitable study site(s) for clinico-pathologic, pharmacologic, immunologic, and entomologic investigations of naturally occurring malaria.

24. (U) Several suitable sites have been established. Rates for drug resistant parasites have been studied, utilizing both in vivo and in vitro techniques. The efficacy of antimalarials other than chloroquine has been evaluated. Circulating malaria antibody is to be examined for its protective effect. Vector populations and climatic conditions are monitored longitudinally at the study sites.

25. (U) 70 07 - 71 06 Chloroquine resistance of Plasmodium falciparum is highly prevalent. The in vitro test, with several limitations, is reliable for detection of resistance. Tetracycline is an effective antimalarial against P. falciparum. Anopheles balabacensis is still the primary vector in malarious areas under study. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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 69 (FOR ARMY USE) ARE OBSOLETE.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 112, Field studies on drug resistant malaria

Investigators.
Principal: Edward J. Colwell, LTC, MC
Associate: Wisit Benjapong, M.D.; Prajim Boonyakanist; Douglas J. Gould, Ph.D.; Bruce A. Harrison, CPT, MSC; Robert L. Hickman, MAJ, VC; Y. M. Huang, Ph.D.; Peter K. Iber, MAJ, MSC; Sanong Kosakal, M.D.; Banharn Laizuthai, MAJ, MC, RTA; Kol Mongkolpanya; Supat Noeypatimanond, M.D.; Walter W. Noll, MAJ, MC; Larp Panthusiri; Katchrinnee Paranand, M.D.; E. L. Peyton; Pung Phintuyothin, MG, MC, RTA (Ret.); Rampa Rattanararithikul; Narong Sadudee, M.D.; John E. Scanlon, Ph.D.; Sunthorn Sirivanakarn; Michael F. Sullivan, CPT, MSC; R. N. Wilkinson, CPT, MSC; Norman E. Wilks, LTC, MSC; Vithune Yuthasas-kosol, M.D.

Investigations of In Vivo and In Vitro Responses to Chloroquine in Thai Nationals with Falciparum Malaria

Principal Investigator: Edward J. Colwell, LTC, MC

Associate Investigators: Pung Phintuyothin, MG, MC, RTA, (Ret)
Narong Sadudee, M.D.
Wisit Benjapong, M.D.
Supat Noeypatimanond, M.D.

OBJECTIVE: To evaluate the reproducibility and reliability of an in vitro technique to detect chloroquine resistant strains of P. falciparum.

DESCRIPTION: An in vitro technique reported by Rieckmann and associates was employed for detection of chloroquine resistant strains among residents of a district in Central Thailand. Their technique consists simply of an in vitro cultivation of infective blood with graded concentrations of chloroquine. The indicator response is the degree of development or inhibition of schizogony in the presence or absence of the drug. In addition to in vitro testing, the clinical responses to conventional chloroquine administration were also determined.

PROGRESS: Both in vivo and in vitro responses to chloroquine were successfully measured in 57 Thai adult and adolescent subjects infected with P. falciparum. Fifty-five of the 57 exhibited clinical resistance following conventional chloroquine administration. In comparison with Rieckmann's studies on the in vitro chloroquine sensitivity of P. falciparum from Uganda, the asexual parasites from all 57 Thai subjects in our study exhibited in vitro chloroquine resistance.

Replicate in vitro examinations performed on blood specimens from 5 subjects demonstrated an acceptable experimental error.

Two major limitations of the in vitro test precluded examinations of many infected individuals. These were high parasite densities (over 20,000 per cmm) and the stage of trophozoite maturity at the time of inititation of the cultures.

SUMMARY: The in vitro chloroquine sensitivity test, with several limitations, was a reliable indicator for detection of chloroquine resistant strains of P. falciparum.

In Vitro Chloroquine Resistant Falciparum Malaria in Thailand

Principal Investigator: Edward J. Colwell, LTC, MC

Associate Investigators: Robert L. Hickman, MAJ, VC
Pung Phintuyothin, MG, MC, RTA, (Ret)
Narong Sadudee, M.D.
Sanong Kosakal, M.D.

OBJECTIVE: To determine the rates of chloroquine resistant falciparum malaria utilizing an in vitro technique.

DESCRIPTION: The simplified in vitro technique described by Rieckmann et al was utilized at three different locations in Thailand for the detection of chloroquine resistant strains of P. falciparum.

PROGRESS: Successful in vitro chloroquine sensitivity tests were performed on blood specimens obtained from 65 residents of Phrabuddhabat in Central Thailand, 39 residents of Trat in the Southeast, and 18 residents of Nong Khai vicinity in the Northwest. All were infected with single P. falciparum infections. In comparison with Rieckmann's in vitro studies on a known chloroquine sensitive strain of P. falciparum from Uganda, only 3 blood specimens from the Thai residents of our study exhibited parasites which were chloroquine sensitive. Two resided in Trat and one resided near Nong Khai.

Two major technical limitations of the in vitro test precluded examination of all infected subjects and resulted in in vitro culture failures of specimens from approximately one third of individuals examined. The failures were caused by high parasite density and an inadequate stage of trophozoite maturity. Attempts to resolve the problem of high parasite densities by means of a normal saline dilution were usually followed by inadequate in vitro parasite maturation responses. Other technical problems were bacterial contamination of cultures and electrical power failures which impaired the in vitro incubation periods.

SUMMARY: The rates for chloroquine resistant falciparum malaria, as detected by a simplified in vitro technique, in 3 areas of Thailand were 100% in Phrabuddhabat, 95% in Trat and 94% in Nong Khai.

Tetracycline Treatment of Asymptomatic and Acutely Ill Subjects with Falciparum Malaria

Principal Investigator: Edward J. Colwell, LTC, MC

Associate Investigators: Robert L. Hickman, MAJ, VC
Sanong Kosakal, M.D.
Pung Phintuyothin, MG, MC, RTA, (Ret)

OBJECTIVE: To evaluate the efficacy of tetracycline in asymptomatic and acutely ill subjects with P. falciparum infections.

DESCRIPTION: Asymptomatic adults with low grade parasitemias were administered 250 mg of tetracycline every 6 hours for 10 days. Acutely ill subjects were alternately assigned to one of two treatment groups. One group received oral quinine, 540 mg base every 8 hours for 3 days, followed by tetracycline, 250 mg every 6 hours for 10 days. The other group received a similar course of quinine followed by a conventional course of chloroquine administration. Followup blood smear examinations were obtained for at least 33 days after initiation of therapy.

PROGRESS: Sixteen asymptomatic subjects were administered the tetracycline regimen. A radical cure was obtained in twelve subjects. In the remaining four subjects, tetracycline treatment was replaced by quinine because of the development of fever, chills and headache within 72 hours of initiation of therapy. The mean parasite clearance time in the twelve treated subjects was 4.6 days (2-6 days).

Twenty-nine of thirty (97%) subjects who completed the quinine-tetracycline regimen were radically cured. Only fifteen of thirty-six (42%) of those who completed the quinine-chloroquine regimen were radically cured. The degree of exposure to reinfection among the subjects within the two groups was believed to be comparable.

In subjects who presented with gametocytemia, tetracycline did not exert a gametocytocidal effect.

SUMMARY: Tetracycline was shown to exhibit antimalarial activity in both asymptomatic and acutely ill subjects infected with P. falciparum.

Pathologic Studies in Cerebral Malaria

Principal Investigator: Banharn Laizuthai, MAJ, MC, RTA

Associate Investigators: Pung Phintuyothin, MG, MC, RTA, (Ret)
Edward J. Colwell, LTC, MC
Sanong Kosakal, M.D.

OBJECTIVE: To examine the pathological manifestations of brain tissue from patients who die of malaria.

DESCRIPTION: Postmortem examinations of tissue from subjects who expired because of cerebral malaria were performed within 15 minutes after expiration. Impression smears for Giemsa staining were accomplished to demonstrate malarial parasites. The tissue was fixed in 10% buffered formalin for further paraffin tissue processing.

PROGRESS: The microscopic examinations of the first two cases revealed similar pathological findings consisting of generalized brain edema, obstruction of capillaries by infected red cells and malaria pigment. Numerous foci of petechial hemorrhage were demonstrated in the cerebral cortex. Endothelial necrosis of the capillaries and thrombosis were also frequently observed. The malarial parasites from impression smears of brain tissue showed occasional schizonts of P. falciparum.

SUMMARY: Pathological involvement in cerebral malaria is due to P. falciparum infected red cells blocking the capillaries and contributing to hypoxia and necrosis of the capillary wall. Intracerebral hemorrhage and thrombosis are probable subsequently related events.

Hemolytic Activity in Malaria Infections

Principal Investigator: Vithune Yuthasastr-Kosol, M.D.

Associate Investigators: Norman E. Wilks, LTC, MSC
Peter K. Iber, MAJ, MSC

Assistant Investigator: Mr. Thamma Sakulkaipeara

OBJECTIVE: To investigate the hemolytic factors associated with human malarial infections with particular emphasis on host complement activity in chloroquine resistant and non-resistant malaria and immunological phenomena producing host cell lysis.

DESCRIPTION: Previous studies at SEATO Medical Research Laboratory (1968, 69) using P. inui and P. coatneyi in monkeys showed marked decrease in erythrocyte survival time in the course of chronic infections with low grade parasitemia or even in the absence of parasites. Studies also showed that inappropriate erythrocyte destruction was mediated by some humoral factor associated with chronic infection. Equally striking is the marked decrease of C' activity in the infected monkeys using the spectrophotometric method for C' assay as described by Hook and Muschel (1964) and Fogel et al at WRAIR (1966).

PROGRESS: Preliminary studies of patients infected with either P. falciparum and P. vivax indicate that there is a pronounced depletion of C' activity in human malaria infections in Thailand. These data are summarized in Fig. 1, which shows the rise in C' activity in four P. falciparum cases and the decrease to zero activity in two terminal cases; in Fig. 2, which shows the rapid increase in C' activity in three cases of P. vivax following chemotherapy; and in Fig. 3, which illustrates the distribution of C' activity levels in malarial patients, normal Thai subjects, and patients with other diseases. The complement activity is being measured in patients from whom it will be possible to obtain follow-up serum specimens after treatment, to test the hypothesis that P. falciparum infections refractory to treatment will persist with less than normal C' activity levels and thus a prediction of recrudescence may be possible.

Studies to detect hemolytic activity associated with an immunological phenomenon have begun, but chronic cases of the two endemic malarial parasites of humans have been few. Observations in this area will increase as the transmission of malaria increases with the onset of the rainy season.

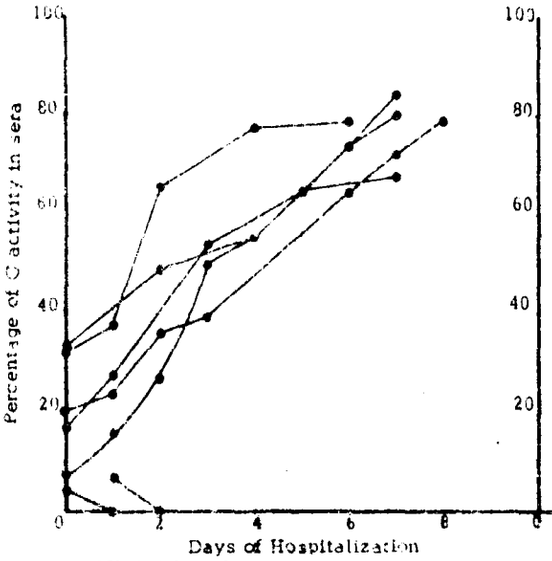

FIGURE 1 - Percentage of C activity in sera of patients infected with Plasmodium falciparum during hospitalization. (Terminal cases, sera were obtained 24 hours before death, and on the day of death).

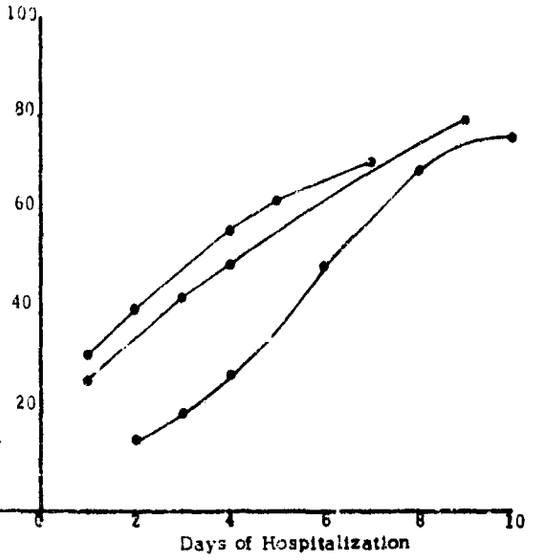

FIGURE 2 - Percentage of C activity in sera of patients infected with Plasmodium vivax.

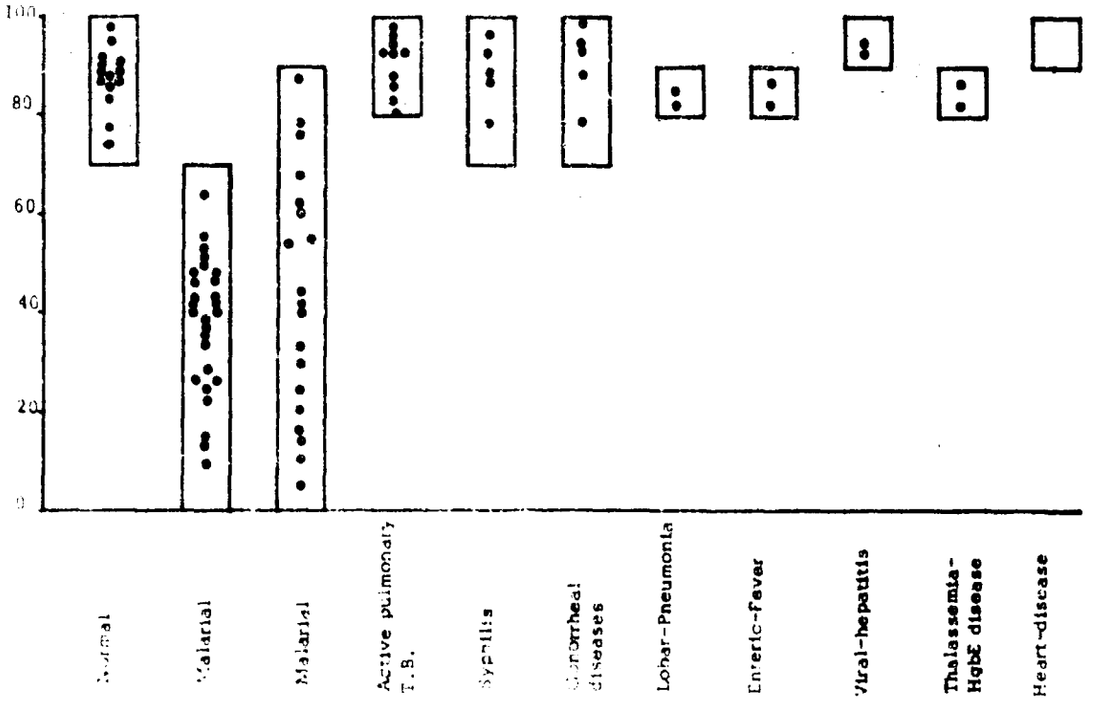

FIGURE 3 - Comparison of distribution of C activity in randomly selected normal Thai (Bangkok) malarial patients and patients with other diseases.

SUMMARY: Complement activity levels of malaria patients have been found to be decreased during infection, and rise to normal levels rapidly after successful chemotherapy. Hemolytic activity associated with an immune process continues under investigation.

Immunochemical Studies of Human Antibodies in Endemic Malaria

Principal Investigator: Peter K. Iber, MAJ, MSC

Associate Investigators: Norman E. Wilks, LTC, MSC
Katchrinnee Pavanand, M.D.

OBJECTIVE: The isolation and characterization of the human malaria antibody from Thais living in a malaria endemic area.

DESCRIPTION: Sera from persons living in an endemic area are to be screened for high antibody titer. Those with high titer will be requested to donate blood for antibody isolation, and a critical study will be made into its mode of action and protective properties with parasites from other areas of Thailand.

PROGRESS:

I. Separation technique: A separation process has been developed for plasma or serum using chromatography on DEAE cellulose followed by rechromatography of CM cellulose. By this mild method, pure gamma globulin is recovered. Although the yield is relatively low, the purity is very high and has been confirmed by immunoelectrophoresis. Presently recovery studies are being made to determine the yield in mg gamma globulin/ml of plasma or serum. Separations to date have been made of plasma supplied by Sri Racha Red Cross Hospital from outdated blood.

