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WRAIR
ANNUAL PROGRESS REPORT

1 July 1967 - 30 June 1968

Volume III

AD

RCS MEDDH-228 (R1)

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

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

Annual Progress Report
1 July 1967 - 30 June 1968

Volume III

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

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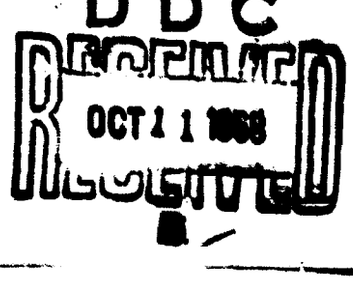

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SUMMARY

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 beginning of each report.

In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care as established by the National Society for Medical Research."

Best Available Copy

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3A635301D829 MALARIA PROPHYLAXIS

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PROJECT 3A014501B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 01
Surgery

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13 (U) METABOLIC PROCESSES ASSOCIATED WITH INJURY

14 START 09 66	15 CONTINUING DA	16 TYPE OF ACTION OTHER			
17 NUMBER MAN YEARS 67	18 PROFESSIONAL MAN YEARS 7	19 FUNDING 245			
20 EXPENDITURES 67	21 PROFESSIONAL MAN YEARS 7	22 FUNDING 245			

23 PERFORMING ORGANIZATION

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26 COORDINATION DA

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30 TECHNOLOGY UTILIZATION: BIOCHEMISTRY PHYSIOLOGY

31 KEYWORDS
TRAUMA, METABOLISM, INFECTION, THERAPY, ENZYMOLOGY, MICROORGANISMS.

32 (U) TECH OBJECTIVE - MAJOR AREAS OF INVESTIGATION - 1. FACTORS RESPONSIBLE FOR MORBIDITY AND MORTALITY ASSOCIATED WITH INFECTIONS AND BACTERIAL TOXINS. 2. METABOLIC ALTERATIONS AND TISSUE DAMAGE CAUSED BY TRAUMA, WHICH INCLUDE BIOCHEMICAL, PHYSIOLOGICAL AND HISTOCHEMICAL STUDIES. 3. EVALUATION OF THERAPEUTIC AGENTS IN BACTERIAL AND ENDOTOXIN SHOCK, AND 4. CLINICAL RESEARCH ON PHYSIOLOGICAL AND BIOCHEMICAL PROBLEMS ASSOCIATED WITH TRAUMA.

33 (U) APPROACH- 1. INFECTIONS ESTABLISHED BY INTRAPERITONEAL INJECTIONS OF MICROORGANISMS ARE STUDIED IN RESPECT TO THE ROLE OF THE BACTERIA, CHANGES IN METABOLIC PROCESSES, AND THE VALUE OF THERAPEUTIC AGENTS. 2. INTRAVENOUS INJECTIONS OF BACTERIAL PRODUCTS USED TO INVESTIGATE METABOLIC ALTERATIONS, TISSUE DAMAGE, THE ROLE OF PHYSIOLOGICALLY ACTIVE SUBSTANCES AND SURGICAL PROCEDURES IN TOXIC SHOCK. 3. BIOCHEMICAL AND HISTOCHEMICAL PROCEDURES ARE DEVELOPED OR MODIFIED TO EVALUATE THE EFFECT OF TRAUMA AND THERAPEUTIC AGENTS ON THE PATHOPHYSIOLOGY OF PATIENTS IN SHOCK. 4. EXPERIMENTAL AND CLINICAL STUDIES ARE CORRELATED TO EVALUATE THE METABOLIC CHANGES ASSOCIATED WITH TRAUMA IN RESPECT TO IMPROVED THERAPY.

34 (U) PROGRESS - JUL 67 THRU JUN 68 1. SEQUENTIAL CHANGES PRODUCED BY BACTERIA AND BACTERIAL ENDOTOXIN, MONITORED BY ACTIVITIES OF SERUM AND TISSUE ENZYMES WERE CORRELATED WITH DEATH OR SURVIVAL. 2. ISOZYME PATTERNS AND ENZYME ANALYSES INDICATED ONLY ONE ORGAN INITIALLY AFFECTED BY ENDOTOXEMIA, AND THAT THIS ORGAN WAS THE MESENTERY OF THE INTESTINES. 3. DEXAMETHASONE WAS THE MOST EFFECTIVE THERAPEUTIC AGENT IN BACTERIAL SHOCK PRODUCED BY A PERITONITIS. A PERITONEAL LAVAGE FOLLOWED BY ANTIBIOTICS ALSO REDUCED MORTALITY. 4. THE ADJUVANT ROLE OF HEMOGLOBIN IN E. COLI PERITONITIS WAS THE INHIBITION OF BACTERIAL PHAGOCYTOSIS, WHICH PERMIT LOCAL BACTERIAL PROLIFERATION AND THE RELEASE OF TOXINS. 5. THE METABOLIC ABNORMALITIES ASSOCIATED WITH HUMAN TRAUMA AND SEPSIS ARE SIMILAR TO THOSE FOUND IN EXPERIMENTAL ANIMALS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A014501B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 091, Metabolic problems associated with injury

Investigators.
Principal: H. Kenneth Sleeman, PhD
Associates: Arthur S. Dobek, PhD; John W. Diggs, BS; Clarence E. Emery, BS; Paul B. Lamborn, CPT, VC; Teruo Matsumoto, LTC, MC; Madiline Bluemle, MAJ, ANC; LaWayne Stromberg, LTC, MC; Irwin R. Berman, CPT, MC; Edward T. Dickson, Cecil Pugh, SFC

Part I

Description.

The Department of Surgical Metabolism conducts research on the metabolic problems associated with injury and provides professional consultation and technical support to other departments of the Division of Surgery and the United States Army Medical Biochemical Research Laboratories. The purpose and major areas of investigation include:

1. Metabolic Alterations Produced by Endotoxemia. Research was continued on the metabolic changes which resulted from endotoxin injection into the dog. Previous studies showed that the activity of selected serum enzymes was elevated during endotoxemia, and that the activity of serum enzymes could be correlated with death or survival. Since these enzymes are released as a result of tissue damage, the patterns of enzyme activity in serum and tissue were studied to determine the specific organ(s) affected by endotoxemia.

2. Effects of Antibiotics, Corticosteroids, and Peritoneal Lavage on Experimental Peritonitis. The value of antibiotics, corticosteroids and peritoneal lavage in the treatment of peritonitis and surgical infections has not been established. Conflicting evidence on the effectiveness of these treatments probably results from differences in dosage and time of initiating therapy following the infection. Using a standardized peritoneal infection in the rat, these therapeutic agents were studied from the initiation of the infection to a time near death. The effectiveness of single treatments and combinations of treatment was evaluated.

3. Factors Responsible for Morbidity and Mortality in Bacterial Shock. Studies were continued on the adjuvant action of hemoglobin in experimental Escherichia coli peritonitis. Previous studies indicated that hemoglobin produced lethality of retarding the clearance of organisms from the peritoneal cavity, hence permitting their proliferation and the release of toxins, which ultimately produces death. Alternative hypotheses of the adjuvant effect of hemoglobin were investigated.

4. Participation in Research Projects with Other Departments. The Department of Surgical Metabolism provides both professional

consultation and technical assistance in support of the research of other activities. Projects in which the department has participated are: (1) the role of lymph drainage in endotoxin and hemorrhagic shock, (2) oxygen toxicity, (3) the role of the central nervous system in endotoxin shock, (4) the evaluation and toxicity of cyanoacrylates of tissue adhesives, and (5) studies on metabolic alteration produced by shock in humans.

Progress.

1. Metabolic Alterations Produced by Endotoxemia. Studies have been continued on the metabolic alterations produced by endotoxemia. Many metabolic processes fail or are vastly altered in the late stages of endotoxin shock, therefore experiments were designed to monitor metabolic changes over the period of time from the initial insult until death or recovery. The approach was the measurement of selected enzymatic activities and substrate concentration in serum. Since serum enzyme activity reflects tissue damage, the enzyme patterns of tissue were determined also in an attempt to relate serum enzyme levels with specific tissue injury.

Previous studies showed that serum glutamic-oxalacetic transaminase (SGOT) and glutamic-pyruvic transaminases were elevated in endotoxemia, and provided an indicator for predicting eventual death or survival. Preliminary studies at that time suggested that lactic dehydrogenase (LDH) activity was a more sensitive indicator for predicting mortality and could be used for indicating possible tissue damage. Therefore, studies were undertaken to evaluate the serum LDH as an indicator of eventual mortality, and to determine if LDH isozyme patterns would suggest specific tissue damage. Concomitant analyses of serum and tissue transaminases and isocitric dehydrogenase, and blood levels of lactate and pyruvate were performed.

Mongrel dogs were anesthetized and placed in a supine position. Cannulae were inserted for injections, sampling, and monitoring arterial pressure. Blood was collected from the femoral arteries, and samples were taken prior to endotoxin injection (control) and 1, 3, 5, and 7 hours following endotoxin injection. Additional blood samples were taken at 24 and 48 hours if the animals survived. Tissues were excised from normal dogs and those that died by 7 hours after endotoxin. One dose of endotoxin (E. coli 0111:B4-Boivin), calculated to produce 70 percent mortality, was injected intravenously. Animals which died within 48 hours after endotoxin administration were considered fatalities.

Serum LDH activity in non-surviving animals increased with time after intravenous injection of endotoxin. By the third hour after endotoxin injection, LDH was increased significantly over controls (t-test, $p < .01$) and high levels of activity were maintained until death. In contrast, the surviving animals had no significant increase in serum LDH activity. The elevation of both LDH and the transaminases, in all instances, indicated a poor prognosis.