II. Immune Sera Collection: Kok Saloong, a village in Lopburi Province, Thailand has been selected for antibody screening based on the age of the village, low population movement, malaria history and lack of easily accessible medical treatment. Initial specimens from patients with low parasitemia and history of prior malaria parasitemias appear promising from immunoelectrophoretic data.

SUMMARY: Separation techniques have been developed and a collection site selected for the study of malaria antibody.

Plasmodium Falciparum Infection Rates in Normal and Enzyme-Deficient Erythrocytes of Glucose-6-Phosphate Dehydrogenase-Deficient Heterozygotes

Principal Investigator: Walter W. Noll, MAJ, MC

Assistant Investigators: Matthew Leatherwood, SFC
Prachar Pooyindee

OBJECTIVE: P. falciparum infection rates in normal and enzyme-deficient erythrocytes of Thai women, heterozygous for glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, will be determined.

DESCRIPTION: Women, heterozygous for G-6-PD deficiency (an X-chromosome-linked trait), are mosaics: approximately half of their red blood cells are normal, the other half are G-6-PD deficient. The two cell populations can be distinguished histochemically by the methemoglobin elution method (Gall et al. 1965). This technique will be applied to blood from Thai women who have malaria and are heterozygous for G-6-PD deficiency. Infection rates in both normal and enzyme-deficient red blood cells will be determined and compared. Hematocrit, reticulocyte count, red blood cell morphology, hemoglobin type, and G-6-PD activity (spectrophotometric assay) will also be determined.

PROGRESS: The study is still in a preliminary stage. Attention has been directed towards perfecting the methemoglobin elution technique and adapting it to the use of small volumes of capillary blood.

SUMMARY: Investigation of P. falciparum infection rates in normal and enzyme-deficient erythrocytes of Thai women, heterozygous for G-6-PD deficiency, is in a preliminary stage.

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*
R. N. Wilkinson, CPT, MSC

Associate Investigators : Prajim Boonyakanist
Kol Mongkolpanya
Larp Panthusiri

OBJECTIVE : To collect, identify, catalogue and redescribe the mosquito species of Thailand. Information is also gathered on the distribution, larval habitats and other aspects of the bionomics of 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 malaria and other arthropod-borne diseases. Additional collections of a specialized nature are made to obtain a correlated series of larvae, pupae and adults for illustration and taxonomic studies. The majority of this material is shipped to the Smithsonian Institution for study by specialists in the Southeast Asia Mosquito Project (SEAMP).

PROGRESS : During the year 765 mosquito collections were made in 3 provinces of Thailand. The majority of the collections were made in Chiengmai province. These collections resulted in 5475 pinned adults, 6553 slide mounts of larvae, larval and pupal skins, 55 slide mounts of terminala and 100 buccopharyngeal slide mounts. Results of the mosquito collections made during this period are given in detail in the following sections.

*Southeast Asia Mosquito Project, Smithsonian Institution, Washington, D.C.

Anopheles : Collections of Anopheles nivipes and Anopheles philippinensis were made from Chiengmai and Chonburi provinces. Siblings from females tentatively identified as either An. nivipes or An. philippinensis were found to be all An. nivipes. Thus, it appears probable that only An. nivipes occurs in Thailand.

Aedes : Taxonomic studies on this genus were concentrated during the period on species of the subgenus Stegomyia which contains such important vector species as Aedes aegypti, A. albopictus and A. scutellaris. The majority of the collections of Aedes (Stegomyia) were of larval stages - both from natural habitats and water-filled bamboo oviposition cups set out at collection sites. Seven closely related species of Stegomyia including A. annandalei, A. craggi, A. desmotes, A. pseudalbopictus, A. gardnerii imitator, A. mediopunctatus and A. patriciae were collected and the progeny reared in an effort to clarify the taxonomy of these species. Laboratory colonies of A. annandalei, A. craggi, A. pseudalbopictus, A. gardnerii imitator, and A. mediopunctatus have been established.

Culex : During this period study of the subgenus Culex was continued as part of a larger study on Japanese encephalitis in Chiengmai province. Data on the larval habitats of the 10 species of Culex (Culex) occurring in the Chiengmai area were collected. Culex annulus, C. pseudovishnui and C. tritaeniorhynchus were the three most common species of the subgroup collected.

SUMMARY : Taxonomic studies on mosquitoes belonging to the genera Anopheles, Aedes and Culex in Thailand were emphasized during this period. Laboratory colonies of five species of Stegomyia from Thailand were established. Data on the larval habitats of Culex species of Chiengmai province were collected in connection with studies of the ecology of Japanese encephalitis in northern Thailand.

Ecology of malaria vectors

Principal Investigators : Douglas J. Gould, Ph.D.
Bruce A. Harrison, CPT, MSC
Michael F. Sullivan, CPT, MSC
R. N. Wilkinson, CPT, MSC

Associate Investigators : Prajim Boonyakanist
Larp Panthusiri

OBJECTIVE : To investigate the bionomics and population dynamics of the species of Anopheles which are vectors of human malaria in Southeast Asia. In addition, the relationship of these species to the dissemination of chloroquine resistant strains of P. falciparum are under study.

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, patterns of biting activity, ovipositional habits and fac influencing egg viability and survival under varying co ns. Studies are also being made in an attempt to determine 1) chloroquine resistant strains of P. falciparum have a greater reproductive potential in anopheline hosts than do chloroquine sensitive strains, 2) if A. balabacensis is more susceptible to infection with chloroquine resistant strains of P. falciparum than are other species and 3) if chloroquine treatment in the human enhances the reproductive potential of chloroquine resistant strains of P. falciparum in the mosquito host. Colonized Anopheles balabacensis and Anopheles minimus are fed simultaneously on patients circulating gametocytes of P. falciparum. The resistance status of the infection is determined prior to treatment with chloroquine by Reickmann's in vitro test and by WHO criteria on days 7, 14, 21 and 28 after treatment. The mean number of oocysts developing in the two vector species are compared with reference to whether the patient was infected with drug sensitive or resistant strains of P. falciparum.

PROGRESS - During this period emphasis was placed on finding a locality with a stable human population and malaria parasite rates of 25% or higher which was accessible during all seasons of the year. During November and December 1970 a total of 97 thick-thin blood films were taken from inhabitants on the island of Koh Chang in Trat province; eighteen (20.6%) were positive for P. falciparum. This area was not considered feasible for field studies because it is inaccessible during part of the year. In January 1971, one hundred and forty six blood films were obtained from school children at two schools in Amphur Chaibadan, Chaiyaphum province; nine (6.1%) of the films were positive for P. falciparum. In addition, two of 15 (14%) films from villagers located near the schools were positive for P. falciparum. On the assumption that malaria rates would rise during the transmission season (June - October) and because of their easy accessibility, these areas were considered as possible sites for long term field studies. The village of Ban Bu Phram in Prajinburi province, which has been highly malarious in previous years, was visited during March 1971. Seventy-six of 211(36%) blood films from this site were positive for either P. falciparum or P. vivax. The rates for P. falciparum and P. vivax were 25.6% and 10.4%, respectively. Infected An. balabacensis have been collected in this area. The area has a stable human population of more than 1000 persons and is easily accessible throughout the year. Consequently, major longitudinal human and vector studies are planned for the coming transmission season.

During January an effort was made to recover viable eggs of An. balabacensis from soil samples collected at suspected oviposition sites in areas of Nakorn Rajasima and Prajinburi provinces where adult An. balabacensis had previously been captured. At the time of collection there had been no precipitation for over two months and conditions were extremely dry. Adults of An. balabacensis, An. kochi, and a member of An. hyrcanus complex as well as adults of three species of Aedes were reared from larvae which emerged when 39 of these soil samples were flooded. In the SMRL laboratory eggs of An. balabacensis have been found viable for up to 18 days when kept on moist filter paper.

Colonized An. balabacensis were fed on patients with falciparum malaria to study growth rates of chloroquine susceptible and resistant strains of P. falciparum in this vector species.

SUMMARY : Sites in Trat, Lopburi, Prajinburi and Nakorn Rajasima provinces were surveyed for feasibility for a long-term study of vector ecology and malaria epidemiology. Viable eggs of three species of Anopheles and three species of Aedes were found in samples of soil taken from dry stream beds in Nakorn Rajasima and Prajinburi provinces. Studies on the growth rate of chloroquine susceptible and resistant strains of P. falciparum in An. balabacensis were initiated.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 112, Field studies on drug resistant malaria

Literature cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6537	71-07-01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'G INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	114
b. CONTRIBUTING				
c. CONTRIBUTING	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 71	9	630
FISCAL YEAR CURRENT 72	9	630

19. RESPONSIBLE DOD ORGANIZATION

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

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Sweeney, Thomas R., Ph.D.

NAME: DA

PII Redacted

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) 70 07 - 71 06 Close supervision, through guidance and integrated evaluation of productivity, was continued for forty-seven contracts in the areas of chemical synthesis, drug preparation and data handling and for twenty biological contracts concerned with the preclinical aspects of antimalarial efficacy and drug safety.

Three new Investigational New Drug (IND) applications and eight IND supplements were submitted and approved, with preclinical work continuing on four additional IND basic documents. Six meeting's 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 70 - 30 Jun 71.

* A suffix No in contractors upon originator's approval

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

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 114, Malaria Program Supervision

Investigators
Principal: COL William E. Rothe, VC
Associates: Thomas R. Sweeney, Ph. D.
LTC Edgar H. Eckermann, VC
Melvin H. Heiffer, Ph. D.

General

The malaria program is a medicinal chemistry research effort encompassing the fields of synthetic organic chemistry, biology and pharmacology. The program involves the ability to coordinate and direct efforts in each of these fields in a manner that allows a constant feedback of data from individual investigators, with necessary program redirection, and culminates in the synthesis and biological evaluation of new antimalarial drugs for troop issue. These objectives are approached through the combined efforts of a team of research managers with the necessary expertise, in-house laboratory support, and extramural consultations.

The Contract Chemical Synthesis Program

During FY-71 there was a maximum of 43 active contracts. In this period 19 contracts expired and 11 new contracts were let. These contracts were with academic institutions, research houses and industry. Table I shows the distribution of contracts among these organizations. On 1 July 1971, there will be 35 active contracts, of which 14 will be with academic institutions, 12 with research houses and 9 with industry.

TABLE I

Number of Synthesis Research Contracts

Organization	In FY-71	Expired in FY-71	New in FY-71	1 July 1971
Academic	20	10	4	14
Research	12	4	4	12
Industry	11	5	3	9
Total	43	19	11	35

In addition, there are four contracts with preparations facilities to synthesize large quantities of selected compounds, one contract to synthesize radioactive tagged compounds for metabolite studies, one contract to analyze compounds and compositions to be used for preclinical and clinical studies, and one contract to study photochemical cyclizations of styryl compounds.

During FY-71 there was a total of approximately 1400 compounds submitted by the contractors; 560 of them were target compounds. As of the end of FY-71 the average cost per target compound is approximately $2500. During the year the number of target compounds requested from the preparations laboratories was 7, 4 and 5 in small, medium and large quantities, respectively. Compounds received numbered 14, 13 and 3 in these respective quantities, some of them carried over from FY-70. Chemical publications and manuscripts submitted numbered 45.

The greatest effort in the synthesis contracts was directed to the synthesis of aminoalcohols. As a percentage of obligated funds, 54% was in this area. Actively pursued synthesis areas can be broken down as shown in Table II.

TABLE II

Area	Percent of Obligated Funds
Phenanthrene Aminoalcohols	34
Anthracene Aminoalcohols	8
Naphthalene Aminoalcohols	4
Fluorene Aminoalcohols	2
Pyridine Aminoalcohols	2
Other Aminoalcohols	4
Total	54

Amino Quinolines	7
Amino Pyrimidines	7
Amino Quinazolines	7
Quinones	4
Thiacyano-subs. Aromatics	3
Miscellaneous:	18
RC-12 Analogs	
Phosphoric Acids Type	
Amino Acids Type	
Phenanthrols	
Solubilizing Compounds	
Benzothiopyrans	
Oxazines	

The main thrust of the synthesis program was with the aminoalcohols and antifols (antimetabolites). The synthetic work in the phenanthrene amino-alcohols is now being deemphasized because the objectives in this area have been largely met. At the same time more attention is being given to the synthesis of potentially prophylactic antimalarials and antimetabolites; in particular, the aminoquinazolines. Research work for finding new methods for synthesizing phenanthrenes has been curtailed; the photochemical approach will be terminated by the end of this year.

Patents

During the year data were assembled in the Division and forwarded to the Command to support patentability searches on 10 classes of compounds and 2 process improvements. In addition, based upon data assembled at WRAIR a patent has been applied for on the pyridyl carbinolamines by one contractor. A patent has been issued, U. S. 3,574,833, on the trimethoprim-sulfalene combination. In compliance with a directive from the Command the compilation of data and requests for patentability searches will be sharply curtailed; only compounds considered to be of utmost importance will be brought to the attention of the Command for possible patent action.

Acquisition of Chemicals

During the report period, approximately 12,000 samples were received. There were no very large industrial submissions (last year one source furnished over 9,000 samples) but commercially discreet submissions still

constitute somewhat over half of the total. About 1400 samples were from the synthesis program and the rest were gifts and purchased samples. A contract to pick up samples expired in November 1970; about 85% of the 15,000 samples picked up during the last 16 months of that contract were arranged for by WRAIR personnel. About 2100 were picked up during the current reporting period. Since the expiration of the contract, WRAIR personnel have picked up 2800 samples.

An active canvass of recently published compounds has proven to be a fertile source of novel structures.

Biology

All compounds received during this period were screened in mice against Plasmodium berghei. In addition, an increased effort in the chick-Plasmodium gallinaceum system permitted the screening of a large number of compounds in an attempt to detect antimalarial activity which may have been missed in the P. berghei screen. Compounds found to be active in the primary screens underwent secondary evaluation for efficacy by oral administration and against drug resistant parasites and pre-erythrocytic forms.

Progress

a. Primary Screens

1. The P. berghei-mouse system and the P. gallinaceum-chick system at the University of Miami with Dr. Leo Rane as principal investigator continued to be the basic screens. 16,379 drugs were evaluated against P. berghei. Of these, 988 were active. There were 282 active compounds found in the 36,665 tested against P. gallinaceum. Development of a sporozoite induced P. gallinaceum-chick system was begun at the University of Miami. The reliability of this system is currently being evaluated, but it is not yet in routine operation. This system is expected to operate as a basic screen with a throughput of 50 compounds per week.

2. The system based on inhibition of sporozoite formation in the salivary glands of mosquitoes maintained at the National Naval Medical Center by Dr. Terzian was discontinued during the report period. Ten compounds were examined prior to termination of that contract.

b. Secondary Screens

1. The antimalarial drug testing performed at the Illinois Institute of Technology Research Institute employs sporozoite induced P. berghei in mice. Throughput was increased in this prophylactic system by utilizing the tissue differentiation test only on active compounds. IITRI also functions as a repository for various strains of malaria and provides these parasites to other investigators when requested. The work conducted during the reporting period is summarized below:

Test	Total Tested Compounds	Active
Prophylactic & Suppressive	18	7
Suppressive	5	0
Prophylactic	146	29
Tissue differentiation	33	24

2. Selected drugs were submitted to the University of Cincinnati, Dr. C. C. Smith, Principal Investigator, for determination of folic acid inhibition activity. The screening performed in this test system during fiscal 1971 is summarized below:

Total Assays	662
Total Compounds	181
Number of compounds with reversed inhibition upon addition of folic or folinic acids	119
Total number of assays conducted against drug resistant lines	153
Total number of compounds tested against drug resistant lines	22
Number of compounds showing cross resistance	19

The sensitivities of individual cells of Streptococcus faecium sensitive and chlorguanide triazine-resistant cultures to chlorguanide triazine was determined by plating aliquots of cultures on series of agar plates containing

various concentrations of drug. This was done to determine whether the cultures are homogeneous and whether any highly resistant cells are present in the parent culture before exposure to the drug. A report of these results is being prepared.

3. The major portion of the testing of compounds against drug resistant P. berghei was done at the University of Georgia under the direction of Dr. Paul Thompson. Drugs were administered orally, providing an estimate of the efficacy of the drug given orally. The following is a summary of work conducted during this report period:

	# Drugs
Sensitive Strain	132
Triazine Resistant Strain	39
DDS Resistant Strain	36
Chloroquine Resistant Strain	84

Two studies were performed using combinations of drugs. Two rate and duration of action studies and one study of the effect of PABA on activity were also conducted.

4. Twenty-one compounds were evaluated against drug resistant P. berghei by Dr. Peters at the Liverpool School of Tropical Medicine. Studies were also conducted on potentiation of drugs used in combination and modes of action.

5. Thirteen compounds were evaluated by Dr. Harry Most, New York University School of Medicine, for activity as causal prophylactics. Studies were also conducted on transfer of drug resistance.

6. The tissue culture systems operated by Dr. Beaudoin at the National Naval Medical Center were used to screen 45 drugs for activity against exoerythrocytic forms of P. fallax. In addition, studies were conducted on site and mode of action of known antimalarial drugs.

Conclusions

All compounds received are being screened promptly. Secondary screening for oral efficacy and efficacy against drug resistant strains is thorough and efficient. Testing against preerythrocytic forms is being conducted on a very limited scale.

Pharmacology and Clinical Program

During the past year the Pharmacology Department supervised the contracts responsible for the preclinical and clinical studies of antimalarial compounds scheduled for clinical trial. Seven contracts included studies on the metabolism of these agents as well as their toxicity, pharmacodynamics and formulation.

During the past year the Department prepared three "Notice of Claimed Investigation Exemptions for a New Drug" as well as eight supplements. Details on each of these drugs are reported in their respective claims.

Phase I studies were completed for WR 6798, 30090, 33063, 38839 and 61112 as well as combination studies on WR 6798 with WR 1544 and WR 6798 with WR 1544 and WR 2975. A comprehensive study was performed on volunteers receiving a three-day course of WR 5949 and WR 4629.

Phase II chemoprophylactic studies were completed on WR 30090, 30063 and 38839. Combination studies were finalized on WR 6798 given with either WR 1544, or WR 1544 with WR 2975, or with 2978.

Phase II efficacy studies were completed on WR 30090, 33063, 38839 and 61112. Combination studies were conducted involving WR 38839 with sulfadiazine, WR 2977 with WR 6527 and WR 5949 with WR 4629. Significant results were also obtained with WR 2976 and WR 4629.

A field trial was conducted in Vietnam using WR 30090 in one group of patients and WR 33063 in another.

Summary and Conclusions

The use of third phase test systems - human plasmodia in the Chicago in vitro test and in the Aotus monkey - has permitted the selection of appropriate chemical development of new structure leads which do not show cross resistance against human strains of interest. These candidate antimalarials are undergoing active preclinical evaluation. The malaria program continues in a balanced fashion. Budget restrictions have reduced the effort slightly but promising drugs have been tried in the field and more potent analogs are under development.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE R/T SUMMARY	REPORT CONTROL SYMBOL
DA 03 6480	71 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
	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	63713A	3A663713D829	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE (Precede with Security Classification Code)

(U) Test System Design and Development (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 07	CONT	DA	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 71	3	105
FISCAL YEAR CURRENT 72	3	105

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington. D.C. 20012

RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

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Division of Medicinal Chemistry

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TELEPHONE: 202-576-2292

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Perrino, P. V.

NAME: Davidson, D. E. DA

PII Redacted

21. GEN'RAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Chemical; (U) Chemistry; (U) Pharmaceutical; (U) Test system; (U) Design; (U) In vitro; (U) In vivo

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

23. (U) To design and develop in vitro and in vivo test systems for evaluation of chemical compounds, and to make modifications when necessary.

24. (U) Appropriate in vitro and animal pilot models are designed to meet a specific need for testing chemical compounds. These models are expanded into operating test systems in-house and utilized to provide guidance to contractors when testing is to be done under contract. When necessary, modifications to existing test systems are designed and developed.

25. (U) 70 07 - 71 06. Design of a system to screen potential antimalarials available in small quantities was begun. The feasibility of utilizing chick embryos infected with Plasmodium gallinaceum is presently being evaluated. Design of an in vitro screen to detect drugs of potential use against oral microorganisms has been completed and is being evaluated. Design of an animal model for further evaluation of active drugs is in the preliminary stages. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*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

Task 00, Malaria Investigations

Work Unit, 122 Test Systems Design and Development

Investigators:
Principal: LTC Edgar H. Eckermann, VC
Associates: LTC David E. Davidson, VC; 2LT Rolla R. Rich, MSC;
2LT James H. Smith, MSC

Description

This investigation is designed to develop and improve test systems for evaluation of antimalarial drugs both in-house and under contract.

Progress

1. Development of a test system to screen antimalarial compounds in embryonated eggs infected with Plasmodium gallinaceum is in progress. Implementation of an experimental design has begun and preliminary results indicate that the design is feasible. The formation of exoerythrocytic forms of the plasmodia in the embryonic tissues raises the possibility that the system might be adapted to testing for causal prophylactic activity. This aspect is being investigated. Such a system would provide a very economical method of screening small amounts of compounds for activity against exoerythrocytic as well as erythrocytic forms.

2. Microorganisms which produce periodontal disease in man can be grown in hamsters in which they produce similar periodontal disease[1]. This has made it possible to establish an animal model to test drugs for effectiveness against periodontal disease. The inventory of drugs built up for the antimalarial drug program provides a variety of compounds of potential interest. In order to screen a greater number of drugs, a preliminary in vitro screen was established using drug saturated discs on nutrient agar inoculated with microorganisms known to be involved in periodontal disease. Drugs which inhibit growth are selected for testing in the in vivo system. The in vivo system is patterned on the system described by Keyes[2] in which hamsters are placed on a high carbohydrate diet and the oral cavity inoculated with the appropriate microorganism. Once the infection is established, drug treatment is begun. Drug activity is determined by comparing the drug treatment groups with untreated controls. All drugs for which IND's have been prepared have been screened in the in vitro system. Those which appeared to be most active are now being tested in the in vivo system.

3. Assistance was furnished antimalarial drug contractors in establishing a sporozoite induced chick test and a test to compare efficacy of drugs

by various routes, regimens, and vehicles. Assistance was also provided a contractor to modify a sporozoite-mouse test.

Project 3A663713D829

Task 00, Malaria Investigations

Work Unit 122, Test Systems Design and Development

Literature Cited.

References:

1. Keyes, P. H.: Dental Caries in the Syrian Hamster. I. The Character and Distribution of Lesions, J. Dent. Res. 25: 341-353, 1946.

2. Keyes, P. H.: Evaluation of two topical application methods used to assess the anti-dental caries potential of drugs in hamsters. J. of Oral Therapeutics and Pharm. 2: 285-294.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6513	71 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
70 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				
XXXXXXXXXX	CDOG1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Biological studies on control of 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 71	1	30
	CURRENT 72	2	30

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
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RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

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NAME: Schiefer, MAJ B.A.

21. GENERAL USE

Foreign Intelligence Not Considered

PII Redacted

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Anopheles; (U) Biocontrol; (U) Colonize; (U) Mosquitoes; (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) 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) 70 07 - 71 06. All mosquito colonies are being maintained by mass rearing procedure except species requiring forced insemination. Density at which anopheline larvae are reared has been shown to have an effect upon the development of malarial parasites in the adult mosquito; mosquitoes reared at low densities show significantly greater malarial oocyst development than those reared at high densities. Anopheles stephensi can be readily infected with a protozoan parasite, Nosema. At low concentrations (10^2 spores/ml) the parasite affects ovarian development and at higher levels (10^5/ml) 95% + mortality occurs. Interactions between Nosema growth and malarial oocyst development in the host are under study. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

1100

*Available to contractors upon certificator'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 123, Biological studies on anopheline vectors of malaria

Investigators
Principal: Ronald A. Ward, Ph.D.
Associate: LTC Bruce F. Eldridge; MAJ Bernard A. Schiefer, MSC;
CPT Edward A. Bergman, MSC; SP5 William N. Palmer;
David E. Hayes, Talmadge J. Neal, B.S.; 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 rearing techniques, the effects of biological agents on anopheline mosquitoes and studies on anopheline behavior.

Progress

1. Laboratory rearing of mosquitoes

A mass rearing procedure has been developed for anopheline mosquitoes which produces a very uniform mosquito and results in a considerable saving of manpower.

The major details of the procedure are as follows: mosquitoes in the stock cage are provided with a blood meal from a rabbit on Monday morning. Oviposition pans are placed in the cage Tuesday afternoon and left overnight. Eggs are collected Wednesday morning and dead adults are separated from the eggs with a strainer. Eggs are concentrated on bolting cloth and rinsed 5 times with distilled water to prevent spread of Nosema. Concentrated eggs are placed in a small pan containing distilled water; the non-viable eggs sink and remaining eggs are transferred to a 1 ml volumetric pipette fitted with a fine screened stopper. Large stainless steel trays containing aged tap water are each inoculated with 0.15 ml of concentrated eggs from the pipette (approximately 12,000 eggs). After the eggs hatch (31 - 40 hrs) the larvae are fed approximately 0.2 gm of a mixture of D&G dog food (ground and sieved through a 40 mesh screen) and liver powder (70% dog food and 30% liver powder by volume). Two days later, late in the 2nd larval stage, the larvae are refed larger amounts of food. To prevent excessive bacterial growth in the pans, pans are only fed when virtually all food is consumed. After the first feeding each pan is inoculated with 500 ml of a hay infusion solution as a supplementary source of food. Pupation usually occurs 7 days after eggs are introduced; at this time the smaller larvae are separated from the pupae and larger larvae by draining the pans through an open chamber fitted with a wire screen (25 mesh size). Water containing the small larvae is returned

*Insects Affecting Man and Animals Laboratory, U.S. Dept. of Agriculture
Gainesville, Florida

to the original pan and pupae are separated from mature larvae with a pupal separator employing a 0.032 inch grid. The larvae are reintroduced into pans for further development and pupae are further separated with the use of calibrated grids. The largest size class, retained by 0.048 - 0.046"grids consists mainly of females, with some males during the first 2 days of pupation. These are kept primarily for breeding stock. The most numerous individuals, largely females, are separated by 0.044 - 0.042" grids and are used for experimental malaria studies. These are counted volumetrically (about 250 - 275 pupae/ml) and placed in pint cartons covered with mosquito netting (250/ carton). After the adults have emerged, the water is poured out of the cartons through the netting and mosquitoes are fed 10% sucrose solution on cotton pledgets placed on the tops of the cartons. The same containers are used for feeding mosquitoes on an infected host and for holding the females for sporogonic development of malaria. The smallest size group, retained by grids less than 0.042", are kept in reserve and are pooled in 2 ft^3 cages. These are mostly males and some are added to stock cages if necessary. Larval and pupal separations are terminated after a 3-day period and remaining larvae in the pans are discarded.

2. Effect of rearing technique upon malaria susceptibility

Previous investigations with Aedes aegypti and Plasmodium gallinaceum indicated that a major component in the susceptibility of this species to malaria infection was genetic and that certain environmental factors such as larval density had no effect upon the level of malarial infection in the host (Ward, 1963). In conducting studies with a simian model of malaria (P. cynomolgi) and Anopheles stephensi it was consistently observed that much of the variation in the susceptibility of this species was related to non-genetic factors (Rutledge, Hayes and Ward, 1970).

In order to determine whether certain aspects of the rearing procedure affected malarial parasite development, larvae were reared in 9" x 12" pans containing 1500 cc water at 3 densities; 150, 300 and 600 larvae per pan. When 4 - 5 days old, adult females were fed upon a rhesus monkey infected with P. cynomolgi, incubated for a week at 27°C and dissected for malarial oocysts (20 per density were dissected). The results indicated (Table 1) that larvae reared at low densities had significantly more oocysts developing on their midgut than larvae maintained under crowded conditions (p values for "t" test between various pairs tested ranged between 0.001 and 0.025). The density factor appears related to the general size of the adult mosquito and relative area of midgut tissue available for oocyst development.

3. Effect of Nosema infection on malaria vectors and transmission of malaria

The collaborative investigations with the U.S. Department of Agriculture Insects Affecting Man and Animals Laboratory are being continued. Spore concentrates of Nosema (a microsporidian parasite of mosquitoes)

are produced at the U.S.D.A. laboratory and standardized spore dilutions are studied at WRAIR in respect to their effect upon anopheline vectors and their concurrent malarial infections.

Previous laboratory studies at Gainesville indicated that Nosema was highly pathogenic to Anopheles quadrimaculatus and A. albimanus, both New World malaria vectors, and that small lab colonies could be eliminated by this pathogen. Various dilutions of a Nosema spore suspension were added to pan containing first stage Anopheles stephensi larvae. There was a proportional increase in larval mortality as the dosage was increased (Table 2) and at a concentration of 7 x 10^4 spores/ml, 36% o the larvae died with an infection rate of 91%. The few adults that emerged from this treatment died 2 - 3 days after eclosion.

The effects of nosematosis upon the development of P. cynomolgi oocysts is shown in Table 3. First stage larvae were exposed to spore suspensions as previously, but higher concentrations were not used due to the greater adult mortality at these levels. The 5.6 x 10^2 spore treatment produced a significantly lower level of oocyst development than the 5.3 x 10^3 treatment or either control. It was expected that the higher concentration would produce a more marked effect to the spore exposure. Examination of individual mosquitoes disclosed a Nosema infection rate of 92% in the 5.6 x 10^2 level and an infection rate of only 50% at the higher treatment level. Apparently, the spore suspension was not uniformly distributed throughout the larval pans at the high concentration and thus produced a differential mosquito infection with the parasite. Mosquitoes from the 5.6 x 10^3 treatment were scored in respect to the degree of visible Nosema infection. Four levels were considered; no infection visible, moderate (1 - 100 spores/microscope field), heavy (100 - 1000) and very heavy (1000+). The mean number of malarial oocysts $\pm$ S.E. for each group was calculated (Table 4) and the results plotted on Figure 1. There was a clear relation between the level of microsporidian infection and malarial oocyst development in the mosquito. Concurrent with the reduction in mean oocyst number there was a visible effect of the Nosema parasite upon ovarian development in the female. In both control groups, the ovaries of all females appeared normal. At the 5.6 x 10^3 dosage level, all ovaries, with two exceptions (2/13 examined) had an amorphous structure with internal morphology absent. The two which had a semblance of normality showed some tissue dissolution in the nurse cells. Females from the 5.6 x 10^3 treatment group showed 13/30 with normal ovarian development. The remaining specimens showed slight to extreme interference with development.

Conclusions and recommendations

1. A semi-automated procedure for mass rearing Anopheles stephensi mosquitoes has been developed. Considerable manpower is conserved and variability of adult mosquito size is reduced. Further analysis of the nutritional requirements of mosquito larvae reared in large pans are required and comparisons between mosquitoes maintained under a variety of environmental conditions remain to be made.

2. The density at which anopheline larvae are reared has been shown to influence the development of malarial parasites in the mosquito host. Mosquitoes reared at low densities show significantly higher malarial oocyst numbers than those reared at high densities. The role of other environmental factors in the susceptibility of anophelines to malaria infection require further analysis.

3. Anopheles stephensi can be readily infected with a microsporidian parasite, Nosema. At low densities (10^3 spores/ml), the parasite affects ovarian development and interferes with the development of malarial parasites in the anopheline host. Exposure of larval mosquitoes to a spore suspension on the range of 10^5/ml produces adult mortality in excess of 95%. Additional laboratory experiments are planned to confirm these preliminary observations. If successful, large scale cage tests should be planned.