Other enzyme activities and substrate concentrations were modified but could not be correlated with death or survival. The serum ICDH was elevated by the 3rd hour after endotoxin injection, and in non-surviving animals attained levels 2 to 3 times that of LDH. However, ICDH activity was elevated also in surviving animals, but to a lesser extent, and remained elevated even 48 hours after endotoxin injection. The ICDH activity was probably related to tissue damage caused by endotoxin. Acid phosphatase activity was slightly elevated by the 3rd hour after endotoxin but usually returned to normal by 7 hours. Lactate and pyruvate concentrations were elevated in non-surviving animals and also in some samples in surviving animals. However, the levels fluctuated during the testing period, and usually returned to normal values by 7 hours whether the animal died or survived. Excess lactate accumulation was found in some samples.

Preheparinization of the animals had no effect on the LDH activity, but did decrease the activity of ICDH and the transaminases. This in vivo action of heparin probably resulted from the effect on cellular permeability, since its in vivo effect on the analyses was slight.

Electrophoretic separation of the serum LDH into the five isozymes revealed a distinctive pattern of increased LDH isozyme activity. The initial rise in total LDH activity was associated consistently with a marked increase in only the 2 and 3 LDH isozymes. (The isozymes were numbered consecutively with the most anodic designated as 1.) Persistently high levels of total LDH activity frequently resulted in increases of isozyme 5 and a lesser extent 4. An increase in isozyme 1, the heart isozyme, concomitant with an increase in isozymes 2 and 3 occurred in only one animal.

The unique isozyme pattern obtained during the endotoxemia suggests that initially only one organ was involved. LDH isozyme patterns on tissue homogenates and blood constituents showed that only the lungs, adrenals, and intestines contained an isozyme pattern which could be equated to that found in the serum. Histological studies on the lung showed no apparent cellular abnormalities, and the ICDH content of the lung was very low, therefore, it is unlikely that enzyme activity found in the serum resulted from the lung damage. Sequential damage to either the adrenal cortex and then the adrenal medulla or to the intestinal serosa, muscularis, and mucosa could account for the observed LDH isozyme pattern in serum. Further studies on the enzyme levels and isozyme content of tissues from normal and endotoxemic dogs are under investigation to determine the organ(s) primarily affected by endotoxin.

2. Effects of Antibiotics, Corticosteroids, and Peritoneal Lavage on Experimental Peritonitis. Studies on the prevention and treatment of bacterial sepsis and shock were continued and expanded. The most common infections, those caused by gram-negative organisms, frequently become refractory to therapy, and despite recent advances in medicine, the mortality rate remains high. The therapeutic agents most frequently

used for these infections are antibiotics, corticosteroids, and in addition for peritonitis, a peritoneal lavage. However, conflicting results as to the effectiveness of these agents have been reported, and these differences probably result from the dosage and the time of initiating treatment following the infection.

The present study was undertaken to study the effects of selected antibiotics, dexamethasone 21-phosphate, and peritoneal lavage on the lethality of a standardized Escherichia coli-hemoglobin peritonitis in the rat. The study was designed to test the effectiveness of these therapeutic agents from the initiation of the infection to a point near the expected time of death.

Table 1 shows the route of administration, the amounts and the effectiveness of each treatment (deaths/group) when given at selected times after inoculation with the E. coli-hemoglobin preparation. This model produces about 70 percent mortality in 24 hours as shown by the control groups. All the antibiotics significantly reduced mortality when given up to 8 hours after the initial infection. However, by 12 hours mortality was not significantly reduced by antibiotics and by 16 hours antibiotics were totally ineffective. At 12 hours, tetracycline, choramphenicol, and neomycin did reduce mortality by about 25 percent which would be considered beneficial although not statistically significant (chi-square). Kanamycin, which has been used extensively in the treatment of peritonitis, was the least effective antibiotic in this study. The ineffectiveness of the antibiotics after 12 hours probably results from the death and lysis of the bacteria. This would produce large quantities of toxins at a time when the resistance of the host was minimal.

Dexamethasone was the most effective treatment in decreasing mortality and was effective even at 16 hours after the inoculation. Dexamethasone, although not shown in Table 1, also afforded protection if given 1 hour prior to the inoculation. The protection provided by dexamethasone probably results from its effect on cellular integrity and cellular permeability.

A peritoneal lavage, which physically removes the organisms and possibly provides some dialysis of toxic materials, was very effective up to 8 hours; at 12 hours, it was totally ineffective. The failure of the peritoneal lavage to protect the host at 12 hours indicates that death at this time did not depend upon a high concentration of organisms in the peritoneal cavity.

Since neither the antibiotics nor peritoneal lavage were effective therapeutic agents at 12 hours, combination of treatments was investigated. When dexamethasone was administered simultaneous with the inoculation or at 4 and 8 hours after the inoculation, and antibiotics were given to the same animals at 8 and 12 hours after the inoculation,

mortality was not reduced significantly over that found with dexamethasone alone. A peritoneal lavage at 12 hours after inoculation followed by dexamethasone, again did not reduce mortality significantly when compared with dexamethasone alone. However, a peritoneal lavage followed by kanamycin did reduce mortality from 77 percent with kanamycin alone to 33 percent in the combined treatment. Therefore, a peritoneal lavage containing kanamycin or followed by kanamycin would be an effective therapeutic regime.

Preliminary studies on the mechanism by which dexamethasone increases survival in this model were considered. It was found that dexamethasone increases peritoneal absorption of dye-tagged protein. Since colloidal substances (protein) and particulate matter (bacteria) are absorbed by the same mechanism, dexamethasone may provide a more rapid clearance and destruction of bacteria, and in this way protect the host. Other factors such as lysosomal integrity and RES function, however, must be considered to fully explain the action of dexamethasone.

3. Factors Responsible for Morbidity and Mortality in Bacterial Shock. Studies were continued on the adjuvant effect of hemoglobin in E. coli peritonitis. Previous studies have shown that hemoglobin inhibits the clearance of bacteria from the peritoneal cavity. The organisms, thus isolated from the host's defense mechanisms, proliferate and ultimately release sufficient toxins to cause death. The present experiments were designed to test alternate hypotheses as to the effects of hemoglobin: (1) Hemoglobin increases the rate of bacterial proliferation. (2) Hemoglobin acts to increase the toxicity of E. coli-endotoxin, and (3) Hemoglobin acts systemically rather than locally.

The rate of bacterial proliferation of two concentrations of E. coli in nutrient broth and in nutrient broth-hemoglobin mixtures was studied. In three replicate experiments, hemoglobin was found to have no effect on the growth of the organism in vitro. The lethality of E. coli was compared in two groups of rats. One group received an intraperitoneal injection of E. coli-hemoglobin which had been incubated for 18 hours, the other group received separate but simultaneous injections of E. coli and hemoglobin. No significant difference in lethality was found between the two groups. The virulence of E. coli therefore was not increased by preincubations with hemoglobin.

The lethality of E. coli and hemoglobin was compared when separate but simultaneous injections into different sites were given. Only when E. coli and hemoglobin were injected intraperitoneally did a significant death (28/38) occur. The combination of E. coli injected subcutaneously and hemoglobin intraperitoneally produced only about 10 percent mortality. All other possible combinations were not lethal. The effects of non-simultaneous intraperitoneal injections of hemoglobin and E. coli on the lethality were studied. A significant number of deaths

occurred only when the injections were separated by one hour or less. If the hemoglobin injections preceded or followed the E. coli injection by more than one hour, mortality was reduced markedly and was proportional to the time interval between injections.

Finally, the effect of intraperitoneal injections of hemoglobin and E. coli endotoxin was examined. Hemoglobin did not increase or modify the mortality of graded doses of endotoxin. The possible systemic effect, RES blockage, of hemoglobin could not be shown. Therefore, hemoglobin appears to act locally by inhibiting the clearance of bacteria and isolating them from the normal defense mechanisms of the host.

4. Participation in Research Projects with Other Departments and Activities. Studies conducted in cooperation with other departments will be included in reports from the respective activities in the Division of Surgery and the United States Army Medical Biomechanical Research Laboratories.

Summary and Conclusions.

The research of the Department of Surgical Metabolism was concerned with metabolic problems associated with injury and included the following areas of investigation: (1) Metabolic alteration produced by endotoxemia was studied by determining selected enzymatic activity affected by endotoxin injection in the dog. Serum elevations of lactic dehydrogenase, glutamic-oxalacetic transaminse, and glutamic-pyruvic transaminase activity were correlated with death or survival. Lactic dehydrogenase isozyme activity indicated that only one organ was primarily damaged by endotoxin, and that organ was either the adrenals or the intestine. (2) The effects of antibiotics, corticosteroids, and peritoneal lavage on experimental peritonitis were evaluated. The corticosteroid, dexamethasone 21-phosphate, was the most effective agent in preventing mortality. The combined treatment with peritoneal lavage and kanamycin was more effective in reducing mortality than either treatment alone. (3) The role of hemoglobin as a lethal adjuvant in E. coli peritonitis was studied. After testing several hypothesis, it was concluded that hemoglobin acts locally by retarding the clearance of organisms from the peritoneal cavity. This permits the proliferation of organism and the release of toxins, which ultimately produce death. (4) Professional consultation and technical assistance were given to support other research activities.

Publications.

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2. Sleeman, H.K., Jennings, P.B., and Hardaway, R.M. Evaluation of

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3. Sleeman, H.K., Jennings, P.B., Diggs, J.W., and Emery, C.E. The Effect of Endotoxemia in the Dog on Lactic Dehydrogenase Activity. Abst. 154th Annual Meeting American Chemical Society, 1967, 219C.

4. Jennings, P.B., Simmons, R.L., Sleeman, H.K., and Hardaway, R.M. Hemodynamic, Biochemical, and Coagulation Alterations in Endotoxin Shock: Modification by Induced Tolerance in the Dog. Ann. Surg. 167:204, 1968.