TABLE 1

Effect of rearing larval Anopheles stephensi at varying densities in relation to subsequent experimental malarial infection. (Malarial infection is assayed in mean number of malarial oocysts per dissected gut)

Experiment	No. larvae/pan	Mean no. oocysts ± S.E. mean
1a	150	9.60 ± 1.48
1b	300	7.80 ± 1.60
1c	600	3.00 ± 0.81
2a	150	398.62 ± 40.03
2b	600	250.15 ± 32.27

TABLE 2

Effect of nosematosis upon mortality of larval *Anopheles stephensi*

Nosema spores/ml	% larval mortality	% infection
0 (control)	7	1
7×10^1	10	1
7×10^2	17	61
7×10^3	16	73
7×10^4	36	91

TABLE 3

Effect of nosematosis upon development of malaria in *Anopheles stephensi*

Nosema spores/ml	% infected with *Nosema*	Mean no. oocysts ± S.E.
0 (control A)	0	23.84 ± 2.88
0 (control B)	0	25.44 ± 2.50
5.6×10^3	92	11.54 ± 4.72
5.6×10^3	50	16.73 ± 3.12

TABLE 4

Relation between level of *Nosema* infection of individual *Anopheles stephensi* and malarial oocyst development

Level of *Nosema* infection	Mean no. malarial oocysts ± S.E.
None visible	23.17 ± 4.71
Moderate	15.83 ± 7.47
Heavy	10.33 ± 6.84
Very heavy	4.83 ± 3.11

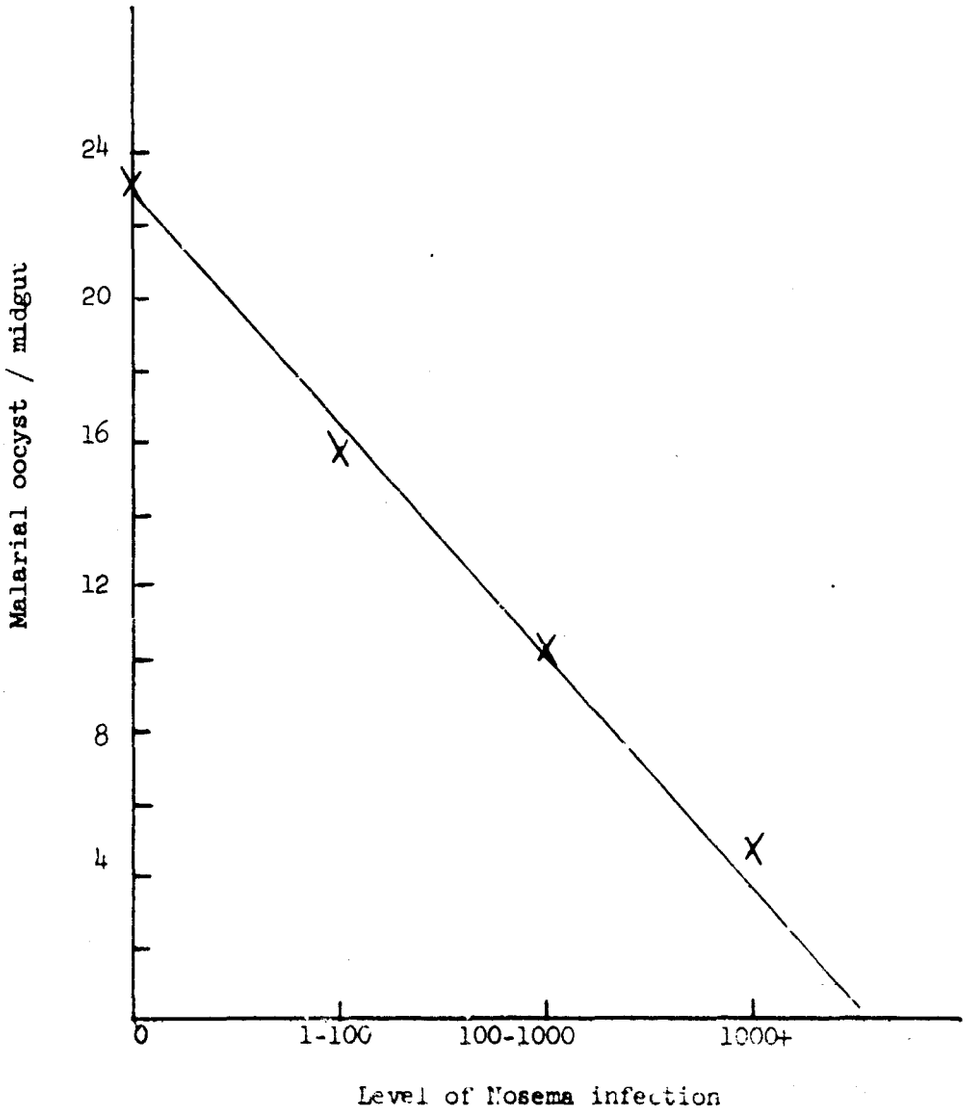
Malarial oocyst / midgut
24
20
16
12
8
4
0
1-100
100-1000
1000+
Level of Nosema infection

FIGURE 1

Project 3A663713L829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 123, Biological studies on anopheline vectors of malaria

Literature Cited.

References:

1. 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. Exp. Parasitol. 27:53-59, 1970.

2. Ward, R.A.: Genetic aspects of the susceptibility of mosquitoes to malarial infection. Exp. Parasitol. 13:328-341, 1963.

Publications: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6514	71 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
70 07 01	D. Change	U	U	NA		☐ 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]	CDOG141241A(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 71	1	35
FISCAL YEAR — CURRENT 72	1	35

19. RESPONSIBLE DOC ORGANIZATION

NAME: Walter Reed Army Institute of Research
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ASSOCIATE INVESTIGATORS
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NAME: Hayes, D. E. DA

PII Redacted

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. 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 infections. Mass selection procedures are used to select more efficient and less efficient vectors of malaria.

25. (U) 70 07 - 71 06. Additional mosquito transmissions of Chesson vivax malaria to Aotus monkeys have been effected. Aotus-adapted Chesson strain was sent to Southern Research Institute for pre-clinical studies on malaria prophylactic drugs. Philippine (Per) drug resistant falciparum malaria successfully adapted to splenectomized Aotus with use of an immunosuppressant agent, Imuran. Gametocytes of the Per strain are infective to Anopheles quadrimaculatus and A. stephensi. This parasite was cyclically transmitted from Aotus to chimpanzee by sporozoite passage. A highly resistant strain of falciparum malaria (Smith) from Vietnam is being adapted to Aotus. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* Available to contractors upon originator's approval.

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 Stephen C. Hembree, MSC; CPT J. Scott Anderson, 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. Plasmodium vivax

Another isolate of Chesson vivax malaria was studied in Aotus monkeys to determine whether the successful adaption reported previous (Ward, Rutledge and Hickman, 1969) could be repeated. Infected blood from a volunteer (Sherman) at Jessup Penitentiary was inoculated into a splenectomized chimpanzee (H954) on 30 November 1970. The animal became patent on 7 December and Anopheles albimanus and A. stephensi mosquitoes were fed on the chimpanzee on 8 December when gametocytes were observed. Dissection of samples of mosquitoes 7 days after the infectious meal indicated a mean oocyst count of 20.6 for A. stephensi and 0 for A. albimanus. Salivary gland dissections of A. stephensi a week later showed numerous sporozoites in the glands. On 21 December these anophelines were fed on 2 splenectomized Aotus. Both monkeys developed a patent infection 20 and 28 days after sporozoite passage. A comparison of the behavior of this isolate indicated that it was very similar to that observed the previous year in sporozoite transmitted infections in monkeys (Table 1).

The constant pattern of infection and mosquito transmission of the Chesson strain indicated that it would be a suitable model for testing the efficacy of candidate prophylactic drugs. The particular strain was of prime significance as the Chesson (New Guinea) strain has been the standard vivax malaria parasite used in the USA for the past 25 years to assess drugs. During the year, the Aotus-adapted strain was sent to an Army contract group (Southern Research Institute) for pre-clinical studies on malaria prophylactic drugs.

2. Plasmodium falciparum

In the 1970 Annual Report, the failure of attempts to infect anopheline mosquitoes with falciparum malaria was discussed. It was postulated that gametocyte immaturity was the responsible factor and that treatment with immunosuppressant drugs or antilymphocyte serum might prolong gametocyte survival until they attained a degree of maturity suitable for infecting mosquitoes.

To test this hypothesis, an experiment was conducted with a commercial preparation of azanthioprine (Imuran) at doses not exceeding the maximum used for immunosuppressant purposes in humans. Nine splenectomized Aotus monkeys were separated into 3 groups: 1) a control group, receiving saline injections, 2) a group receiving Imuran at a dosage of 5m/kg for a 17 day period, beginning two days prior to infection, and 3) a group receiving 5m/kg two days prior and 7 days subsequent to infection, then 2 mg/kg for the succeeding 8 days. All drugs were administered in saline solution by the intraperitoneal route. The animals were examined for a period of 35 days following inoculation of P. falciparum (Per strain) from an infected Aotus. All animals became patent within a period of 9 - 14 days irrespective of treatment (Table 2). There was a marked difference in the level of maximum parasitemia achieved, with untreated controls attaining only 10% of the level of treated monkeys. Gametocytes were far more abundant in animals receiving immunosuppressants and only in these individuals were mature gametocytes observed. Similarly, only in treated monkeys was it possible to infect Anopheles stephensi and A. quadrimaculatus mosquitoes with this parasite. Although oocyst counts were low, the appearance was normal and some salivary gland infections were observed.

Infected anophelines from this experiment were permitted to feed upon 2 splenectomized Aotus and one splenectomized chimpanzee to determine whether these sporozoites were virulent. Within two weeks, the chimpanzee exhibited parasitemia while the Aotus did not become positive over a 90 day observation period. The failure to establish a patent infection in the Aotus has been attributed to the inability of the liver of this host to sustain complete exoerythocytic development of P. falciparum.

Conclusions and recommendations

1. Additional mosquito transmissions of Chesson vivax malaria to Aotus monkeys have been effected. The Aotus-adapted Chesson strain was sent to Southern Research Institute for pre-clinical studies on malaria prophylactic drugs.

2. The use of an immunosuppressant agent, Imuran, permitted P. falciparum gametocytes to attain a suitable level of maturity in the Aotus monkey so that mosquitoes could be infected with this parasite. Falciparum malaria was transmitted cyclically to the chimpanzee but not to Aotus. This procedure will be applied to other parasite strains of falciparum malaria in adapting them to subhuman primates for cyclical transmission.

TABLE 1

Comparison of cyclically passaged Chesson vivax malaria in splenectomized Aotus trivirgatus (mean values

	Date of Isolate	
	1969 (n = 4)	1970 (n = 2)
Day of first gametocytes	40	26
Day of 1st mosquito infection	40	32
Day of peak gametocytemia	52	35
Day of peak mosquito infection	42	35
Peak gametocyte count/mm^3 blood	2680	6480
Highest mean oocyst count/ mosquito	11	12

TABLE 2

Effect of an immunosuppressant upon Plasmodium falciparum (Per strain) infection in Aotus trivirgatus (splenectomized)

Treatment	Animal #	Day patent	Dey highest parasitemia	Maximum no. parasites/mm^3	Day of first gametocytes	No. days gametocytes observed / No. days examined	No. mosquito pools infected / No. pools fed
Control	67	12	33	14,760	23	2/14	-[1]
	234	13	13	120	-[2]	0/13	-[1]
	250	14	26	33,960	26	3/17	0/3
Imuran (5mg/kg x 17 days)	251	12	16	340,800	22	7/18	0/7
	252	11	19	187,680	19	11/22	2/17
	395	12	20	211,680	20	6/18	0/2
Imuran (5mg/kg x 8 days, 2mg/kg x 9 days).	253	12	26	95,760	19	5/16	0/4
	254	9	19	457,560	19	16/24	14/23
	396	13	19	31,200	-[2]	0/17	0/2

[1]Feeding trials not attempted; no gametocytes observed.

[2]Gametocytes not observed

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 124, Biological studies of mosquito malaria infection and transmission

Literature Cited

References:

1. Ward, R.A., Rutledge, L.C., and Hickman, R.E.: Cyclical transmission of Chesson vivax malaria in subhuman primates. Nature 224:1126-1127, 1969.

Publications: None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6515	71 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
70 07 01	D. Change	U	U	NA	NL	XX 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. [redacted]	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

a. DATES/EFFECTIVE: Not Applicable 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 71	1	35
FISCAL YEAR CURRENT 72	1	35

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
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NAME: Harrison, CPT B. J. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Anopheles; (U) Aedes; (U) Mosquitoes; (U) Malaria; (U) Arboviruses; (U) Disease 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) 70 07 - 71 06. Study of anopheline vectors of malaria from Southeast Asia has continued. A. stricklandi reported from Thailand for first time. Two previously unrecognized forms discovered in Anopheles aitkenii complex. Study of Anopheles (Myzomyia) series has shown that only 5 species of this group occur in Thailand: aconitus, culicifacies, jeyporiensis, minimus, and pampanai. This study showed tnat previous records of filipinae, fluviatilis and varuna from Thailand are in error. Revision of Aedes (Christophersiomyia) of Southeast Asia completed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* Available to contractors upon originator's approval.

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 Bruce A. Harrison, 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. Short term field studies are conducted in endemic areas for vector incrimination and collection of specimens for laboratory analysis. 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 work only.

Progress

1. Anopheles of Thailand

Portions of the text of Anopheles mosquitoes of Thailand that are in final or rough draft form are currently being edited and nearly 200 plates are undergoing final preparation and correction. Examination of recently accrued specimens is in progress and keys to the immature stages are being prepared.

Examination of the Anopheles aitkenii complex revealed the first specimens of A. stricklandi Reid from Thailand. In addition, 2 previously unrecognized "forms" have been found in the aitkenii complex. The status of these "forms" is not yet resolved.

* University of Texas, School of Public Health, Houston
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Preliminary examination of nearly 18,000 sibling and individually reared specimens of the Anopheles (Myzomyia) series suggests only 5 species occur in Thailand, i.e., aconitus, culicifacies, jeyporiensis, minimus and pampanai. Previous records of adult filipinae, fluviatilis and varuna from Thailand can now be disregarded, since specimens identical to these were infrequently obtained from larvae and sibling series of wild-collected aconitus and/or minimus. No immatures of filipinae, fluviatilis or varuna were found in Thailand.

2. Aedes of Southeast Asia

Study has been completed on the subgenus Christophersiomya and the taxonomic revision is undergoing final preparation as the illustrations are being prepared. Publication is expected in early FY 72.

3. Field studies in Northern Afghanistan

During 1968, the Johns Hopkins University Geographical Epidemiology Unit (USAMRDC Contract MD-2658) conducted field studies in northern Afghanistan which indicated the presence of a focus of vivax malaria at Bulla Quchi. In this village of 361 inhabitants, who were of Uzbek origin, six persons between the ages of 1 to 16 years exhibited Plasmodium vivax parasites in peripheral blood smears. Limited entomological surveys conducted in the village with CDC light traps resulted in the collection of several hundred specimens of A. hyrcanus (Pallas)[1] and a small number of A. pulcherrimus Theobald in the period between 8-20 September. Surprisingly, no specimens were found of A. superpictus, which has always been considered to be the only vector of malaria in Afghanistan. Since these investigations were conducted towards the end of the transmission season the incrimination of a vector could not be achieved. Several possibilities existed: 1) The vector was an anopheline which was present earlier in the transmission season, 2) A. hyrcanus was the vector, 3) The vector was A. pulcherrimus or, 4) More than a single species served as a vector.

A follow-up study by a field team from the Johns Hopkins University School of Hygiene and Public Health and the Walter Reed Army Institute of Research[2] visited northern Afghanistan during the height of the malaria transmission season in order to incriminate the mosquito vector(s), collect additional specimens for taxonomic study and examine the incidence of malaria in several villages.

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[1]The literature on malaria in Afghanistan refers to this species as A. hyrcanus var. pseudopictus Grassi or A. hyrcanus var. sinensis Wiedemann. A study of the Palearctic members of the hyrcanus group indicates that the Afghanistan populations should be designated A. hyrcanus (Pallas)

[2]The field team comprised: Alfred A. Buck, M.D. (Epidemiologist), Robert I. Anderson, Ph.D. (Parasitologist), Kay Buck (Nurse-technician) and Ronald A. Ward, Ph.D. (Medical entomologist).

Entomological and epidemiological studies were conducted during July 1970 in Anguor Baugh (10 km S of Kunduz, near the Kunduz River) and in Khana Qua (a small village 5 km S of Imam Saiyid and 15 km from the Tadzik S.S.R.-Afghan border) while only entomological studies were made in Bulla Quchi. Parasitological surveys of the Malaria Institute indicated that approximately 20 percent of individuals in Anguor Baugh had malarial infections (cumulative total through 14 July 1970), 55 percent in Khana Qua and about 10 percent in Bulla Quichi. In all instances, Plasmodium vivax was the only parasite observed.