TABLE 1
Effectiveness of treatments (deaths/group) when given at times (hours) following E. coli-hemoglobin inoculation

Treatments	Amount	Hours after inoculation 0	4	8	12	16	Untreated Controls	12 hr deaths vs untreated controls*
Tetracycline	25 mg/kg	1/12 (8%)	3/18 (17%)	2/18 (11%)	5/12 (42%)	8/12 (67%)	12/18 (67%)	n.s.
Chloramphenicol	25 mg/kg		0/12 (0%)	1/12 (8%)	5/12 (42%)	7/12 (58%)	9/12 (75%)	n.s.
Neomycin	40 mg/kg	2/24 (8%)	2/24 (8%)	7/24 (29%)	13/24 (54%)	15/24 (62%)	16/24 (67%)	n.s.
Kanamycin	40 mg/kg	3/10 (30%)	7/22 (32%)	10/22 (45%)	20/28 (71%)	16/22 (72%)	20/26 (72%)	n.s.
Dexamethasone	8 mg/kg	2/26 (8%)	4/42 (10%)	6/34 (18%)	6/48 (13%)	22/56 (40%)	104/129 (80%)	$p < .001$
Peritoneal lavage	100 ml saline		0/6 (0%)	1/6 (16%)	6/6 (100%)		14/20 (70%)	

*Chi-square statistics; n.s. = not significant

Part II

Description.

1. Survey of Quantitative Antibiotic Sensitivity of Isolates from Infected Wounds in Patients at WRGH. The object of this study was (1) to determine quantitatively in vitro the minimum inhibitory concentration for eight standard antibiotics for the microorganisms from infected wounds and (2) as a continuation and extension of a similar project reported in the 1966-67 annual progress report. An analysis of 134 broth cultures, provided by Miss Maddox of the WRGH bacteriology laboratory from patients with infected wounds, was performed in this study.

2. Therapeutic Regimens in Contaminated Soft Tissue Wounds of Rabbits and Guinea Pigs. This study is a continuation of the one reported in the 1966-67 annual progress report. As before the objects of this study were primarily (1) to compare infection promoting properties of several soils from Vietnam with an increased sampling of animals and (2) to evaluate antimicrobial regimens used prophylactically in the management of wound infections. This study was conducted in conjunction with the research project of LTC Matsumoto.

3. Endotoxin Assay of Lymph and Serum from Dogs in Hemorrhagic Shock. The object of this study was to determine the lethality of lymph and serum from shocked dogs via chick embryo assay. Protection of the embryo by pretreatment was also investigated. This study was conducted in conjunction with the research project of CPT Berman.

4. Bacteriological Monitoring of Suctioned Material from Four Tracheostomized Patients (Neurosurgery, WRGH). The object of this study was to determine what influence the one-catheter versus the two-catheter suctioning procedure had upon the microflora of the trachea and nasopharynx in selected tracheostomized patients. This study was conducted by MAJ Bluemle as a research project in conjunction with her military nursing research program. The Department of Surgical Microbiology provided professional guidance and research facilities.

5. The Effect of X-irradiation Followed by Dermal Wounding on the Occurrence and Extent of Microbial Invasion in the Adult Rat. The object of this study was to determine what effect x-irradiation,

followed four days later by a standard skin disc removal, would have upon the occurrence and extent of microbial invasion in various organs and tissues of adult rats. This study was conducted on an interdivisional level with LTC Stromberg et al. of the Division of Nuclear Medicine.

Progress.

1. Broth cultures obtained from infected wounds were periodically collected over several months from the WRGH bacteriology laboratory. Pure cultures were selectively isolated, identified and tested against chloramphenicol, tetracycline, cephalothin, bacitracin, neomycin, penicillin, polymyxin B and lincomycin individually in a concentration range of 200 to 0.1 μg or units per ml medium in a two-fold dilution series. The procedure used was the agar plate dilution technique with 4 hr broth cultures applied via the Steeris inoculator.

Tables 1a and 1b summarize the pertinent data. The data indicate that tetracycline was inhibitory against some S. aureus, S. epidermidis and streptococcus cultures. Neomycin showed a moderate degree of inhibitory activity, especially against the Klebsiella-Aerobacter group, as well as against S. aureus and S. epidermidis. Bacitracin was very inhibitory for S. aureus and streptococcal cultures. Cephalothin and lincomycin inhibition of S. aureus varied, being either very effective or only moderately inhibitory. Penicillin can be placed in a similar category for both species of staphylococcus, but in contrast was quite inhibitory toward the streptococcal cultures. Antibiotics which are at the same time very inhibitory and moderately inhibitory tend to encourage the development of resistance in such a varied bacterial population.

Chloramphenicol and polymyxin B have generally no inhibitory effect on the microorganisms tested.

As might be expected Pseudomonas cultures were quite resistant to the antibiotics tested, with only bacitracin showing a slight inhibitory activity for some cultures. The same general situation occurred with the Klebsiella-Aerobacter, the Proteus and the E. coli-Citrobacter groups, but in this case with neomycin.

2. During the year 1967-68, 25-30 rabbits per week and later the 60 guinea pigs per week were monitored bacteriologically following the infliction of a standard crush wound of the thigh muscle and contamination with 0.75 g of soil from Vietnam and 1.0 ml of a mixed 24 hr culture of Staphylococcus aureus, group D streptococcus, and Pseudomonas. The rabbits were monitored at 3, 12, 24 and 48 hr after wounding and the guinea pigs at 5, 24 and 48 hr.

The wound area was swabbed, the swab immersed in broth and the broth streaked for colony count using the Lindsey technique onto plates of mannitol salt agar, MacConkey's agar, SF agar and blood azide egg yolk

agar. With these selective media, wounds were monitored primarily for S. aureus, S. epidermidis, streptococci, lactose fermenting and non-fermenting enterobacteria, Pseudomonas, Proteus, Clostridium perfringens and other clostridium species.

Depending upon the particular experiment, the rabbit and guinea pig wounds were treated at various time intervals after wounding with an antibiotic or combination of antibiotics. Summarization of the data on the basis of high bacterial counts of the major microorganisms monitored at 24 hrs after wounding is presented in Table 2. The groups were divided into survivors and nonsurvivors for the antibiotic-treated and saline control groups.

The 24 hr monitoring period was selected as representing the general trend in bacterial quantitation of microorganisms isolated from the wounds. If we further select counts of 10^6 cells (or greater) per ml of wound exudate, a general pattern of microorganism concentration is apparent. There appears to be a general decline in the percent of 10-day-survival animals with high bacterial counts of streptococci compared with the nonsurvivors at 24 hrs after wounding. The remaining bacteria show no such general trend, but it should be noted that Pseudomonas remain quite persistently high among survivors and non-survivors regardless of the antibiotic given. However, the effect on survival is not apparent at this monitoring period. High staphyloccal counts tend to remain fairly consistent among survivors and nonsurvivors. A low percent of animals with high bacterial counts for clostridia was found. This percent may have increased with survival time, but at 24 hrs after wounding the aerobic microorganisms are predominant, as would be expected. The rapidly proliferating and antibiotic resistant Pseudomonas already are most predominant among the aerobes.

3. The assay was accomplished by injecting 0.1 ml of a filter-sterilized sample or a dilution thereof into a prominent vein of a 10-day-old chick embryo and recording embryo mortality for ten days following injection. Control eggs were inoculated with 0.1 ml sterile saline.

The lethality of serum and lymph from dogs in hemorrhagic shock appears to be related to the degree of acidosis and duration of shock. The pretreatment of the chick embryo with steroid has afforded no protection.

4. Tracheal catheter suction specimens were taken every two days with a total of eight specimens from each patient. One patient expired after the fifth specimen. At the time of each tracheal sampling the nasopharynx was swabbed for culture. The tracheal secretion was streaked for bacterial counting on mannitol salt agar, MacConkey's agar and two blood agar plates after appropriate tube dilution in brain heart infusion broth. One blood plate was incubated anaerobically. For comparison the Lindsey streaking technique for microbial quantitation was

performed on ten-fold diluted secretion. The nasopharyngeal swabs were incubated in brain heart infusion and in thioglycollate broths for 24 to 48 hrs. The resultant cultures were streaked for single colony isolation on the aforementioned plated media for identification.

An overall survey of the quantitative results indicates that tracheal colony counts showed marked variation among the patients. Two possible explanations for the variation are: (1) the fluctuation in volume and density of the secretions, and (2) the influence of antibiotic chemotherapy. There was no apparent relationship between bacterial counts and the type of suctioning procedure used.

The qualitative data for tracheal cultures are presented in Table 3 and for nasopharyngeal cultures in Table 4. These tables indicate that S. epidermidis and Diplococcus were predominant in throat cultures, whereas Klebsiella and Proteus were most frequently found in tracheal cultures. Pseudomonas, Proteus, Diplococcus, Klebsiella and Aerobacter were quite prevalent in both types of cultures. (Table 5)

5. Four days after adult rats (175-200 g) were x-irradiated (800 R) the dorsal skin was shaved and a full-thickness disc (3.0 cm diameter) of skin was removed. The rats were individually housed and the wound left untreated and opened. Control groups included irradiated non-wounded and nonirradiated wounded rats.

A representative number of rats from each group was sacrificed at various intervals after treatment and a standard amount of tissue was ground in a Tenbroeck tissue grinder with 4 ml of trypticase soy broth after being aseptically removed from each of the following anatomical areas: heart, spleen, liver, lung, kidney, back muscle beneath the wound site and leg muscle. A 5 ml aliquot of heart blood was used to inoculate the standard blood culture bottle. The homogenized material was streaked onto plates of blood agar using the Lindsey technique for bacterial cell count. Duplicate plates were incubated anaerobically. In addition broth cultures (brain heart infusion broth and reinforced clostridium medium) were included for comparison.

The data indicate that those irradiated wounded rats from which positive cultures were obtained yielded primarily nonhemolytic streptococci, Aerobacter, E. coli, and Proteus. An occasional Pseudomonas and alpha-hemolytic streptococcus were isolated. Among the very few positive cultures from control groups, alpha-hemolytic and nonhemolytic streptococci were almost exclusively present.