The three villages were similar in size with populations between 500-1000. All homes were constructed of mud or sun-baked brick and situated in courtyards surrounded by high mud walls. Cattle and domestic animals were housed in adjoining buildings in the courtyards, cooking and sleeping (during the summer) usually occurred outdoors in the yards. Both Bulla Quchi and Khana Qua were surrounded by rice and melon fields; most dwellings were within a distance of 150 to 300 meters from a rice field. The rice fields were approximately 1 km distant from Anguor Baugh. However, in this instance, the village was situated on a small bluff overlooking the Kunduz River, several hundred meters away. Rainfall is virtually non-existent during the summer and most of the water is supplied from narrow irrigation ditches which are usually contaminated. Within the villages little natural vegetation remained with the exception of weeds, small fruit trees such as apple and pomegranate and artifically planted shade and lumber trees.

Mosquito collections were made from the villages using standard survey procedures, i.e., human-biting and resting collections, larval surveys and use of the CDC light traps. Anopheles pulcherrimus was the commonest anopheline encountered and comprised 95 percent of the adults collected. In habits, this species appeared to be exophilic and partially anthrophilic. Biting collections indicated that female A. pulcherrimus confined most of their blood-feeding to the period 1930 - 2200 hours, usually outside the houses. A biting collection at Bulla Quchi during the evening of 23 July collected 4.3 anophelines and less than 1 culicine per man hour from human bait. A similar biting rate was observed from cattle several hundred meters from the nearest dwelling. No anophelines were observed within houses or stables in the villages, either at night or during the day. However, during the evening of 24 July this species was observed feeding indoors in a house on the outskirts of Kunduz.

Light trap collections were made during three nights at Anguor Baugh (18-19, 19-20, 21-22 July) and one evening at Bulla Quchi (22-23 July). Although the total number of mosquitoes collected was relatively low (Table 1), a large proportion of the catch consisted of engorged Anopheles pulcherrimus. The only adult A. hyrcanus encountered during the study came from a light trap in Bulla Quchi suspended from a tree near several Tethered cows and a resting collection from a wall at the edge of a rice field in Khana Qua. Culicine mosquitoes were extremely scarce during the period surveyed and no additional anopheline species were encountered in a variety of habitats examined.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OA 6516	71 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. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	126
b. CONTRIBUTING				
c.	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 71	1	30
	CURRENT 72	2	30

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

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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) Malaria; (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) 70 07 - 71 06. By employing a new culture medium and modifying the trypsin treatment of the primary explants it is now routinely possible to subculture cells from various anopheline and culicine species after seven to fourteen days in vitro. Comparable progress previously required three weeks to three months. Isolation of Plasmodium cynomolgi sporozoites from mosquito tissues and the separation of the various erythrocytic stages of the same parasite by density gradient centrifugation have not been entirely successful. In both instances, the high osmolarity of the gradient medium, a mixture of Renografin and serum albumin, has been somewhat detrimental to the parasites. Exhaustive dialysis of both components is being carried out to reduce the osmotic pressure to well below isotonic levels. The osmolarity is then readjusted to physiological levels by the addition of salts. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71

*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

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 126, In vitro cultivation of mosquito tissue and malarial parasites

Investigators
Principal: Imogene Schneider, Ph.D.
Associate: David H. Chen*, SP/5 William N. Palmer

Description

This investigation involves three major areas: (1) the use of an in vitro system for the collection of large numbers of malaria 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. The sporozoites obtained directly or indirectly through the first two approaches outlined above are to be used as the source of malarial antigen for immunological studies. The mosquito cell lines are to be utilized in both malaria and arbovirus research.

Progress

1. Cultivation of malaria parasites and mosquito tissues

Prior work on culturing the mosquito phases of malaria parasites centered on using the oocyst stages. Little information was gained by doing so. With a reasonably adequate culture medium the older oocysts, having already differentiated to some extent, matured in vitro and released their sporozoites within a period of 24 hours. Placing younger oocysts in vitro, while theoretically desirable as more stringent demands would be made on the culture system, was impractical due to the technical difficulties involved in dissecting such small bodies from the midgut of the mosquito host. It therefore seemed advisable to culture the initial stages, e.g., the gametocytes, assuming they could be obtained in sufficient numbers. Efforts are being made to separate the gametocytes from the other erythrocytic stages as well as uninfected blood cells by the technique of density gradient centrifugation (see section 2a).

The culture chambers were microtest tissue culture plates, prepared one day in advance by filling 30 of the wells with 15 µl of medium and the remaining wells with an equal amount of medium containing Anopheles stephensi cells (5×10^5 per ml). Previous work had employed cells from an established line which had been in existence for approximately three years. More recently, cells from actively proliferating primary cultures were used since the latter would be expected to reflect more accurately

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During the day a search was made for adult anopheline resting sites. The interior and exterior of houses and stables did not disclose mosquitoes. At Anguor Baugh numerous A. pulcherrimus were observed resting on the walls of mud fisures or "caves" in a small bluff between the Kunduz River and the village proper. The "caves" were 2 - 4 meters high, 0.3 - 1 meter wide and 0.6 - 2 meters in depth. This type of microniche was fairly extensive along the shores of the river. Often as many as 25 anophelines were observed in a single fissure. During a 45 minute period, between 0600 - 0645 hours on 22 July, 53 A. pulcherrimus were collected by one individual. These included 10 ♀♀ (freshly engorged), 1 gravid ♀ and 42 ♂♂. Engorged females from this and other collections were preserved for precipitin testing to determine the source of the blood meal. At Khana Qua, no natural resting sites were observed. Careful searching of the outer walls of the village disclosed female A. pulcherrimus resting along the sides of small cracks in the mud surface. Similarly, a low wall adjacent to a rice field contained many mosquitoes. A 20 minute collection recovered 34 anophelines (32 A. pulcherrimus [7/8 ♀♀ engorged and 22 ♂♂] and 2 ♀♀ A. hyrcanus).

Anopheline larvae were observed in two rather distinct situations. In Anguor Baugh, the rice and melon fields adjacent to the village were dry. Numerous seepages were observed at the base of the previously mentioned bluffs. These contained slow flowing water which was contaminated with cattle droppings. In this situation as many as 15 second and third stage anopheline larvae could be found in a single larval dip. Other potential breeding sites in this village such as the irrigation ditches, borrow pits and the pond in the mosque courtyard were negative for mosquito larvae. The second anopheline habitat was observed both in Bulla Quichi and Khana Qua where extensive larval breeding was observed in the flooded rice fields. Larvae usually only occurred in well-seasoned water which had been standing in the fields for a period of 3 weeks or longer. Under such conditions, over 100 anopheline larvae were found in a single dip. As with the adult collections. A. pulcherrimus was the dominant species (80 - 90 percent) with A. hyrcanus as the residuum.

Precipitin testing of engorged female A. pulcherrimus from Anguor Baugh indicated that bovine animals were the main host (Table 2) and that 64 percent of the meals were derived from this source. Horses and humans were the other common sources of blood meals. Unidentified mammals accounted for a residual 15 percent. Approximately 75 parous females of this species were dissected for malarial oocysts and/or sporozoites. All specimens were negative.

The entomological evidence indicates that Anopheles pulcherrimus is probably responsible for the transmission of Plasmodium vivax to to man in the Kunduz - Imam Saiyid area of northern Afghanistan during the peak of the transmission season in July and August. The ecology of this species is such that it has a close association with man in its breeding sites, feeding habits and resting sites - all attributes of a good malaria vector. This was confirmed by A.S. Badawi, WHO entomologist who found 6/540 (1.1 percent) of A. pulcherrimus dissected during

July and August 1969 with infected salivary glands. 93 additional specimens dissected during June and September were negative.

The importance of A. hyrcanus as a secondary vector requires further study. Since it has breeding habits similar to the former species it may be postulated that it becomes significant in late August or September when pulcherrimus populations decline drastically and hyrcanus assumes 95 percent incidence. Badawi found 2/304 (0.7 percent) of dissected A. hyrcanus with positive salivary glands during August and September 1969 but observed no infections from 866 females examined during June, July or October. He pointed out that no infected anophelines were seen after Septebmer 15th. According to his observations some of these A. hyrcanus may survive for a sufficient period to undergo full sporogonous development in the early fall. The feeding habits of these late seasonal mosquitoes are not known so it is not possible to ascertain their epidemiological significance.

Residual spraying of houses with DDT has occurred on a fairly regular basis in the Kunduz area since 1950. Until 1968, both the above species were susceptible to this insecticide. However, during 1969, A. hyrcanus developed DDT-resistance. It is evident the use of residual DDT house-spraying is not the solution to anopheline vector control here. A number of alternate procedures such as the use of larvicides, applied either from back-pack sprayers or aircraft, spraying of adult resting sites (which appear to be so clearly defined) and changing irrigation practices in the rice-growing areas should materially reduce anopheline density to such a level that malaria transmission will be drastically reduced.

The disappearance of A. superpictus from Kunduz Province is apparently related to ecological changes in the Kunduz Valley which are associated with the flooding of tremendous tracts of land for the cultivation of rice. In addition, with the increase in human and cattle populations, natural sources of water, which were formerly the breeding sites of A. superpictus have become contaminated and can no longer support this species. On the other hand, pulcherrimus and hyrcanus have invaded this habitat and the rice field environment to produce extensive breeding sites.

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

Mosquito Light Trap (CDC) Collections in Kunduz Province, Afghanistan[1]

Mosquitoes	Date and Location				
	Anguor Baugh			Bulla Quchi	
	18-19 July	19-20 July	21-22 July	22-23 July	TOTALS
Anopheles pulcherrimus	0	2 engorged ♀♀	20 engorged ♀♀ 2 ♂♂	2 engorged ♀♀ 3 ♂♂	29
Anopheles hyrcanus	0	0	0	1 ♀, 2 ♂♂	3
Culicines[2]	0	1 engorged ♀ 3 ♀♀	5 engorged ♀♀ 1 ♀	3 ♀♀, 1 ♂	14
TOTALS	0	6	28	12	46

[1]Four light traps were operated daily from 1800-0600 hours.

[2]Aedes caspius, Culex theileri and Culex sp.

TABLE 2

Precipitin Testing of Blood Meal Source of *Anopheles pulcherrimus* from Anguor Baugh

Host of blood meal	Source of Collection			Totals	
	CDC traps in house doorways or courtyards	CDC trap in cottonfield	Resting in "caves"	Number	Percent
Bovine	9	3	18	30	63.8
Horse	1	0	4	5	10.6
Goat	0	0	1	1	2.1
Human	0	0	4	4	8.5
Other mammal	3	0	4	7	15.0

Project 3A663713D829, MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 125, Taxonomy and Ecology of disease bearing mosquitoes of Southeast Asia

Literature Cited.

References: none

Publications:

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2. Tyson, W.H.: Contributions to the mosquito fauna of Southeast Asia. VII. Genus Aedeomyia Theobald in S.E. Asia. Contr. Amer. Entomol. Inst. 6(2):1-27, 1970.

3. Tyson, W.H.: Contributions to the mosquito fauna of Southeast Asia. VIII. Genus Aedes, Subgenus Mucidus Theobald in S.E. Asia. Contr. Amer. Entomol. Inst. 6(2):28-80, 1970.

the properties of cells within the intact insect. One µl of blood forms was added to each of the wells the following day. In no instance was the separation of the gametocytes and schizonts complete; the latter always appeared in greater numbers in the samples. Also there was evidence that the blood cells containing the parasites had been adversely affected by the gradient medium. In none of the control or experimental cultures (6 series of 60 microtest cultures each) was there any evidence of further development on the part of the gametocytes.

2a. Isolation of the gametocyte stage from the other blood forms

Investigators from another laboratory 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 older trophozoites banded in between. Gametocytes were found in the upper half and uninfected cells in the lower half. Attempts to use identical gradients for isolating the various blood stages of P. cynomolgi were unsuccessful. Not only was the separation poor but all of the blood cells, regardless of their final position in the gradient, showed morphological abnormalities when examined under phase contrast. The damage suffered by the cells was attributed to the high osmotic pressure of the gradient medium. Therefore, density gradients had to be prepared in which the osmolarity of the fractions was controlled.

To determine the specific density of an average red blood cell of the Rhesus monkey a 6 ml sample of blood was withdrawn from the femoral artery and defibrinated with glass beads. The blood was then mixed with 30 ml of cold buffered saline-glucose solution (BSG) having a pH of 7.4 and an osmolarity of 291 mOsm/l. It was then filtered twice in the cold through 14µ Millipore filters, washed twice with cold BSG and then packed by centrifugation at 1400xg in a Sorvall refrigerated centrifuge. 47.5 mm of packed red cells were then introduced into 92 mm capillary tubes previously filled with 20 mm of phthalate ester having densities varying from 1.066 to 1.114 gm/cm^3. The density of the average blood cell could be determined by noting in which of the tubes 50 percent of the blood cells had moved through the gradient. This proved to be between 1.086 and 1.090 gm/cm^3.

In order that the infected cells be retained in the upper half of the gradient, the uninfected heavier red cell must reach equilibrium below the center of the gradient. This was accomplished by using a discontinuous gradient consisting of five fractions in increments of 0.008 gm/cm^3, the lightest and heaviest fractions having densities of 1.068 and 1.108 gm/cm^3, respectively.

To prepare bovine serum albumin gradients with the proper density and osmolarity, the albumin is first dissolved in deionized water (30% by weight) and dialized in the cold. An Amicon PM 30 dialysis membrane,

which allows molecules of under 30,000 molecular weight to pass through, was employed. Dialysis of the commercially obtained bovine serum albumin was necessary because it has an osmolarity of over 4,000 mOsm/l at a concentration of 30 gms percent; the physiological level of red blood cells is approximately 290 mOsm/l. After exhaustive dialysis (30 hours) the osmolarity was lowered to 43 mOsm/l. The albumin after being centrifuged and filtered to remove bacterial growth was weighed and the volume estimated from the weight. The volume of available water for binding with salts was estimated and then the following salts were added in serial order to raise the osmolarity to approximately 237 mOsm/l: $MgCl_2 \cdot 6H_2O$, NaCl, KCl, $NaHCO_3$ and finally glucose. The osmolarity is then adjusted to 290 by adding the appropriate amount of NaCl (100 mg of NaCl/100 ml of solution raises the osmolarity by 31.5 mOsm/l). The specific density of the albumin solution is determined with a Lipkin bicapillary pycnometer.

Gradients are prepared by layering the five fractions of differing densities in a 40 ml cellulose nitrate tube. One ml of defibrinated P. cynomolgi infected blood cells are layered on top of the gradient and spun at 16,000xg for 60 minutes. The majority of gametocyte-infected cells are found at the interface of the two lightest fractions. Separation of the gametocytes and schizonts is not completely successful but all of the blood cells retain their normal morphology both under phase contrast and in Giemsa stained preparations. Continuous linear gradients are now being prepared in an effort to obtain greater separation between the gametocytes and schizonts.

2b. Isolation of malaria sporozoites on density gradients

In a previous study, P. gallinaceum sporozoites were routinely isolated in large numbers (10^7/ml or more) from homogenates of infected Aedes aegypti mosquitoes by the use of a density gradient system. The gradient medium was composed of Renografin (methylglucamine diatrizoate plus additives) and bovine serum albumin-Fraction V. Linear gradients were prepared from 15% BSA/ 20% R(1:1, density = 1.061 gm/cm^3) and 30% BSA/56% R (1:1, density = 1.153 gm/cm^3). Infective sporozoites were routinely recovered from gradient fractions 11 and 12 with an average density of 1.120 gm/cm^3.

P. cynomolgi sporozoites were isolated from An. stephensi mosquitoes in an identical manner. Although the sporozoites banded in approximately the same fractions they were neither infectious nor viable. The most plausible explanation is that the P. cynomolgi sporozoites are much more sensitive to osmotic changes than are the P. gallinaceum sporozoites. In an effort to negate this factor, a number of gradient runs were made in which the concentrations of Renografin and serum albumin were varied while the densities of the fractions remained constant. No improvements were noted in the appearance of the sporozoites.

Consequently, present attempts are centered on using a gradient composed solely of BSA which has been exhaustively dialized (see preceding section) and in which the osmolarity has been adjusted to

physiological levels. The osmolarity of An. stephensi hemolymph has been estimated to be approximately 380 mOsm/l whereas that of monkey blood is about 290 mOsm/l. With appropriate salts and sugars the osmolarity of the different gradient fractions are adjusted to levels within this range. Initial trials with such gradients have been very encouraging: motile, viable sporozoites have been recovered from the peak fractions. However, infectivity of the sporozoites has yet to be tested.

3. Establishment of cell lines from Culex tritaeniorhynchus and Culex salinarius

One of the most striking features regarding the establishment of insect cell lines has been the long period of adaptation required between the initiation of the primary cultures and the subsequent transition to subculturing. This process has often taken three or more months with some lines requiring as long as nine months. Most of the dipteran lines established, currently numbering about 15, are being used for the propagation of arthropod-borne viruses and for studies of host cell-pathogen interactions involving various parasitic protozoans. Such prolonged periods of adaptation undoubtedly compromise the value of these cell lines for such studies.

In an earlier report, the initiation and development of cell lines from neonate larvae of C. tritaeniorhynchus and C. salinarius was described. Intervals of 2-3 months were required before the cells were capable of being subcultured. Additional lines have since been established in which the transition period from primary culture to subculture was reduced to a maximum of 8-14 days.

Five primary cultures for each species were initiated during March and April, 1970. Methods for the collection, surface sterilization and subsequent handling of the embryos were identical to those used for initiating cell lines from An. stephensi. Two changes were made in the culturing procedure: (1) the culture medium designed by S.H. Hsu for C. quinquefasciatus was utilized and (2) the length of time the larval fragments remained in the trypsin solution was doubled. Outgrowth of cellular spheres from the larval fragment ends, so commonly seen in primary cultures of earlier dipteran lines, was virually absent. Instead, small colonies of cells were seen attached to the bottom of the culture flasks within 2-4 days after the cultures had been set up. Multiplication of the cells in the colonies, particularly in the C. tritaeniorhynchus cultures, was very steady with no indication of a lag in growth. All 5 primary cultures of C. tritaeniorhynchus were subcultured within 8 days of being initiated. Although growth in the C. salinarius cultures was neither as rapid or extensive, the cells could nonetheless be subcultured within 10-14 days.

The cells of the C. tritaeniorhynchus line grow in a monolayer but have a pronounced tendency to pile up at central foci as the cell density increases. There is considerable variation with respect to both size and shape of the cells. The most prevalent cell type is spindle-shaped

and varies from 7-15μ in diameter and 35-55μ in length. The chromosome number is predominently diploid (2n = 6) with 10-15% of the cells being tetraploid. Higher polyploidy is rare. During the logarithmic growth phase, the population doubling time is approximately 21 hours.

C. salinarius cells also grow in a monolayer but unlike those of the C. tritaeniorhynchus line have no tendency to cluster. The great majority of cells are polygonal in shape and vary from 10-20μ in diameter and 30-70μ in length. Approximately 65% of the cells possess a diploid chromosome set; the other 35% are either tetraploid or occasionally octaploid. Upon seeding into new flasks, the cells initially decrease in number but by day three are multiplying quite rapidly. They never, however, attain the density found in the C. tritaeniorhynchus line.

Conclusions and recommendations

1. Attempts to culture the mosquito phases of malaria parasites in medium alone or in media containing cells from primary or established cultures of the mosquito host have not been very successful. The use of the more advanced stages is not very meaningful as differentiation of the parasite has by and large been completed and the medium need serve only for maintenance rather than growth of the parasite. Younger oocysts would prove more sensitive indicators of the adequacy of the medium but it is not feasible to dissect them in any great number from the midgut. The gametocyte stage might prove the most valuable for assessing the culture system were it possible to obtain this stage free and undamaged from the other blood stages.

2. Initially two problems were involved in attempting to separate the gametocytes from the other blood forms; namely, separation between schizonts and gametocytes was not completely satisfactory and secondly, all of the blood cells, infected as well as uninfected, were damaged to some extent by being on the gradient. The latter problem has been resolved by using gradients in which the osmolarity of the fractions is rigidly controlled. Emphasis should therefore be placed on varying the densities of the intermediate fractions in order to achieve finer separation between gametocytes and schizonts.

3. P. cynomolgi sporozoites proved to be much more fragile than P. gallinaceum sporozoites with respect to their ability to withstand adverse changes in the osmotic pressure of the gradient medium. This problem has apparently been solved by adjusting the osmolarity to physiological levels. This technique provides for the mass isolation of sporozoites with a minimum of extraneous debris in the parasite suspension. As such it should prove invaluable for studies requiring large numbers of clean sporozoites.

4. Cells from the neonate larval stage of any mosquito species can probably be placed in culture with a good chance of surviving and eventually developing into a cell line. The interval between initiating the primary culture and the first subculture depends upon the

length of time the larval fragments are treated with trypsin and on the adequacy of the culture medium. More emphasis should now be placed on culturing tissues from the adult insect since it is this stage which serves as a vector for many viruses and parasitic protozoans.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6518	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORG'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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. CONTRIBUTING	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
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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 71	2	70
	CURRENT 72	2	

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: MOON, A.P. DA

NAME:

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. (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 physidiological 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) 70 07 - 71 06 Hematologic, biochemical and parasitologic parameters of Aotus trivirgatus were studied. Serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase values were greater than those previously reported for man or old world non-human primates. Thirty percent of serum electrophoretic patterns had double albumin components. Most animals had high alpha-2 globulin levels. No natural malarial infections were found, but some monkeys had microfilariae or trypanosomes. Giardia sp. was the most common intestinal parasite. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* 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 127, Test systems for Plasmodium falciparum

Investigators
Principal: E. H. Sadun, Sc.D., Lib. Doc.
Associate: CPT A. J. Johnson, VC; H. R. Langbehn; J. S. Williams

1. Hematologic, biochemical and parasitic parameters of the night monkey, Aotus trivirgatus.

The reports that the night monkey, Aotus trivirgatus, is susceptible to infection with human Plasmodia initiated interest in this animal as a model for experimentation with human malarial parasites. Although many laboratories have studied Aotus monkeys in various aspects of malarial research, little information has been published until recently on the normal hematologic, biochemical or parasitologic parameters of these animals. An early report of hematologic findings in a few Aotus monkeys has been followed by more substantial hematologic studies. Some serum and red blood cell biochemical values have been reported and a number of monkeys have been examined for naturally occurring malarial infections.

This report compiles hematologic, biochemical and parasitologic observations made on Aotus monkeys during a period of one year.

Animals

The Aotus monkeys used in this study were imported from Colombia and quarantined for three or four weeks during which time they were tuberculin tested. After quarantine the monkeys were transferred to a room kept at a temperature of 80-85°F and were maintained on a diet of monkey chow and fresh fruit supplemented three times a week with live neonatal mice. Most of the monkeys weighed between 400 and 900 grams. At least two definite color variations were seen in the population, the majority being light to dark brown with a white underbelly while the major variant had grey to black coloring with a yellow-orange underbelly. The range, habitat, and a description of behavior have been reported by Napier and Napier.

Collection of materials

Blood and fecal samples from apparently healthy adult animals were obtained in the morning and all hematologic procedures were completed on the same day. Blood obtained from the femoral vein was mixed with disodium ethylenediaminetetraacetate (EDTA) for hematologic procedures. Serum was collected from clotted blood and frozen at -70°C within two hours after the blood samples were taken. Thick and thin blood films were also made from each animal.

Hematology

Erythrocytes and leukocytes were counted in a Neubaur chamber using Hayems solution as diluent for erythrocytes and 1% acetic acid solution for leukocytes. The microhematocrit method was used to determine packed cell volumes. Hemoglobin concentrations were determined by the cyanmethemoglobin technique. Reticulocytes were stained with brilliant cresyl blue by mixing two drops of blood with two drops of 1% stain in saline citrate. After 10 minutes of staining the contents of the tubes were mixed, blood films were made and the percent of reticulocytes in 1,000 RBC's was determined. Platelet numbers were determined by phase microscopy according to the method of Brecher using 1% ammonium oxalate as the diluting fluid. Thin blood films were fixed in methanol and along with thick films were stained with Geimsa stain. Differential white cell counts were done by classifying 100 leukocytes on thin blood films stained with Wright's stain.

Serum chemistry

Serum glutamic pyruvic transaminase (SGPT), glutamic oxaloacetic transaminase (SGOT), glucose, and total protein levels were determined by the ultramicro methods described by Williams et al. Uric acid determinations were performed by an ultramicro modification of Carraway's method. Levels of urea nitrogen were determined by an ultramicro modification of the urease method. Serum electrophoresis on cellulose acetate strips was performed by the Beckman Microzone technique using Beckman B-2 buffer.

Parasitology

Two hundred oil immersion fields of Giemsa stained thick blood smears from each monkey were examined under oil immersio.. for natural blood parasites.

Fecal samples were examined in saline and Lugol's iodine preparations and by the formalin-ether concentration technique.

All values are results from individual male or female monkeys and not repeated samplings from a small number of animals.

Maintenance

Aotus monkeys do not adapt readily to captive conditions and are known to be susceptible to a number of viral agents, at least one of which may be associated with exposure to man. During the quarantine period losses averaged 35% of the monkeys received and were usually attributed to respiratory tract infections in which pneumonoccal, streptococcal, staphylococcal, Klebsiella and Pasteurella species have been isolated. A few fatal generalized viral infections occurred

in monkeys in which intranuclear inclusion bodies commensurate with those caused by herpes viruses were observed. After the quarantine period losses were markedly reduced and relatively little overt disease or mortality occurred in our laboratory. The introduction of a more constant heat supply may have in part accounted for the improvement in the health of the colony. Newly arrived monkeys avidly consume fresh fruit and most of them eat neonatal mice but do not readily accept the commercial Purina monkey chow, although their acceptance of this food increases gradually. Monkeys maintained for relatively long periods (1-2 years) eat the monkey chow satisfactorily.

Hematology

The data obtained from monkeys during the past year are summarized in Table 1. In general our results agree with those obtained by Porter for Panamanian monkeys except for the mean erythrocyte counts. This difference is reflected in the lower mean corpuscular volume reported in our data when compared to that reported from the Panamanian monkeys. Since hematocrit values were similar in both groups, this difference is probably due to variation in RBC counting techniques between the two laboratories rather than a real difference in cell volume. Reticulocytes were commonly found, although nucleated red blood cells were seldom seen in the peripheral blood. Platelet numbers, which had a fairly wide range, were in agreement with those previously reported. Total leukocyte counts were similar to those reported for monkeys maintained in the laboratory for a period of one to two years. Polymorphonuclear neutrophils were usually present in the highest percentage and they often contained hypersegmented nuclei. A relatively large percentage of eosinophils was found in many monkeys with the upper limit of the range reaching 37%. The mean values are somewhat higher than those previously reported, although Porter's values for monkeys newly arrived at the laboratory ranged to 19%. Whether these high percentages of eosinophils are a reflection of a disease process is not known. Basophils were found only rarely. Cells of the lymphocytic and monocytic series were combined under the heading of mononuclear lymphoid cells. Small lymphocytes, many displaying prominent stippling, were the predominate cell. Large mononuclear cells were extremely fragile and were often ruptured, making differentiation difficult. However, it appeared that mature monocytes were present at low levels.

Serum proteins

The results of total protein and electrophoretic determinations are listed in Table 2. The serum of thirty percent (33 of 110) of the monkeys possessed a definite double or split albumin band on electrophoretic patterns (Fig. 1). This bisalbuminemia is a rare genetic trait in man although some lower animals frequently have albumin polymorphisms. No reference to this occurrence could be found for sub-human primates.

Relatively high levels of alpha-2 globulins were found in most Aotus monkeys and in general the amount of total globulins was greater than albumin. As Bier suggests, the low albumin and high globulin levels found in monkeys in captivity may reflect the relatively poor adaptation to cage life.

TABLE 1

Hematologic findings in Aotus monkeys

	Number animals	Mean	S.D.*	Median	Range
RBC (/cu mm^3)	157	5.17	(.84)	5.04	3.50– 7.74
PCV (%)	157	42.0	(5.4)	42.0	31.0 –56.0
Hemoglobin (g%)	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 (10^3x/cu mm)	63	397.1	(109.4)	390.0	204–734
WBC (10^3/cu mm)	157	12.7	(4.7)	11.7	3.2 –28.5
Differential (%)					
Neutrophils	157	55.4	(7.6)	58.0	13–91
Eosinophils	157	9.6	(9.2)	7.0	0–37
Mononuclear lymphoid	157	35.5	(18.3)	35.0	5–80
Basophils	157	< 0.1	–	0.0	0–1

*Standard deviation

TABLE 2

Serum proteins (g/100 ml) of Aotus monkeys

	Number animals	Mean	(S.D.)	Median	Range
Total protein	75	7.0	(1.2)	7.0	4.9–10.2
Albumin	75	2.7	(0.7)	2.7	1.2– 4.2
Alpha-1 globulins	75	0.3	(0.1)	0.3	0.2– 0.5
Alpha-2 globulins	75	1.3	(0.3)	1.3	0.7– 2.4
Beta globulins	75	1.0	(0.2)	0.9	0.6– 1.5
Gamma globulins	75	1.8	(0.5)	1.7	1.0– 2.9

Serum chemical values

Since only microliter amounts of serum are needed to perform analysis by the ultra-micro method, individual sera could be assayed without testing sera pooled from several animals or of severely stressing the individual monkeys by excessive bleeding. The results of serum chemical tests (Table 3) show that both SGOT and SGPT levels were elevated when compared to values obtained for man or old world primates in our laboratory by the same procedures. Other authors have noted increased levels of transaminases in the serum of Aotus as well as other new world monkeys. Whether these higher enzyme levels are a genetic trait or are a result of infections or other stress factors brought about by captivity is not known. Urea nitrogen values were consistent within a relatively short range, while uric acid values were found at low levels except in a few animals.

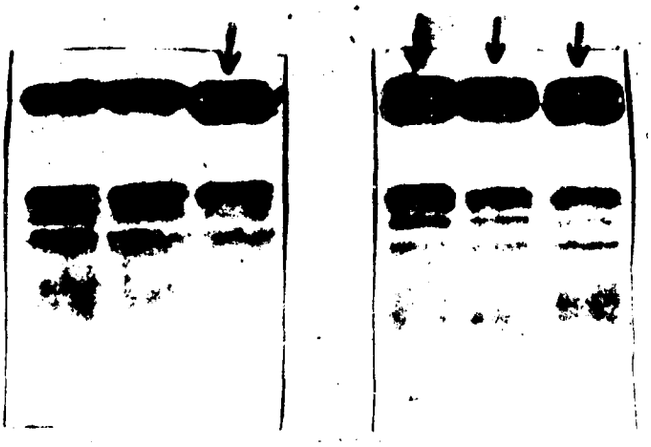

Fig 1. *Cellulose acetate electrophoretic patterns contrasting sera from 2 Aotus monkeys having a single albumin component with sera from 4 monkeys which have double albumin components (see arrows). US Army photograph.*

TABLE 3

Serum chemical values of Aotus *monkeys*

	Number animals	Mean	(S.D.)	Median	Range
Transaminase*					
SGOT	75	153	(71)	131	49–323
SGPT	75	47	(23)	39	21–121
Urea nitrogen mg%	56	14	(3)	13	7–26
Uric acid mg%	56	0.5	(0.4)	0.3	0–2
Glucose mg%	43	113	(40)	106	27–220

* Reitman-Frankel Units

Glucose levels were similar to those previously reported for Aotus and other new world monkeys.

Parasitology

No naturally occurring malarial parasites were found in any of the 181 monkeys examined. This finding agrees with other results with Panamanian monkeys and Colombian monkeys. Serum from normal Aotus monkeys did not react with malarial antigens in either the indirect fluorescent antibody test or a micro hemagglutination test. Microfilariae, however, were found in 2 monkeys. One trypanosome was found in the blood of each of two monkeys and repeated examination of blood smears obtained from these animals failed to reveal other trypanosomes. The incidence of trypanosomiasis is probably greater than that observed considering the small numbers of trypanosomes detected in the peripheral blood of these monkeys.

The results of stool examinations of 60 Aotus monkeys are shown in Table 4. The most commonly found protozoan was Giardia sp. Six of the nine monkeys harboring nematodes were infected with pinworms and stools from the other three animals contained a hookworm-like egg measuring approximately 44 x 77 microns. Stools from two monkeys contained operculated trematode eggs approximately 18 x 29 microns in size. A fluke identified as Athesmia foxi was found in a bile duct in a histological section of liver from one monkey. Cestode eggs were found in the feces of one monkey. No eggs or parasites were seen in the stools of 16 monkeys.

TABLE 4

Intestinal parasites of 60 Aotus monkeys

Protozoa	Number infected
Giardia sp.	27
Trichomonas sp.	7
Entamoeba sp.	7
Iodamoeba sp.	1
Chilomastix sp.	1
Endolimax sp.	1
Unclassified	2
Helminth eggs	
Nematodes	9
Trematodes	2
Cestodes	1

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 127, Test systems for Plasmodium falciaprum

Literature Cited.