The bacterial counts were in the range of 10^5 to 10^7 cells per ml of sample for nonhemolytic streptococci and E. coli; 5×10^7 cells per ml of Aerobacter were found. The Proteus isolated was a swarming type.

Of the organs and tissues cultured, the liver, lung, kidney and back muscle most frequently yielded positive cultures. The spleen was positive less frequently. Positive cultures were isolated inconsistently from heart blood. This may have been due to the time after irradiation and wounding chosen for bacteriological monitoring.

Summary and Conclusions.

1. Bacitracin, tetracycline, cephalothin, penicillin, lincomycin and neomycin showed varying but generally satisfactory degrees of inhibitory activity in vitro toward some cultures of S. aureus, S. epidermidis and streptococcus. Chloramphenicol and polymyxin B were ineffective. None of the antibiotics tested were inhibitory toward the gram negative microorganisms.

2. At 24 hrs after wounding high cell counts of Pseudomonas are predominant among the aerobes in both surviving and nonsurviving animals regardless of antibiotic chemotherapy. Among survivors there is generally a lower percent of animals with high streptococcal counts. The low percent of animals with high clostridia counts was expected at 24 hrs after wounding, when aerobes predominated.

3. The lethality of serum and lymph from dogs in hemorrhagic shock may be related to the degree of acidosis and duration of shock. This study is incomplete.

4. There was an evident degree of fluctuation in bacterial cell counts among the tracheal cultures. Pseudomonas, Proteus, Diplococcus, Klebsiella and Aerobacter were frequently isolated from both trachea and nasopharynx. The type of suctioning procedure used had no effect on the microbial flora in the trachea or nasopharynx.

5. Rats exposed to x-irradiation and wounding showed a more frequent microbial invasion of internal organs and tissues than did the controls. Streptococci (nonhemolytic and hemolytic), Aerobacter, E. coli and Proteus were the predominant invasive microorganisms of the liver, lungs, kidneys and muscle beneath the wound site. The spleen and heart blood were invaded less often.

TABLE 1a: RANGES OF ANTIBIOTIC SENSITIVITIES OF CULTURES FROM WOUNDED WRGH PATIENTS

Organism	Total *	Tetracycline (ug/ml)	**	Chloramphenicol (ug/ml)	**	Cephalothin (ug/ml)	**	Bacitracin (units/ml)	**
S. aureus	39	> 200-200	17	> 200-200	37	100-50	10	12.5-3.1	29
		3.1-1.6	21			0.8-0.4	23	1.6-0.4	6
S. epid.	21	> 200-100	8	> 200-200	13	> 200-0.2	21	12.5-6.2	18
		12.5-3.1	7	100	6				
Strep.	24	> 200	16	> 200-200	21	100-50	17	6.2-3.1	12
		12.5-1.6	5					1.6	12
E. coli-Citrobact.	22	> 200	19	> 200	21	100-50	15	> 200	21
Pseud.	11	> 200-200	11	> 200	11	> 200	11	> 200-200	5
								100-50	6
Kleb-Aero	14	> 200	11	> 200	14	> 200	12	> 200	14
Proteus	2	> 200-200	2	> 200	2	> 200	2	> 200	2

* Total number of each bacterial species or group tested
** Number of bacterial species in each group sensitive to a particular concentration or range of concentrations

TABLE 1b: RANGES OF ANTIBIOTIC SENSITIVITIES OF CULTURES FROM WOUNDED WRGH PATIENTS

Organism	Total *	Neomycin (µg/ml)	**	Penicillin (µg/ml)	**	Lincomycin (µg/ml)	**	Polymyxin (µg/ml)	**
S. aureus	39	> 200	10	> 200-100	15	50-25	11	> 200	39
		25-12.5	17	0.4-0.1	11	1.6-0.8	20		
S. epid.	21	> 200	5	> 200-50	5	> 200	5	> 200	20
		1.6-0.4	7	3.1-1.6	11	1.6	7		
Strep.	24	> 200	23	1.6-0.8	21	> 200	6	> 200	24
						100-25	16		
E. coli-Citrobact.	22	> 200	7	> 200	16	> 200	19	> 200	10
		50-25	9	100	6	50	3	50	9
Pseud.	11	> 200	11	> 200	11	> 200	4	> 200	8
						50	7		
Kleb-Aero	14	> 200	5	> 200	14	> 200	10	> 200	13
		50-12.5	8			50	4		
Proteus	2	100-50	2	> 200	2	> 200	2	> 200	2

* Total number of each bacterial species or group tested

**Number of bacterial species in each group sensitive to a particular concentration or range of concentrations

TABLE 2: COMPARISON OF ANTIBIOTIC TREATMENT AMONG SURVIVING- AND NONSURVIVING-WOUNDED RABBITS AND GUINEA PIGS WITH HIGH BACTERIAL CELL COUNTS DETERMINED 24 HRS AFTER WOUNDING

Animals	Treatment Groups	Survival (days)	% Surviving of 260 Animals	% of Animals in each Treatment Group with 10^6 cells or more bacterial count per ml wound exudate at 24 hrs after wounding			
				Staph	Strep	Pseud	Clost
Rabbits Total: 260	Saline Controls	less than 10	14	5	38	47	10
		10	12	7	29	41	23
	Neosporin*	less than 10	8	0	28	53	19
		10	23	14	23	34	29
	Terramycin	less than 10	10	4	40	44	12
		10	33	6	26	56	12
Guinea Pigs Total: 260	Controls	less than 10	15	25	30	30	15
		10	45	29	19	44	8
	Neosporin*	less than 10	6	30	26	24	20
		10	13	35	26	35	4
	Terramycin	less than 10	0	0	0	0	0
		10	6	27	25	25	23
	Neomycin	less than 10	4	0	44	50	6
		10	11	24	24	39	13

* Neosporin: Bacitracin, Neomycin, Polymyxin B.

TABLE 3

Isolation Frequency of Microorganisms in 29 Tracheal Cultures

Organism	Isolation Frequency	
	Number	Per Cent
Staph epidermidis	23	79
Diplococcus	19	65
Alpha hemolytic strep	16	55
Pseudomonas	16	55
Bacillus	15	52
Proteus	13	45
Staph aureus	12	41
Aerobacter aerogenes	12	41
Klebsiella	11	38
E. coli	7	24
Citrobacter	7	24
Serratia	5	17
Arizona	3	10
Yeast	2	7
Alcaligenes	2	7
Moraxella	1	3
Providence	1	3
Beta hemolytic strep	1	3

TABLE 4

Isolation Frequency of Microorganisms in 29 Nasopharyngeal Cultures

Organism	Isolation Frequency	
	Number	Per Cent
Klebsiella	21	72
Proteus	19	65
Aerobacter aerogenes	16	55
Staph aureus	16	55
Diplococcus	16	55
Bacillus	12	41
E. coli	12	41
Pseudomonas	12	41
Citrobacter	10	34
Alpha hemolytic strep	10	34
Yeast	7	24
Staph epidermidis	7	24
Arizona	6	20
Edwardsiella	2	7
Providence	1	3
Alcaligenes	1	3
Herrelleae	1	3

TABLE 5

Isolation Frequency of Microorganisms in
Both Tracheal and Nasopharyngeal Cultures

Organism	Number of Isolations
Proteus	12
Pseudomonas	10
Diplococcus	10
Klebsiella	9
Aerobacter aerogenes	9
Staph aureus	7
Staph epidermidis	6
Bacillus	6
E. coli	5
Alpha hemolytic strep	5
Citrobacter	4
Yeast	4
Arizona	1

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 046527	CSCRD-163

4. FISCAL YEAR	5. KIND OF RPT./DOC.	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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10b. PRIOR NUMBER/CODE
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11. TITLE
(U) INTENSIVE STUDY AND TREATMENT OF SHOCK IN MAN

12. SUBJECT AREA	13. START DATE	14. CMPL. DATE	15. FUNDING AGENCY
12500 CLINICAL MEDICINE 012500 PHYSIOLOGY	07 65	NA	OTHER DA

16. PERFORMING METHOD	17. CONTRACT/GRANT		18. RESOURCES EST.	19. PROFESSIONAL MAN-YEARS	20. FUNDS (In Thousands)
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			CURRENT FY 69	6	150

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23. TECHNOLOGY UTILIZATION
SURGERY

24. COORDINATION
NA

KEYWORDS: WOUNDS, RESPONSE TO TRAUMA, PULMONARY INSUFFICIENCY, COAGULATION PARAMETERS, SERUM ENZYMES, CARDIAC OUTPUT, BLOOD VOLUMES.

25.

(U) TECH OBJECTIVE - THE INTENTION OF THIS PROJECT IS TO EVALUATE CHANGES IN ARTERIAL BLOOD GASES, COAGULATION PARAMETERS, SERUM ENZYMES, CARDIAC OUTPUT AND BLOOD VOLUME IN COMBAT CASUALTIES IN VIETNAM.

(U) APPROACH- PREVIOUS WORK IN THIS AREA WAS CARRIED OUT IN ANIMALS. THIS PROJECT IS THE EXTENSION OF THESE STUDIES TO HUMANS. A CLINICAL UNIT IN NRGH WAS SET UP FOR THE INTENSIVE STUDY AND TREATMENT OF REFRACTORY SHOCK. THE BASIC ASSUMPTION IS THAT THE COMMON DENOMINATOR FOR ALL TYPES OF SHOCK IS A DIMINISHED CAPILLARY PERFUSION CAUSED BY HYPOVOLEMIA, VASOCONSTRICTION AND EXPANSION OF THE VASCULAR SPACE. THIS LEADS TO ACIDOSIS AND HYPERCOAGULABILITY OF BLOOD. LEFT UNTREATED THIS PROGRESSES TO COAGULATION OF BLOOD WITHIN THE VASCULAR TREE. THIS ENTIRE SEQUENCE OF EVENTS CAN BE REVERSED AND PREVENTED IN MOST CASES BY ADEQUATE VOLUME ADDITION. IF THERE IS PERSISTENT VASOCONSTRICTION THIS MAY NEED TO BE RELIEVED BY VASODILATORS.

(U) PROGRESS - JUL 67 THRU JUN 68 THE DATA OBTAINED IN VIETNAM DURING THE MONTHS OF AUGUST 1967 THROUGH JANUARY 1968 ARE AT PRESENT BEING COLLECTED AND CORRELATED FOR THE BASIS OF THE FOLLOWING REPORTS- 1. EFFECT OF WOUND LOCATION AND SHOCK ON ARTERIAL HYPOXEMIA. 2. RECOVERY PATTERNS OF PATIENTS WHO ARE HYPOXEMIC. 3. A REVIEW AND ANALYSIS OF PATIENTS WITH ACUTE PULMONARY EDEMA. 4. AN ANALYSIS OF THE PATIENTS WITH HEAD INJURY WHO DIED OF PULMONARY EDEMA. 5. A REVIEW OF THE PATHOLOGY OF LUNGS IN PATIENTS WHO DIED OF WOUNDS. 6. A STUDY OF THE CLINICAL CARE OF ACUTE PENETRATING CHEST WOUNDS. 7. A STUDY OF THE EFFECT OF TRAUMA, HEMORRHAGE, MASSIVE TRANSFUSION ON MULTIPLE COAGULATION PARAMETERS. 8. THE EFFECT OF TRAUMA AND SHOCK ON THE SERUM ENZYMES OF COMBAT CASUALTIES. 9. A STUDY OF POST-RESUSCITATIVE BLOOD VOLUMES IN COMBAT CASUALTIES. 10. A STUDY OF ACID-BASE DISTURBANCES IN COMBAT CASUALTIES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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34. [illegible]	35.

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Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 01, Surgery

Work Unit 092, Intensive study and treatment of shock in man

Investigators.
Principal: CPT Arthur M. Martin, MC
Associate: CPT Henry B. Soloway, MC; LTC Teruo Matsumoto, MC

Description.

During the past year the Department of Surgical Pathology has continued its studies into the role of the cardiopulmonary system in hemorrhagic shock and trauma, both clinically and experimentally. In this regard, detailed analysis of autopsies of combat fatalities was carried out, a pathology section was added to the Trauma Study Section, U. S. Army Medical Research Team (WRAIR) Viet Nam, and experimental studies of pulmonary oxygen toxicity were initiated. This Department has continued to work in collaboration and support of other departments in the Division of Surgery, especially the Department of Human Studies, Department of Experimental Surgery, and Department of Anesthesia and Resuscitation.

A. Pathology Section, USAMRT (WRAIR)V: For several years the Division of Surgery, WRAIR, has maintained in Viet Nam the Trauma Study Section, USAMRT(WRAIR)V, formerly the Surgical Research Team. Through the clinical studies of this group, attention has been focused on the clinical problems of the pulmonary complications after combat injury. The Pathology Section was established in November 1967 to work in conjunction with this Trauma Study Section in the performance of autopsies on patients dying of combat injury and to study tissue or organs that may be removed at surgery on such cases.

B. Autopsy Review of Combat Deaths: In order to set guidelines for the Pathology Section, USAMRT (WRAIR)V, a review was made of the autopsy reports in the files of the U. S. Army 9th Medical Laboratory, Viet Nam. Particular emphasis of this review was directed toward determining the extent of pulmonary findings in these cases.

C. Pathology of the Heart in Clinical Shock: In experimental animals specific cardiac damage, myocardial zonal lesions, is a consistent finding at autopsy following hemorrhagic and septic shock. This type of damage has not previously been identified in man. Autopsies were performed in Viet Nam on combat fatalities including specific examination of the hearts for these lesions.

Progress.

A. Pathology Section, USAMRT (WRAIR)V

In November 1967, the Pathology Section was added to the U. S. Army Medical Research Team (WRAIR) Vietnam. This section was established to work in conjunction with the Trauma Study Section in order to permit clinicopathologic correlations of the problems in management and treatment of combat injuries. An autopsy facility was established at Long Binh Post, RVN, in the morgue of Graves Registration. From the middle of December 1967 to the end of January 1968, eleven autopsies were performed on combat fatalities dying in hospital and one surgical specimen (lung) was received. The material is presently under study and analysis.

B. Review of Autopsies of Combat Deaths

To obtain a better understanding of the pathological response to trauma, the autopsy files of the U. S. Army 9th Medical Laboratory, RVN, were reviewed. Approximately 800 autopsy reports were screened for the period from July 1966 to January 1968. From this file 100 reports were selected for detailed analysis. Selection was made with the following criteria: (1) death occurred following trauma, (2) except for three cases, all patients lived long enough to receive resuscitative care at a hospital installation, and (3) the autopsy reports were sufficiently complete, usually with microscopic study, to permit detailed analysis.

The results of this review showed a high incidence of pulmonary changes at autopsy in deaths due to trauma. This was especially true of pulmonary edema and congestion, pleural effusion, and, interestingly, an 11% incidence of pulmonary hyaline membranes. With the exception of hemothorax and atelectasis which were more common after thoracic injury, there was no significant difference in the incidence of pulmonary findings between thoracic and non-thoracic injury. This review emphasizes the need for more detailed study of the lungs to permit better understanding of the clinical problems of pulmonary complications and to provide for more effective therapeutic management of the seriously injured patient.

C. Pathology of the Heart in Clinical Shock

Myocardial zonal lesions are a consistent result of experimental hemorrhagic and endotoxic shock but had not been demonstrated in man. During the operation of the Pathology Section, USAMRT (WRAIR)V, a young soldier was autopsied who had died shortly after arriving at the hospital. The cause of death was due to acute exsanguination from a perforated femoral vein. At autopsy, marked subendocardial hemorrhage was present, and, with special histochemical methods, extensive myocardial zonal lesions were observed throughout both ventricular walls and interventricular septum.

The full significance of this finding is not known. Myocardial zonal lesions appear quite early during experimental hemorrhagic shock and become more extensive as the period of shock is prolonged. To a large extent the heart itself is responsible for production of these lesions in that the heart must be in shock for these lesions to occur. Hypovolemic hypotension of the systemic circulation alone does not cause these lesions to develop. The etiology of these lesions is related to the cardiac response to shock. If either tachycardia is eliminated from the shock syndrome (as with complete heart block) or a positive inotropic response is prevented (as occurs after beta sympathetic blockade with pronethalol), cardiac damage will not develop. Studies are required to define the effects of such anatomic change on ventricular function. The finding of a similar damage to the heart in humans as well as the experimental animal permits a more direct extrapolation of laboratory results to man in understanding the role of the heart in clinical shock.

Summary and Conclusions.

A. A pathology section has been added to the U. S. Army Medical Research Team (WRAIR) Viet Nam. This section will work in conjunction with the Trauma Study Section in evaluating the clinicopathologic findings in combat injury. Particular attention is being devoted to the problem of the pulmonary complications of non-thoracic injury as well as thoracic injury.

B. Review of autopsy reports of deaths due to trauma indicates a high incidence of pulmonary findings at necropsy. This is especially true for pulmonary edema and congestion, pleural effusion, and a significant incidence of pulmonary hyaline membranes. Except for hemothorax and atelectasis, the incidence of pulmonary changes is similar for thoracic and non-thoracic injury.

C. Myocardial zonal lesions have been identified in a human case of hemorrhagic shock. This finding will permit a more meaningful extrapolation to man of information obtained in laboratory studies of shock and thereby permit a better understanding of the role of the heart in clinical shock.

Publications.

None

PROJECT 3A014501B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 02
Internal Medicine

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
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11. TITLE
(U) VASCULAR COMPONENTS OF CARDIORESPIRATORY DISEASE

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002400 BIOENGINEERING 002300 BIOCHEMISTRY	OP 68	NA	OTHER DA

16. PHYSICAL METHOD	17. CONTRACT/GRANT		18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN-YEARS	20. FUNDING ESTIMATE
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	a. NUMBER NA	c. DATE	FY 69	11	200
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21. GOVT. LAB/INSTALLATION/ACTIVITY	22. PERFORMING ORGANIZATION
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23. TECHNOLOGY UTILIZATION MEDICINE	24. COORDINATION NA

25. KEYWORDS
CARDIOVASCULAR SYSTEM, CIRCULATION, HEART, BLOOD, CORONARY VESSELS, MYOCARDIUM, OXYGEN.

26.
(U) TECH OBJECTIVE - DEVELOPMENT OF STANDARDIZED BIOLOGICAL PREPARATIONS FOR LONG TERM HEMODYNAMIC AND BIOCHEMICAL STUDIES OF THE CONTROLS OF THE HEART AND CIRCULATION IN THE NORMAL STATE AND UNDER THE INFLUENCE OF ABNORMAL AND PATHOLOGICAL STRESSES.

(U) APPROACH- ENERGY METABOLISM OF THE HEART HAS BEEN STUDIED AT THE SARCOSOME LEVEL. IDENTITY AND CONTROL OF CORONARY ARTERY GENETIC PATTERNS HAS BEEN ATTEMPTED. REGULATION OF REGIONAL BLOOD FLOW HAS BEEN STUDIED IN THE UNANESTHETIZED DOG WITH CHRONICALLY IMPLANTED ELECTROMAGNETIC FLOWMETERS AND SPECIAL TUBES IN THE AORTA, CORONARY SINUS AND CORONARY ARTERY. THE PREPARATIONS AND STRESSES USED INCLUDE THE BLOCKED AND PACED HEART, THE SURGICALLY DENERVATED HEART, CHRONIC CORONARY INSUFFICIENCY WITH CORONARY COLLATERALS AND OTHER COMPENSATIONS.