Publications:

Merrill, Thomas G. and Wellde, Bruce T.: The fine structure of the heartof owl monkeys (Aotus trivirgatus) infected with human malaria (P. falciparum). Reprinted from 28th Annual Proceedings EMSA.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	3. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6519	71 07 01	DD-DR&E(AN)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
70 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	128
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1412A(2)			

11. TITLE: (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 71	2	70
FISCAL YEAR CURRENT 72	2	

19. RESPONSIBLE DOD ORGANIZATION
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TELEPHONE: 202-576-3551

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NAME: SADUN, E. H., Sc.D.
TELEPHONE: 202-576-3308
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: MOON, A. P. DA
NAME:

PII Redacted

21. GENERAL USE
Foreign intelligence not considered

22. KEYWORDS (Provide each with security classification code.)
(U) Malaria; (U) Antibody; (U) Rodents; (U) Susceptibility; (U) Immunity; (U) Plasmodium; (U) Irradiate

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) 70 07 - 71 06 Since irradiated Plasmodium berghei infected erythrocytes stimulate a strong protection to challenge in mice, experiments are in progress to isolate a protective substance from infected erythrocytes. Results to date indicate that neither irradiated parasitized cells subjected to 20,000 lbs. sq. in. pressure in a French pres nor irradiated parasitized cells frozen and thawed 3 times in a carbon dioxide-ethanol bath can stimulate a protective immunity. For technical reports see Walter Reed Army Institute of Reserach Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* Available to contractors upon withdrawer'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 128, Natural and acquired immunity in rodent malaria

Investigators
Principal: E. H. Sadun, Sc.D., Lib. Doc.
Associate: LTC C. L. Diggs, MC; CPT R. O. McAlister, MSC; B. T. Wellde

1. Isolation of protective antigens from irradiated Plasmodium berghei

Since irradiated Plasmodium berghei infected erythrocytes stimulate a strong protection to challenge in mice, experiments are in progress to determine whether a protective substance can be isolated from the infected erythrocytes. Results to date show that neither irradiated parasitized cells subjected to 20,000 lbs./sq in. in a French press nor parasitized cells frozen and thawed 3 times in a Co_2-ethanol bath can produce a protective immunity.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 128, Natural and acquired immunity in rodent malaria

Literature Cited.

Publication:

Sadun, E. H., Wellde, B. T., and Hickman, R. L.: Immunization in rodent and human malaria by the use of irradiated plasmodia. International Atomic Energy Agency, Vienna. (IAEA - PL - 339/13):97-98, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION: DA OA 6520	2. DATE OF SUMMARY: 71 07 01	REPORT CONTROL SYMBOL: DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	129
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 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
64 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 71	2	70
	CURRENT 72	2	70

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

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NAME: Walter Reed Army Institute of Research
Division of CD&I
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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:

PII Redacted

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 radiosotope-labelled amino acids, study the levels of enzyme activity in tissue extracts and alterations in protein and free amino acid contituents 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) 70 07 - 71 06 Irradiated blood forms of Plasmodium falciparum were used to immunize Aotus trivirgatus monkeys. Control animals developed a parasitemia by the fourth day and all died of malaria by the eleventh day. Of the immunized animals, one never became patent. One became patent on the fourth day and had a low grade parasitemia for 50 days and none thereafter. Another became patent on the fourth day with a gradually increasing parasitemia until death from malaria on the 37th day. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon originator's approval

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 129, Host responses to malaria

Investigators

Principal: E. H. Sadun, Sc.D., Lib. Doc.
Associate: CPT J. S. Anderson, VC; LTC C. L. Diggs, MC; B. T. Wellde

1. Resistance in owl monkeys injected with Plasmodium falciparum

An acquired resistance to homologous infection with P. falciparum developed in owl monkeys after 4 weekly immunizations with irradiated malaria parasites. Recent experiments were conducted to extend these findings and to determine whether the immunization process with irradiated blood forms of P. falciparum produced detectable disease in the animal.

Two of five monkeys receiving the irradiated parasites died during the immunization period. These monkeys developed progressive weakness, anorexia, anemia, leucopenia, and thrombocytopenia, but no parasitemia.

The three remaining immunized animals and five control monkeys were given challenge infections with blood forms of P. falciparum (Camp strain). All of the control animals developed a parasitemia on the 4th day and all died of malaria by the 11th day. The immunized monkeys had varying degrees of resistance. One animal never became patent; a second became patent on the 4th day and had a low grade parasitemia for 50 days and no parasites thereafter, and a third also became patent on the 4th day with a progressively increasing parasitemia and died on the 37th day after challenge.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 129, Host responses to malaria

Literature Cited.

Publications:

1. Scheibel, L. W. and Pflaum, W. K.: Carbohydrate metabolism in Plasmodium knowlesi. Comp. Biochem. Physiol. 37:543-553, 1970.

2. Scheibel, L. W. and Pflaum, W. K.: Cytochrome oxidase activity in platelet-free preparations of Plasmodium falciparum. J. Parasit. 56: 1054, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6525	71 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
70 07 01	D. Change	U	U	NA	NI.	☒ 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. [redacted]	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 71	2	70
	CURRENT 72	1	35

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202: 576-3551

20. PERFORMING ORGANIZATION

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Division of Medicine

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NAME: Canfield, LTC C. J.

TELEPHONE 202: 576-3268

SOCIAL SECURITY ACCOUNT NUMBER [redacted]

ASSOCIATE INVESTIGATORS

NAME: Hall, LTC A. P.

NAME: DA

PII Redacted

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) - 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) 70 07 - 71 06. A total of 12 patients at Walter Reed General Hospital with recrudescent falciparum malaria have now been treated with WR-33063. All were cured despite repeated drug failures with previous courses of all standard antimalarial agents. A preliminary field trial of this drug has recently been completed in Vietnam in 25 patients with acute falciparum malaria. Preliminary results suggest that this drug is without significant side effects and more effective than any single agent presently in use for treatment of drug-resistant infection from S. E. Asia. WR-30090 has been similarly used at Walter Reed General Hospital in 4 patients and 26 patients in Vietnam. This new drug is also superior to any single agent available in Vietnam and is nearly as effective as WR-33063. One new strain of resistant falciparum malaria from Cambodia has been introduced into the testing program. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon originator's approval

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 132, Clinical studies of human malaria

Investigators.
Principal: LTC Craig J. Canfield, MC
Associate: LTC Anthony P. Hall, 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. Results of previous evaluations on the response of vivax malaria to suppressive amounts of chloroquine (300 mg base) were published.[1]

b. P. falciparum. Results of various treatment regimens used in Vietnam for drug-resistant falciparum malaria and complications of the disease were assessed and published.[2]

Results of a field trial in Vietnam and the experience at Walter Reed General Hospital in the treatment of patients with recrudescent falciparum malaria with trimethoprim/sulfalene were tabulated and published.[3] Although this combination of drugs has a rapid schizonticidal action, the cure rate was only about 75 per cent. However, they are useful in some patients who have failed to respond to standard antimalarial drugs.

A total of 12 patients with recrudescent falciparum malaria has now been treated at Walter Reed General Hospital with WR-33063. All were cured.

This new agent has also been evaluated in Vietnam in 25 patients with acute malaria. There was one R-1 response and one R-2 response with an overall cure rate of 92 per cent. Speed of action was remarkable. The average interval for the patients to become afebrile was 48 hours (compared with 70 hours for the combination of quinine, pyrimethamine and dapsone). There were no side effects.

Four patients with recrudescent falciparum malaria have been treated at Walter Reed General Hospital with WR-30090. All were cured. This drug has also been evaluated in Vietnam in 26 patients with acute falciparum malaria. There were three R-1 responses with an overall cure rate of 88 per cent. Speed of action was somewhat slow, 80 hours to become afebrile. Side effects were insignificant.

Previous work on the erythrokinetic response of patients with acute falciparum malaria was published.[4] Patients with acute malaria who are anemic should probably receive folinic acid concurrently with standard antimalaria drug therapy which includes pyrimethamine to hasten hematologic recovery.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 132, Clinical studies of human malaria

Literature Cited.

Publications:

1. Hiser, W.H., MacDonald, B.S., Canfield, C.J., and Kane, J.J. Plasmodium vivax from Vietnam: Response to Chloroquine-Primaquine. Amer. J. Trop. Med. & Hyg., 20:402, 1971.

2. Neva, F.A., Sheagren, J.N., Shulman, N.R., and Canfield, C.J. Malaria: Host-Defense Mechanisms and Complications. An. Int. Med., 73: 295-306, August 1970.

3. Canfield, C.J., Whiting, E.G., Hall, W.H., and MacDonald, B.S. Treatment of Acute Falciparum Malaria from Vietnam with Trimethoprim and Sulfalene. Am. J. Trop. Med. & Hyg. 20:524-526, 1971.

4. Canfield, C.J., Keller, H., and Cirksena, W. Erythrokinetics During Treatment of Acute Falciparum Malaria. Mil. Med. 136:354, No. 4, April 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6534	71 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
70 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	63731A	3A663713D829	00	135
b. CONTRIBUTING				
c. [redacted]	cpOG 1412A(2)			

11. TITLE (Precede 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: Not Applicable
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 71	2	30
CURRENT FISCAL YEAR 72	2	30

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 202-576-3551

21. GENERAL USE

Foreign Intelligence Not Considered

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Experimental Pathology
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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

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Plasmodial Protein; (U) Plasmodial Ribosomes; (U) Plasmodial Microsomes; (U) Plasmodial Lipids

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 metabolic pathways in malarial parasites maintained in vitro. To isolate, purify and identify by chemical, physical and morphologic means the sub-cellular components of the malarial parasites, including cellular localization of anti-malarial drugs.
24. (U) Biophysical, biochemical and morphologic techniques are employed.
25. (U) 70 07-71 06 The experimental pathology of infections with Plasmodium falciparum in the owl monkey, Aotus trivirgatus, is described by Doctor Helen Jervis in a joint study carried out with the Division of Medical Zoology. This work is at the manuscript stage. The studies of lipid metabolism in Plasmodium knowlesi carried out in this department during the past two years have also been completed and are described in three manuscripts, the first of which has been submitted for publication. The titles of these papers are as follows: "Incorporation of 33P-orthophosphate into membrane phospholipids of Plasmodium knowlesi and host erythrocytes of Macaca mulatta," by R.C. Rock, J. Standefer and W. Little; "Incorporation of 14C-labelled non-lipid precursers into lipids of Plasmodium knowlesi in vitro," by R. C. Rock; and "Incorporation of 14C-labelled fatty acids into lipids of Rhesus erythrocytes and Plasmodium knowlesi in vitro," by R. C. Rock. Our studies have been directed towards working out the interrelationships between the extremely active lipid biosynthetic activities of Plasmodium knowlesi and the relatively limited lipid metabolism of its host erythrocyte. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

*Available to contractors upon originator's approval

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PII Redacted

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 Robert C. Rock, MC

Problem

Details of the lipid metabolism of malarial parasites are largely unknown and yet lipids form an indispensable component of every living cell including plasmodia. If we could devise techniques to separate parasite from host cell lipid in sufficient purity and amounts, we would then be in the position to analyze and compare the lipid constituent classes. We could then gain an idea of the kinetics of lipid synthesis in the parasite, of the sequence of metabolic steps in parasite lipid metabolism, and to which extent complete lipids, lipid precursors and lipid building blocks are furnished by the host plasma, by the host erythrocyte or synthesized by the parasite itself. If we could gain this information and unravel the pathways of lipid metabolism of the intra-erythrocytic parasite, we hope to block a vital enzymatic step of lipid synthesis within the parasite and abort the infection by a specific agent.

Background

Our earliest ultrastructural studies of plasmodia convinced us that lipid containing membranes and organelles were extremely rapidly synthesized and transformed[1,2,3]. We then set out by a combined biophysical, biochemical and electronmicroscopic study to isolate lipid components of the parasite. This proved to be a formidable undertaking which eventually was crowned with success.[4] In fact, we were able to further refine our methodology subsequently by arduous experimentation.[5] To the best of our knowledge the parasite separation and lipid extraction procedures are the most accurate available at this moment.

The next task of great complexity was the quantitative analysis of relatively minute samples of purified parasite lipid. This effort eventually was also crowned by success by a combinedanalytic chemical and biochemical approach.[6] We were greatly helped in our lipid research methodology by the 3 month visit of Doctor Rokus A. de Zeeuw, State University, Groningen, The Netherlands.[7] Doctor de Zeeuw is an expert in analytical chemistry and he in turn obtained the advice of Professor van Deenen, State University, Utrecht, The Netherlands. Professor van Deenen is considered the foremost authority on lipids in the world today. It has been very reassuring to us that parasite lipid analysis performed independently by Doctor de Zeeuw in Groningen gave results identical to those obtained by Major Rock at WRAIR. In order

to assure the widest possible dissemination of our results, it was decided to submit the main body of our results to the Journal of Comparative Biochemistry and Physiology. This journal is critically edited and has an international reputation. Unfortunately, there is a delay of nearly one year between acceptance and publication. For this reason, out of the four papers submitted and accepted for publication only one so far has appeared in print and will be discussed more fully under Results. In addition, a fifth paper dealing with biophysical properties of plasmodial ribosomes has been accepted for publication by the same journal. The papers in press are listed as references 8, 9, 10, and 11.

Results

We suspected that differences in lipid composition would exist between the parasite and its host red cell. The results of our work proved our hypothesis:

1. Plasmodium knowlesi has significantly more phospholipid and less cholesterol (Phospholipid: cholesterol ratio = 5.2) than the host erythrocyte membranes of the Rhesus monkey (phospholipid): cholesterol ratio = 2.15).

2. Although both parasite and host red cell have the same major phospholipid components (Phosphatidylcholine and phosphatidylethanolamine) other phospholipids differ significantly.

3. The red cell contains 12-14 per cent sphingomyelin and 12 per cent phosphatidylserine, whereas the parasite contains only 2-3 per cent sphingomyelin, 8-10 per cent phosphatidylinositol, and barely detectable levels of phosphatidylserine.

Due to the departure of the principal and the associate investigator our research had to stop at a point where we were beginning to elucidate the interrelationships between the extremely active lipid biosynthetic activities of the malarial parasite and the relatively limited lipid metabolism of its host erythrocyte. While in-house research under work unit 135 has been discontinued, it is fortunate that we will be able to assist Doctors Aikawa, Cook, Rock and de Zeeuw who will be carrying on the research extramurally.

In conclusion, in a few short years research performed under this work unit has achieved international acclaim. A number of promising researchers were enticed to explore the experimental pathology of malaria. In alphabetical order they are: Aikawa, Cook, Hepler, Jervis, Ladda, MacCallum, MacComber, Sesta and Terzakis. None had any significant exposure to malaria research before they came to WRAIR, but a modern history of malaria research cannot be written without taking cognizance of their contributions.

Project 3A663713' '9 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 135 Experimental pathology and metabolism of plasmodia

Literature Cited.

References:

1. Aikawa, M. The fine structure of the erythrocytic stages of three avian malarial parasites, Plasmodium fallax, P. lophurae, and P. cathemerium. Am. J. Trop. Med. Hyg. 15: 449-471.

2. Hepler, P. K., Huff, C. G., and Sprinz, H. The fine structure of the exoerythrocytic stages of Plasmodium fallax. J. Cell Biol. 30: 333-358, 1966.

3. Ladda, R., Aikawa, M., and Sprinz, H. Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. J. Parasitology 55: 633-644, June 1969.

4. Cook, R. T., Aikawa, M., Rock, R. C., Little, W., and Sprinz, H. The isolation and fractionation of Plasmodium knowlesi. Mil. Med. 134: 866-883, Sep 1969.

5. Rock, R., Standefer, J. C., Cook, R. T., Little, W., and Sprinz, H. Lipid composition of Plasmodium knowlesi membranes: comparison of parasites and microsomal subfractions with host rhesus erythrocyte membranes. Comp. Biochem. Physiol. 38B: 425-437, 1970.

6. Rock, R. C., and Standefer, J. Membrane metabolism and phospholipid biosynthesis in Plasmodium knowlesi. J. Parasitology 56: 287-88, 1970.