(U) PROGRESS - JUL 67 THRU JUN 68 CERTAIN GUANIDINE DERIVATIVES SERVE AS SPECIFIC INHIBITORS TOWARD ENERGY YIELDING SITES OF THE ELECTRON TRANSFER CHAIN. A MECHANISM FOR TRANSFERRING REDUCING EQUIVALENTS ACROSS SARCOSOMAL MEMBRANE, UTILIZING THE MALATE-OXALACETATE COUPLE, HAS BEEN DEMONSTRATED. FOUR ANATOMIC CORONARY ARTERY PATTERNS HAVE BEEN FOUND IN RATS. THESE CAN BE ALTERED AND CONTROLLED BY BREEDING. IN PACED HEARTS, CORONARY FLOW CONTINUES TO RISE AFTER CARDIAC OUTPUT HAS REACHED AN OPTIMUM AT HIGH VENTRICULAR RATE. PACED HEARTS AT FIXED HEART RATES (UP TO 150) RESPOND WELL TO EXERCISE, EXCITEMENT, HYPOXIA, WITH LARGE INCREASES IN STROKE CORONARY FLOW AND CARDIAC OUTPUT. IN PACED AND UNPACED HEARTS, ALPHA AND BETA RECEPTORS HAVE BEEN FOUND IN THE CORONARY CIRCULATION AND THEIR INFLUENCE HAS BEEN STUDIED WITH VARIOUS STRESSES. LONG TERM REVERSIBLE LEFT CIRCUMFLEX CORONARY INSUFFICIENCY (0-50 PER CENT OF NORMAL CORONARY FLOW) ABOLISHES REACTIVE HYPEREMIA, AND INCREASES DESCENDENS CORONARY FLOW, PERIPHERAL CORONARY PRESSURE AND CORONARY COLLATERAL FLOW (XE-133 CLEARANCE). ACUTE CORONARY OCCLUSION RAPIDLY INCREASES PERIPHERAL CORONARY PRESSURE AND CORONARY COLLATERAL FLOW. VASODILATORS DO NOT AFFECT COLLATERAL FLOW DURING ACUTE OCCLUSION. FOLLOWING CARDIAC NEURAL ABLATION, THE RESPONSE OF THE CORONARY CIRCULATION DEVIATES FROM THAT OF THE NORMALLY INNERVATED HEART BY A RISE IN MYOCARDIAL OXYGEN EXTRACTION AND A MARKED INCREASE IN STROKE CORONARY FLOW. P. KNOWLESI AND P. COATNEYI MALARIA IN RHESUS MONKEYS IS CHARACTERIZED BY CURTAILMENT OF RENAL SODIUM AND WATER EXCRETION AND INCREASES IN HEART RATE IN ORDER TO MAINTAIN CARDIAC OUTPUT AND SYSTEMIC BLOOD PRESSURE. THESE ANIMALS DEVELOP AZOTEMIA AND OLIGURIA DESPITE NORMAL RENAL BLOOD FLOW RATES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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27. GOVERNMENT DISCIPLINE	28.	29. DOD CODE	30. BUDGET CODE
☐ A. SPONSOR ☐ B. OTHER		BR	1
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Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 085, Vascular components of cardiorespiratory disease

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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.

Development of instruments and methods for cardiovascular research.

A switching device has been designed to permit the measurement of temperature in up to four different regions of the body simultaneously, and through the same amplifier. This arrangement makes it possible to use only one channel of the recorder for four variables, and to have the very same gain for all thermistors used. The "on" time for each thermistor is about 20% of a cycle, so that between the temperature samples a base line is obtained. This base line is the amplifier output with no transducer connected and yields a continuous check on amplifier drift. The device has been used successfully in the measurement of temperature in various layers of the kidney in the monkey, and of the heart in the dog, for blood flow determinations in these organs by the heat dilution technique.

Effects of exercise and its cessation on the heart and its blood supply.

Studies were carried out in rats of different ages on the effects of daily and intermittent exercise on heart size, cross-sectional areas of extracoronary collateral arteries, and capillary-ventricular muscle fiber ratios. Cardiac hypertrophy occurred in young exercised animals and was absent in adult exercised animals. Old exercised rats had an actual loss of heart weight. Collateral blood supply to the heart increased in exercised animals of all ages. Capillary-fiber ratios

increased in young exercised animals and were associated with an increase in the total number of capillaries. In old exercised animals, the number of capillaries remained constant; thus, the increased capillary-fiber ratio was secondary to a loss of myocardial fibers. The effects of exercise are dependent not only on the amount of exercise but also on the age of the animal.

Since relative hypoxia had been suggested as the stimulus for certain changes observed in exercise, quantitative histologic studies were undertaken in chronically exercised animals to see if their organ and cellular development might be similar to that observed in animals residing in a hypoxic environment. The rate of body growth was reduced in exercised animals. Cardiac hypertrophy was proportionate to the severity of exercise. Cellular compositions of the liver, kidney, adrenals, and spleen of exercised animals were similar to those reported in animals residing in a hypoxic environment. However, cellular changes of hearts of exercised animals were different from those reported in hypoxic animals, suggesting the influence of factors other than relative hypoxia.

Similar quantitative histologic studies were conducted in rats of different ages which were subjected to different amounts of swimming. In young animals, exercise caused a retardation of over-all growth proportionate to the severity of the exercise. Body and organ weights of the exercised animals were less and associated with a reduced cell number in the various organs. Cardiac hypertrophy was also present in this age group. In adult animals, exercise appeared to have little effect on body and organ weights. The cell numbers of the heart and liver of adult exercised animals were less than controls, but the cytoplasmic mass of the individual cells of these organs was slightly greater than controls. In old animals, exercise elicited a catabolic response. There was an actual loss of body weight and organ weight proportionate to the severity of the exercise. It is evident from these findings that when analyzing exercise studies, the age of the subject should be taken into consideration.

In other experiments carried out in cooperation with the Division of Biochemistry, WRAIR, tissue and blood enzyme changes (lactic dehydrogenase (LDH) and its isoenzymes) and blood lipids and lipoproteins have been measured in rats subjected to varying amounts of acute exercise and training. Severe exercise (4 hours of swimming) produced a marked rise of enzymatic activity in untrained animals. Training by swimming one hour daily for 1-10 weeks provided little or partial protection against severe exercise. Training 4 hours daily for 6-8 weeks prevented the enzymatic changes observed after severe exercise. By isoenzyme analysis, the rise of plasma LDH activity was associated with the kidney, heart, liver, and muscle; they further showed that adaptation to exercise by training varies in the different organs.

These enzyme studies and quantitative histologic studies were undertaken in rats which were trained (swimming 4 hours daily for 10

weeks) and then subjected to acute exercise (4 hours swimming) at different times after the cessation of training. At the end of 10 weeks training, no enzymatic changes were observed after acute exercise. Cardiac hypertrophy, indicated by an increased heart weight-body weight ratio, was associated with an increased number of myocardial fibers. Two weeks after the cessation of training, heart weights of the post-trained rats were less than controls. When these animals were acutely exercised, plasma LDH reached levels nearly five times that observed in the controls. These values were also significantly higher than those observed in untrained rats after acute exercise. Quantitative histologic analysis at two weeks after the cessation of training showed smaller nuclei and a loss of myocardial fibers while the amount of sarcoplasm in each myocardial fiber was increasing. These changes suggested that a change in membrane permeability of the myocardial fiber may be responsible for the marked enzymatic activity response to acute exercise in these post-trained animals. Four weeks after the cessation of training, cellular measurements of the post-trained animals and their enzymatic response to acute exercise were similar to those observed in controls.

Studies of the effects of training and acute exercise on plasma lipids and lipoproteins were carried out in rats. In the untrained rat following acute exercise (4 hours swimming), plasma cholesterol and non-esterified fatty acids (NEFA) were significantly elevated while plasma triglycerides were significantly decreased. There was no significant change in the plasma levels of cholesteryl esters and phospholipids after acute exercise. Training (4 hours swimming daily) for four weeks reduced plasma cholesterol to one-half the control level. There was no significant change of plasma cholesterol in the trained rat following acute exercise. The level of plasma triglycerides was also reduced in the trained rats. A further reduction of plasma triglycerides was observed after acute exercise in the trained animals. Training caused no significant changes in NEFA, cholesteryl esters and phospholipids from control levels. However, when trained animals were exercised, NEFA were elevated immediately thereafter.

Inheritance of coronary artery anatomical patterns and the relationship to coronary artery disease.

Our previous work showed that coronary artery patterns in rats are subjected to polygenetic inheritance. Quantitative genetic analysis demonstrated that the genetic contribution to coronary variability was 20%-25%, with the remainder of variability being due to prenatal environmental circumstances. These findings suggested that it may be possible to increase the frequency of "favorable" coronary patterns in offspring by manipulating the prenatal maternal environment. In an initial test of this hypothesis, pregnant female rats were subjected to prolonged exercise, either treadmill or swimming, during periods when the fetal heart is known to be developing. No significant differences in the frequency distribution of coronary patterns were observed when the resulting offspring were compared to those from control mothers.

Since the variability of coronary artery anatomical patterns present in these rats is similar to that seen in man, we have subjected rats of different familial backgrounds with different patterns to atherogenic ("high fat") diets in order to produce coronary artery disease. Animals were sacrificed at regular intervals and their hearts removed for gross and microscopic examination. Histological sections were coded so that their origins were unknown to observers, and the coronary artery lesions were assigned quantitative scores according to the method of Wissler. Preliminary observations indicate that the severity of the coronary artery lesions more closely correlated with the familial background of the animals rather than specific anatomic configurations of the coronary arteries.

Other rats of similar familial background, but of different ages, have been subjected to daily exercise while on atherogenic diets, in order to determine if exercise reduces the severity of coronary arterial atherosclerosis. Quantitative histologic studies are now being conducted on these animals.

Effects of Dimethyl sulfoxide (DMSO) on experimental myocardial necrosis.

The effects of DMSO on the healing of isoproterenol-induced myocardial lesions have been determined. The various stages of healing of myocardial lesions were quantitated according to the method of Gal and colleagues. These preliminary observations reported last year were confirmed, i.e., DMSO treated animals had myocardial lesions that were quantitatively less severe; less myocardial fiber necrosis was present with the lesions consisting chiefly of stromal alteration; and residual myocardial fibrosis was less. Additional series of animals were completed in order that appropriate statistical analysis could be carried out. These studies are now being prepared for publication.

Hemodynamic and metabolic studies in simian malaria.

Studies of the hemodynamic and metabolic responses to P. knowlesi and P. coatneyi malaria infections in rhesus monkeys have been completed. In 5 animals, blood pressure and cardiac output were maintained throughout infection, but at the expense of a marked increase in heart rate. Right atrial pressures were low. Renal blood flow remained at normal levels until hemoglobinemia and hemoglobinuria developed, whereupon it virtually ceased. At the time renal blood flow fell, all the animals had been oliguric and azotemic for as long as 36 hours. Water, sodium, potassium, and nitrogen metabolism were studied by balance technique in 4 monkeys. There was curtailment of renal sodium and water excretion and increased renal excretion of potassium and total nitrogen. Fecal electrolyte and nitrogen excretion also increased. Plasma electrolyte studies suggest that the salt and water retention was isotonic. Studies of intrarenal blood flow distribution and blood volume distribution have been severely hampered by technical difficulties, mainly related to the small size of the animals, and to abnormal vascular permeability.

Regulation of the coronary collateral circulation.

Experiments were continued to study in chronic dogs the relationship of collateral flow during the development of the peripheral circumflex pressure secondary to chronic constriction, but the experiments to date have not been successful owing to technical failures. In view of the difficulties encountered, these experiments were discontinued for the time being and Dr. Pasyk was instructed in the use of the coronary tube in chronic experiments. It appears the method can be used by others; no trouble was encountered with the method of implanting the tube, however, there were some complaints about the method of sealing it off. It appears that the flared portion of the tube does not stand up to repeated usage. Also, the method of bringing it out through the ribs and skin can be improved. Dr. Pasyk's suggestion of bringing it out more posteriorly, as opposed to the side of the chest, is worth pursuing. The work of the previous year was written up and published.

The effect of vasodilators on the coronary circulation before, during, and after coronary artery occlusion.

Studies have been made of the effects of the coronary vasodilators, nitroglycerine, and persantin, on the coronary circulation and on the coronary collateral circulation in intact, unanesthetized, fully conscious, trained dogs. Estimates of coronary blood flow were obtained from electromagnetic flow transducers implanted on the left circumflex branch and from precordial measurements of myocardial clearance of the isotope Xe-133. The isotope was given intracoronarily via a modified Barger tube implanted in the left circumflex branch. The vasodilators were given either intravenously or through the coronary tube. In addition, nitroglycerine was administered sublingually and orally.

When 1-8 μg doses of nitroglycerine were given instantaneously into the coronary artery, transducer flow did not change from the control level. However, Xenon clearances were higher than control values and were associated with a small change in phasic coronary flow pattern. This dissociation in response of transducer flow and clearance suggests a redistribution of flow. At dose levels of 15 μg and more of nitroglycerine, Xenon clearances and transducer flows increased in parallel. A peak response was elicited with 30 μg nitroglycerine given intracoronarily with no systemic effect occurring. The response occurred immediately after injection and was transient in nature. Intracoronary persantin gave a similar response but the increase in flow was much larger and maintained.

When nitroglycerine was given as a continuous intravenous infusion or sublingually, the observed changes in isotope clearance and transducer flow were similar to those obtained after intracoronary injection. At low doses there was a 20%-40% increase of isotope clearance while transducer flow remained unchanged. At higher dose levels, a marked increase in both transducer flow and isotope clearance was noted. Following sublingual administration, coronary flow increase began at 30

seconds, peaked at 1½ minutes, and continued for 3 minutes. With continuous intracoronary and intravenous administration, coronary flow remained elevated as long as the infusion continued. Large coronary flow responses could be obtained following intravenous slug or continuous injection with minimal effects on heart rate, cardiac output, and blood pressure. Up to 50 mgm of nitroglycerine prepared for oral administration did not affect the coronary or systemic circulations.

The ability of the coronary vascular bed to dilate was also measured by determining peak reactive hyperemia following short occlusions of the left circumflex branch. In the normal animal, it was observed that peak reactive hyperemia increased proportionate to the time of occlusion, i.e.,a 40 second occlusion resulted in a higher peak reactive hyperemia than did a 10 second occlusion. With low doses of nitroglycerine and persantin administered during the occlusive period, reactive hyperemia was increased over the value obtained by occlusion only for the same time interval. When vasodilators were given in massive doses, the reactive hyperemia response to short occlusions was abolished indicating the coronary vascular bed was maximally dilated.

Estimates of the coronary collateral circulation were obtained by measuring the residual pressure in the circumflex branch distal to the point of its temporary occlusion (peripheral coronary pressure or PCP) and by measuring isotope clearance during the temporary occlusion. In the normal dog, PCP remained at a constant level for days even though multiple short temporary occlusions had been performed. The level of PCP was partially dependent on the level of systemic arterial pressure. Vasodilators decreased the PCP pressure pulse but not the mean PCP.

After completion of these studies in the normal dog, the left circumflex branch was occluded for 24 hours. The development of the coronary collateral circulation was observed. Vasodilators were given into the coronary bed distal to the site of occlusion, and also intravenously in order to ascertain their effects on coronary collateral blood flow. It is of interest that coronary collaterals appear to develop rapidly during acute occlusion as evidenced by an increase of PCP to levels 4-5 times control values within 8 hours of the onset of occlusion. Isotope clearances showed a similar directional change. Nitroglycerine and persantin appeared to have no significant effect on coronary collateral blood flow during this period. ECG recordings during the course of acute occlusion showed transient ischemia initially. After 3-4 hours of occlusion, evidence of transmural infarction with conduction disturbances appeared.

When the occlusion of the left circumflex branch was released at the end of 24 hours, the reactive hyperemia response was greatly diminished. The response to nitroglycerine and persantin was also less than that observed in the control period. The reactive hyperemia response and the response to vasodilators gradually increased over the ensuing days and approached control levels 5 days after the release of occlusion. After the release of the 24 hour occlusion, ECG's

demonstrated a return to sinus rhythm within 48 hours. However, evidence of myocardial infarction persisted.

The coronary circulation in the conscious dog with cardiac neural ablation.

The controls of the left coronary circulation and myocardial energetics in the well-trained, unanesthetized dog were delineated by our earlier studies. The present study was designed to test the importance of the external innervation of the heart to such regulation under the influence of normal and abnormal stresses. The hearts of 10 dogs were denervated by a stripping technique. Electromagnetic flow transducers were implanted on the ascending aorta and left circumflex coronary artery. A pneumatic cuff was placed distal to the coronary transducer for temporary and permanent coronary artery occlusion. Tubes were placed in the aorta and coronary sinus for pressure recording and for biochemical studies. Finally, a tube was chronically implanted in the left circumflex for drug injection and for measurement of two indices of the state of the collateral circulation. These were the level of residual pressure (peripheral coronary pressure) and Xe-133 clearance during temporary and permanent occlusion of the circumflex branch. Studies were made for 8-9 weeks on 5 of the 10 dogs. Their clinical status was excellent. Their acid-base balance and electrolytes (K and Na) were normal. Their ability to respond after the operation to stresses such as exercise was equal to that before denervation. Pharmacological, biochemical and physiological tests for cardiac innervation were negative throughout the study. The systemic and coronary circulations did not respond to large intravenous doses of tyramine and atropine; stimulation of the stellate ganglia and their cardiac fibers just before sacrifice did not affect the heart; the hearts after sacrifice contained only negligible amounts of catecholamines.

At rest, circumflex flow (20-30cc/100gm/min) and myocardial oxygen usage (3-4cc/100gm/min) were lower and myocardial oxygen extraction (14-15 vol.%) higher than in the normal dog. Exercise and excitement increased greatly the mean cardiac output and coronary flow and stroke coronary flow. In the denervated heart, heart rate increase was delayed and only moderate so that the circulation relied for an adequate response mainly on a large increase in cardiac output and coronary flow per heart beat. This contrasted with the dog with intact cardiac nerves in which aortic and coronary flow responses were obtained mainly by a large increase in the number of heart beats per minute with only a small increase in stroke flows even under very heavy stress. Tilting (head up) also greatly increased mean and stroke coronary flow. Exercise increased oxygen extraction whereas excitement and tilting decreased greatly oxygen extractions, the coronary sinus oxygen saturation at times increasing to as high as 70%, thus indicating massive active vasodilation and a plethora of blood flow.

After permanent coronary occlusion, the findings that pain and arrhythmia did not develop during the first 3-4 hours were similar

to those in the innervated heart of the conscious dog (Federation Proc. 27:632, 1968). K efflux into the coronary sinus was also delayed 3-4 hours. Within the first hour, there was a transient rise in circulating norepinephrine and, in one dog, the indices of collateral development, Xe-133 clearance, and peripheral coronary pressure rose considerably.

Myocardial metabolism.

In addition to the findings reported last year on the guanidine and biguanide derivatives, we now have evidence for specificity to all three phosphorylation sites by some of these blocking agents. Most of these studies have been done on a system utilizing the energy-linked reduction of NAD^+ by either succinate or tetramethyl-p-phenylenediamine (reduced by ascorbate). This reaction is allowed to proceed in the presence of cyanide with energy supplied by exogenous ATP. Under these conditions, high energy intermediates are generated at all three phosphorylation sites along the electron transfer chain. As long as the two reduction reactions are not so tightly coupled as to inhibit electron flow when one or more of the energy transfer intermediates are bound to the blocking agent, high energy intermediates formed at any of the three sites can drive reversed electron flow along any segment of the respiratory chain. By the use of multiple additions of the various guanidine derivatives, it has been possible to titrate the sequential phosphorylation sites and determine the major target of these inhibitors. These multiple titrations have been carried out on several of these compounds and several more are in the process of being done.