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11. Cook, R. T., Rock, R. C., Aikawa, M., and Fournier, M. J. Ribosomes of the malarial parasite, Plasmodium knowlesi. I. Isolation activity, and sedimentation velocity. Comp. Biochem. Physiol. (In press)

Publications:

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2. Rock, R. C., and Standefer, J. Membrane metabolism and phospholipid biosynthesis in Plasmodium knowlesi. J. Parasitology 56: 287-88, 1970.

3. Rock, R. C., Standefer, J., Cook, R. T., Little, W., and Sprinz, H. Lipid composition of Plasmodium knowlesi membranes: comparison of parasites and microsomal subfractions with host Rhesus erythrocyte membranes. Comp. Biochem. Physiol. 36, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6535	71 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
70 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. CONTRIBUTING	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)
PRECEDING FISCAL YEAR 71	2	60
CURRENT FISCAL YEAR 72	2	60

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

PII Redacted

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) - Measure the effect of antimalarial drugs on morphologic growth, lactate production, 14-C methionine or 14-C orotic acid incorporation during in vitro schizogony and observe utilization of metabolic precursors of nucleic acids: to measure folic acid reductase in parasite suspensions.

25. (U) 70 07 - 71 06 In vitro culture of P. knowlesi parasites has continued. Using 14-C orotic acid incorporation into DNA as a sensitive indicator, known and candidate antimalarial drugs and derivatives and analogues of them have been tested for antifol activity. In this system, the hydroxylamine derivative of DDS evidenced the same activity as the parent antimalarial drug, DDS.

Drug synergism is being investigated. Combinations of sulfalene and trimethoprin have shown potentiation of the in vitro antifol activities of these drugs, while combinations of quinine and chloroquin have shown only additive effects. These findings correspond to those expected on the basis of theory and clinical experience and suggest that this system may yield information useful in prediction of potentiating drug combinations for clinical applications in treatment of malaria. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon originator's approval

1156

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 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. and Gloria P. Willet

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.

A description of the in vitro P. knowlesi malaria system has been published (1). In this system, young ring forms of the parasite develop into mature schizonts during 18-20 hours of incubation in a nutrient medium. Dose response curves of known antimalarial drugs were obtained using as parameters morphologic change, production of lactic acid and incorporation of 15C-methionine into protein.

The in vitro system is being used in the investigation of anti-folic acid acitivity of drugs, based upon the inhibition of incorporation of 14C-orotic acid into DNA during the incubation. Known and candidate antimalarial drugs have been tested. In this system, the hydroxylamine derivative of DDS evidence the same activity as the parent antimalrial drug. During studies of the mode of action of antifols, it was found that both folic and folinic acids, while stimulating 14C-orotic acid incorporation at lower concentrations, were inhibitory at high concentration (0.2 mM), folinic acid being the more inhibitory. In studies with drug WR 40070, structurally related to but less effective than pyrimethamine as an inhibitor in this system, both folic and folinic acids reduced the drug's inhibition by 50% when present in equimolar concentration to a 100% inhibitory drug concentration (5×10^{-3}mM). Since in previous studies folic and folinic acids had little or no effect against pyrimethamine, this result suggested that differences in degree or mode of activity of drugs of this type may be studied with this system.

Application of the *in vitro* system to the study of synergism between drugs has received considerable developmental work. Potentiation of effect has been demonstrated with combinations of sulfalene and trimethoprim, while quinine and chloroquin showed only additive effects. These results correspond to those expected on the basis of theory and clinical experience and suggest that this *in vitro* system may yield information useful in prediction of potentiating drug combinations for clinical applications. Combinations of other drugs are being tested.

5-Fluor-orotic acid, previously found to be an effective inhibitor in the *P. knowlesi in vitro* system, was found to be effective *in vivo* against *P. berghei* in mice. Single doses at 20 to 640 mg/kg body weight extended the average life in groups of infected mice from 7 days (untreated) to 16 to 20 days. Drug toxicity was seen at 1280 mg/kg.

Investigation of 2,3-diphosphoglycerate levels in red blood cells of *P. knowlesi*-infected Rhesus monkeys continues. The two-fold elevation of infected levels over uninfected control levels was not significantly different from the elevation found in anemic uninfected animals. Effect of chloroquin treatment of the animals is being studied. Studies of possible correlation between 2,3-DPG levels and hemoglobin oxygen affinities are planned.

A previously completed investigation of sodium transport was published (2).

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 136, Metabolic and enzymatic studies of normal and malaria infected red cells

Literature cited.

Publications:

1. Canfield, C. J., Altstatt, L. B., and Elliot, V. B.: An *in vitro* system for screening potential antimalaria drugs. Am. J. Trop. Med. and Hyg. 19, 905-909 (1970).

2. Dunn, M. J.: The effect of transport inhibitors on sodium outflux and influx in red blood cells: evidence for exchange diffusion. J. Clin. Invest. 49, 1804-1814 (1970).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6482	71 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	GP	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713 A	3A663713D829	00	171
b. CONTRIBUTING				
c. [redacted]	CDOG 1L12A(2)			

11. TITLE (Provide with Security Classification Code)

(U) General Pharmacology of Antimalarial Drugs (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 07	CONT	DA	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 71	9	350
FISCAL YEAR CURRENT 72	8	350

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted] DA

ASSOCIATE INVESTIGATORS

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NAME: Einheber, A., PnD, Rozman, R., PhD

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Provide each with Security Classification Code) (U) Pharmacodynamics; (U) Pharmacokinetics; (U) Toxicity; (U) Drug Metabolism; (U) Antimalarial Drugs; (U) Preclinical Pharmacology

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

23. (U) The technical objective is to develop and to exploit animal models for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarials in man. The intended purpose of these studies is to provide a basis for predicting the response of soldiers to antimalarials in a military environment and to fulfill requirements for submission of IND for clinical trials of new antimalarials.

24. (U) The approach will be to study the effects of antimalarial drugs in healthy animals and to study the handling of antimalarial drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The effects of antimalarial drugs in diseased or injured animals will be studied in order to determine the effect of the drugs on disease and injury. The handling of antimalarial drugs by diseased and injured animals will be studied in order to determine the effect of disease or injury upon pharmacokinetics in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).

25. (U) 70 07 - 71 06. Pharmacokinetics of four new phenanthrene methanol antimalarials has been described in healthy rodents. Rodent malaria models are being tested for applicability to the study of pharmacodynamics and pharmacokinetics of new antimalarials in the disease state. The pharmacodynamics of WR 113,618.$2H_2SO_4$ has been described in rodents and anesthetized dogs. The acute toxicity of WR 113,618.$2H_2SO_4$ in dogs has been accomplished in preparation for submission of an IND. Therapeutic measures to reverse cardiotoxicity in animal models are being studied. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

* 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 171, General pharmacology of antimalarial drugs

Investigators.
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC Gale E. Demaree, MSC; Robert S. Rozman, Ph.D.; CPT Robert W. Caldwell, MSC; Albert Einheber, Ph.D.; Ann Berman, M.S.

PRECLINICAL PHARMACOLOGIC STUDIES OF CANDIDATE ANTIMALARIAL DRUGS

BACKGROUND: Statutory requirements for clinical testing of new drugs include acute and subacute toxicity and pharmacodynamics as well as absorption, distribution and excretion of these chemicals in animals.

METHODS: Acute pharmacodynamic responses to new drugs are evaluated following intravenous and intraperitoneal injections in anesthetized rats and following intravenous and intraduodenal injections in anesthetized dogs. Cardiac effects are investigated in the isolated, perfused dog heart. Absorption, distribution and excretion of new drugs are studied following intraperitoneal, oral or subcutaneous administration of the drugs in mice; the radiolabelled drugs are suspended in either peanut oil or aqueous methyl cellulose solution. Tissues and fluids are examined at various times following drug administration. Urine and feces are continuously collected and examined for excretion of labelled drug.

RESULTS: WR 113,618.2H_2SO_4 produces respiratory depression, hypotension, bradycardia, and reversible EKG voltage changes in the anesthetized dog or rat following intravenous injection. Intraduodenal administration of the new drug produced no cardiovascular effects in the dog. The drug does not affect the responses to histamine, norepinephrine, serotonin or acetylcholine at doses up to 20 mg/kg in the rat. WR 113,618.2H_2SO_4 depresses heart rate and contractility as well as producing reversible EKG voltage changes in the isolated dog heart.

DISCUSSION: These findings emphasize the importance of close scrutiny of EKG's during clinical studies since WR 113,618.2H_2SO_4 appears to have a direct deliterious effect on the heart.

ADJUNCTIVE THERAPY OF EXPERIMENTAL MALARIA

BACKGROUND: The course of human malaria is accompanied by a phase which is described as "vasomotor inadequacy". Other forms of cardiovascular failure such as endotoxin and hemorrhagic shock and traumatic injury

have responded favorably to treatment with WR 2823 or WR 149,024. The more potent agent, WR 149,024, was tested for its potential effect to influence favorably the terminal course of P. berghei infection in mice. The underlying assumption being that prevention of vascular disturbances or improvement of such states would be manifest in prolonged survival of the infected animals.

METHODS: This preliminary experiment involved 40 mice infected with P. berghei at a normally 100% lethal infection, mean time to death of 6.5 days. Half of the animals received WR 149,024 (25 mg/kg i.p.) on days 3 and 5 of infection; the remainder served as controls receiving simultaneous water injections. Mortality and parasite levels were observed. These studies were done in cooperation with the Division of Biochemistry.

RESULTS: There were no differences in mortality or parasitemia in the two groups.

DISCUSSION: These data show that the terminal stages of the disease do not respond to WR 149,024 alone by this schedule of administration; however, significantly, malaria-infected mice can tolerate doses of WR 149,024 that are efficacious in other forms of shock. The approach still has considerable merit to attempt to speed recovery from the effects of malaria and hasten return to duty. We propose to investigate other dosage schedules and combinations with antimalarial drugs.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 171, General pharmacology of antimalarial drugs

Literature Cited.

Publications:

Rozman, R.S., Berman, A., Hutchinson, A. and Cintron-Molero, G.: Uptake and Excretion of Antimalarial Phenanthrene Methanols. Fed. Proc. 30:335, 1971.

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 OB 6441	71 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
70 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. / CODES:	PROGRAM ELEMEN	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A062110A830	00	055
b. CONTRIBUTING				
c. CONTRIBUTING	CLOG 1412A(2)			

11. TITLE (Procede 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. STAFF 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)
FISCAL YEAR PRECEDING 71	3.5	225
FISCAL YEAR CURRENT 72	4	240

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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NAME: Wilke, CPT W.L.

NAME: Scalera, 1LT S.E. DA

PII Redacted

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 temperamentally 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 accomplish the objective.

25. (U) 70 07 - 71 06 Sixty five litters produced 369 puppies weaned. Present kennel population of 299 dogs includes 257 German Shepherd Dogs, 8 German Shorthaired Pointers, 8 Pointer-Shepherd crosses, and 25 Drahthaars. An additional 268 were shipped to other activities including Land Warfare Laboratory, Fort Benning Military Dog Committee (Mine/Tunnel Dogs); WRAMC Provost Marshal, Customs Bureau and WRAIR. Evaluation program of young dogs was begun. The following improvements to the physical plant were undertaken: a pier was built to accustom the dogs to water; flourescent lighting was installed in three buildings; tighter security measures were instituted; a new office was installed, and a confidence course was installed. Puppy evaluation procedures were altered. Consultant visits to this facility were made by national authorities. Contractor problems concerned with the permanent facilities were resolved. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 70 - 30 June 1971.

(Available to contractors upon sponsor's approval)

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

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 Jeffrey M. Linn, VC; CPT Willard L. Wilke, VC; 1LT Stephen E. Scalera, MSC

OBJECTIVE: To develop a more intelligent and sensually acute dog which is physically and temperamentally 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. Sixty four whelpings produced 371 weaned puppies.

2. Present kennel population is as follows:

German Shepherd Dog	288
German Wirehaired Pointer	13
German Shorthaired Pointer/German Shepherd Dog cross	8

3. Two hundred and sixty four dogs were transferred to other activities:

Walter Reed Army Institute of Research	218
Fort Benning, Georgia	37
Provost Marshall Office, WRAMC	2
US Bureau of Customs	2
Texas A&M University	5

4. Puppy evaluation: Computer review of our puppy evaluation procedures revealed that the stair test and the decoy test were of little value as indicators of a puppy's future potential. These two tests have been dropped. The come and sit tests have value but are not as indicative as the rag play, fetch, and maze tests. A new experience test suggested by our behavioral consultant, Dr. M.W. Fox, is now being used to indicate a puppy's inherent boldness and curiosity.

B. Special Projects

1. The use of field dogs such as the German Shorthaired Pointer and crossbreeds between the Pointer and the German Shepherd has been dropped. The advantages of the typical bird-dog point and field quartering are largely negated by the difficulty encountered by Fort Benning in training these dogs to sit-alert on mines and trip wires. The remaining individual puppies of these breeds will, however, continue to be evaluated.

2. It has been noted that almost all of our best dogs are "fetch happy". Because of this we have recently incorporated fetch in our socialization program and are exploring the possibility of converting the fetch desire to the finding of mines and booby traps. This was suggested by one of our consultants, Mr. W.R. Koehler, as a result of our attempts to find a motivation stronger than pet and praise, and/or food reinforcement as a training method. Specifically he recommended the use of forced retrieve in combination with pet and praise. This is being accomplished in the case of a single trial dog. Results are not obtained as quickly as with food reinforcement, but the dog seems to enjoy her work and, since hunger is not the underlying motivational element, she stays in excellent physical condition. We believe that the act of fetching is manifestation of the hunting instinct. The appeal to this instinct may have greater motivational value than a pat on the head or a handful of food especially if the dog is hot and tired.

3. Until recently those of our dogs which radiographically demonstrated the slightest degree of hip dysplasia were eliminated from the program and transferred to the Walter Reed Army Institute of Research. A recent report* based on data accumulated on more than 300 dogs on which tenomyectomy of the pectineus muscle had been performed indicates that this procedure is effective in maintaining or restoring functional normality in effected dogs. Certain of our more highly rated but effected dogs are being thusly operated with the expectation of halting the progress of the conditions to enable subsequent military use of the dog. These animals will not of course be used as breeders.

4. A breeding index was established for each animal in our breeding colony. It was recently revised and is based upon the following data accumulated from each litter:

a. Percentage of hip dysplasia
b. Average of puppy evaluation scores
c. Temperament of litter
d. Size of litter at birth
e. Number weaned
f. Percentage of longhaired puppies
g. Percentage of floppy-eared puppies

C. Consultant Visits: During the past year this Division was visited by its canine behavioral consultant, Dr. M.W. Fox, DVM, by Dr. Wayne H. Riser, DVM, who monitors our hip and elbow dysplasia control program and by Mr. L. Wilson Davis, our evaluation and training consultant.

D. Facilities: A three hundred gallon dog dipping tank was constructed to facilitate control of external parasites. A thirty foot pier was constructed in adjacent Lauderick Creek to permit swimming the dogs.

E. Equipment: An electric welder and related hand tools were obtained in a "self-help" repair and maintenance program of kennels and facilities. An improved lighting and heating system was installed in our indoor winter kennels.

DISCUSSION: The genetic refining of the German Shepherd Dog to produce a more intelligent, dysplasia free, temperamentally sound dog for military purposes is progressing well. Almost half of our present German Shepherd population are F_2 generation while the remainder are combinations of F_1 and original stock. The problem of hip and elbow dysplasia is responding most gratifingly to selective breeding. Improvement of intelligence and temperament is, as expected, less rapid and will require several generations to be of marked significance. As an example of the complexities of this area of our work, some of our earlier puppy evaluation procedures were such that the more aware puppies scored higher in the tests; and a timid puppy is usually an aware puppy. This is the reason for the introduction of Dr. Fox's new test mentioned in paragraph 4 above. We expect it to assist us in differentiating between the aware, timid puppy and the aware, curious puppy. These changes plus elimination of timid breeding animals and even more emphasis on early socializing of the puppies are producing a bolder more self-confident dog than heretofore.

CONCLUSION: Because of the previous accomplishments in the field of animal genetics in improving quantity and quality of animal products, there is no reason not to expect the development of a markedly improved military dog within the next decade.

RECOMMENDATIONS: None

Project 3A062110A830 BIO SENSOR SYSTEMS

Task 00, Biosensor Systems

Work Unit 055, Development and evaluation of improved biological sensor systems

Literature Cited.

References:

1. Staff Report: Selected Topics, Hip Dysplasia, Mod Vet Pract 52 (June 1971): 29-30.

END