We now have direct evidence for the transfer of reducing equivalents from the inside to the outside of heart sarcosomes via the malate-oxalacetate couple. These experiments were carried out with rat heart mitochondria using succinate to furnish the reducing equivalents. If, after the addition of succinate to the system, cyanide, NAD^+, and ATP are introduced into the reaction mixture, there is no significant reduction of the exogenous pyridine nucleotide. However, when malic dehydrogenase is then added to the system, there is a short lag phase followed by a slow reduction of the added NAD^+. The reaction is faster in rat liver mitochondria than in the heart sarcosomes but this may reflect differences in isolation conditions. We plan to further study this reaction utilizing the PEP carboxykinase of the mitochondria to furnish malate from added pyruvate. Since this enzyme is present in some species and absent in others, it should provide a good internal control for the role of the malate-oxalacetate couple.

Summary and Conclusions.

A heat dilution technique is being evaluated for regional blood flow estimation in various layers of the heart and kidney of conscious animals.

Studies in rats have delineated the effects of exercise and training on the heart, cardiac blood supply, organ and cellular development,

LDH and its isoenzymes, and plasma lipids and lipoproteins. It is evident that the response and adaptation to exercise vary in different organs and are dependent on the severity of exercise and the age of the subject.

Genetic studies in rats have shown that prolonged exercise during pregnancy has little effect on the frequency distribution of genetically determined coronary artery patterns. The severity of experimentally induced coronary artery disease is related to the animals' familial backgrounds. Furthermore, exercise, while on atherogenic diets, appears to reduce the severity of coronary artery disease.

Dimethyl sulfoxide (DMSO) administration to rats with isoproterenol-induced myocardial infarct-like lesions altered the healing of such lesions. The severity of these lesions was quantitatively reduced in DMSO-treated animals when compared to untreated controls.

The renal response to P. knowlesi and P. coatneyi malaria in rhesus monkeys is characterized by the development of oliguria and azotemia in the face of normal renal blood flow, intact concentrating ability, and renal restriction of sodium excretion.

Improvements have been made in the method of chronic coronary artery intubation.

Studies have been made of the effects of coronary vasodilators on the normal coronary circulation and on the coronary collateral circulation in intact, conscious dogs. During coronary occlusion of 24 hours duration, coronary collateral circulation increased rapidly as indicated by peripheral coronary pressure measurements and isotope clearances. Vasodilators did not change collateral flow during acute occlusion. Reactive hyperemia and the response to vasodilators were reduced after release of the 24 hours occlusion, and approached control levels some five days later.

Chronic cardiac denervation decreases resting coronary flow and myocardial oxygen consumption. Adequate response of coronary flow to exercise, excitement, and positional change is attained by a much larger increase in stroke coronary flow than in the normal dog. In tilt and excitement, coronary flow is so high that oxygen extraction across the myocardium is greatly reduced. Early after coronary occlusion, circulating norepinephrine and indices of collateral development rise, but pain, arrhythmias, and K efflux into the coronary sinus do not occur before 3-4 hours.

Characterization of several inhibitors of energy metabolism which show specificity for certain phosphorylation sites has been carried out with the aid of a multiple titration technique. Direct evidence for the transfer of reducing equivalents across the mitochondrial membrane via the malate-oxalacetate couple has been obtained. This system may be of value for studies on the role of high energy intermediates in intact mitochondria.

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION DA	REPORT CONTROL SYMBOL CSGRD-193
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25.

(U) TECH OBJECTIVE - BASIC OBSERVATIONS OF HEMATOLOGIC RESPONSES TO DISEASE AND INJURY, IMPROVE THE ARMY TRANSFUSION SERVICE, STUDIES OF BLOOD, BLOOD PRODUCTS AND BLOOD SUBSTITUTES FOR THE TREATMENT OF CASUALTIES AND THE PREVENTION OF DISEASE.

(U) APPROACH- STUDIES OF COAGULATION ABNORMALITIES IN VARIOUS DISEASE AND PHYSIOLOGIC STATES. STUDIES OF THE MECHANISM OF BONE MARROW REPOPULATION FOLLOWING IRRADIATION. IMPROVED METHODS OF BLOOD BANKING. CLARIFICATION OF THE BLEEDING ABNORMALITIES WITH PLASMA EXPANDERS.

(U) PROGRESS - JUL 67 THRU JUN 68 ACCELERATED INTRAVASCULAR COAGULATION WAS OBSERVED IN HUMANS WITH KOREAN HEMORRHAGIC FEVER AND MENINGOCOCCEMIA AND ANIMALS WITH YELLOW FEVER AND ARGENTINE HEMORRHAGIC FEVER. THESE OBSERVATIONS WOULD EXPLAIN MANY OF THE COMPLICATIONS WHICH COULD BE AVERTED WITH ANTICOAGULATION THERAPY. CHILDREN WITH CYANOTIC HEART DISEASE WERE SHOWN TO HAVE SEVERE COAGULATION ABNORMALITIES WHICH WERE RAPIDLY CORRECTED BY HEPARIN THERAPY. STUDIES OF MONKEYS MADE ERYTHREMIC IN A HYPOBARIC ENVIRONMENT SIMULATING 17,500 FEET ALTITUDE SHOWED DISSEMINATED INTRAVASCULAR COAGULATION WHICH SEEMED RELATED TO THE ERYTHROCYTOSIS. SUCCESSFUL TRANSPLANTS OF BONE MARROW WERE MADE INTO MARROW CAVITIES OF LONG BONES AFTER THE FIBROSIS CAUSED BY RADIATION WAS EXCISED. A METHOD FOR FREEZING PLASMA AND STORING RED BLOOD CELLS THAT PERMITTED RECONSTITUTION OF WHOLE BLOOD WITHIN A CLOSED SYSTEM WAS DEVISED. FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

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Project 3A014501B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Surgery

Work Unit 086, Blood and blood disorders

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Description.

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

Progress.

Patients dying with an infectious disease frequently succumb with findings of hemorrhage and thrombosis in post mortem specimens from multiple body organs. These combinational findings suggest the occurrence of disseminated intravascular coagulation in which accelerated utilization of coagulation factors occurs during the formation of thrombi and causes a depletion of essential coagulation factors from the blood with the subsequent development of hemorrhagic phenomena. The importance of this abnormality is that it can frequently be averted or lessened in severity by prompt anticoagulant therapy. During the last year, we have undertaken studies of humans and animals with viral, bacterial and parasitic diseases in which hemorrhage and thrombosis have been observed as complications of the disease state.

Serial studies of rhesus monkeys infected with a virulent yellow fever virus (Asibi strain) showed coagulation abnormalities within 72 hours after injection. At that time and coincident with the onset of clinical illness there was a prolonged one stage prothrombin time and prolonged partial thromboplastin time, reflecting measured deficiencies of factors II, V, VII, VIII, IX, X, and XI. Prolongation of both the euglobulin lysis time and thrombin time suggested that there was a depression of plasminogen activation and accumulation of fibrinogen degradation products. The latter was confirmed by examination of

sera with a fibrinogen antisera. Early in the incubation period the platelet count and fibrinogen levels were normal and did not become abnormal until the animals were terminally ill. Since the coagulation changes occurred coincident with detectable viremia and before alterations in liver function tests and evidence of hepatic necrosis in liver biopsy specimens, there is convincing evidence that hemorrhagic manifestations are caused by a consumptive coagulopathy in addition to hepatic failure.

Studies in guinea pigs infected with Argentinian hemorrhagic fever (Junin) showed these animals died with widespread evidence of thrombosis and hemorrhage. Coagulation tests become abnormal approximately seven days after viral inoculation and just prior to the onset of clinical illness and worsen when the animals become moribund. These animals show a marked thrombocytopenia and leukopenia in addition to deficiencies of factors V, VII, VIII, IX, X, XI and XII with evidence of fibrinogen degradation products in sera.

Studies of the consumptive coagulopathy observed in malarious rhesus monkeys and reported during the previous fiscal year were continued. Heparinization of monkeys not only prevented the occurrence of marked changes in coagulation but also depressed the number of circulating parasites in the blood of anticoagulated animals. The effect of heparin seemed dissimilar to the effects of other antimalarial drugs because parasitemia recurred and the animals died within a few days after cessation of therapy. We believe the heparinization produces alterations in the host rather than a pharmacologic effect upon the parasite either by making the invasion of red blood cells less successful or by decreasing the availability of substrates which are required for optimal parasitic growth.

Coagulation studies have been performed in humans with chloroquine resistant P. falciparum malaria, fulminant meningococcemia and Korean hemorrhagic fever. In each of these diseases coagulation abnormalities exist which suggest the occurrence of accelerated intravascular coagulation. Only severely ill patients with marked coagulation abnormalities have been heparinized. In malarious patients heparin therapy seemed to have a demonstrable salutory effect. Evidence is less convincing in the patients with meningococcemia and Korean hemorrhagic fever. Whether patients who are moribund with a consumptive coagulopathy are beyond help with anticoagulation therapy or whether they should be administered fresh plasma to replace depleted coagulation factors coincident with the administration of heparin demands further investigation.

Since the advent of corrective surgery for patients with congenital cyanotic heart disease, disordered hemostasis has been recognized in those with marked secondary erythrocytosis. Hemorrhage and thrombosis are frequently observed as complications of surgery and thrombocytopenia has been reported in many children with a hematocrit greater than 65 per cent. The referral of a cyanotic child with transposition of the great vessels because of a platelet count of 17,000 per mm^3 led to the performance of coagulation studies. These tests showed deficiencies of the labile clotting factors (V and VIII) with evidence of excessive accumulation of fibrinogen degradation products in serum specimens. Heparinization markedly improved these abnormalities, indicating that they were caused by excessive destruction and not by impaired production of coagulation factors. This correction of severe coagulation abnormalities permitted the performance of corrective surgery. Subsequently, we have performed coagulation studies in additional patients with cyanotic congenital heart disease and found evidence of a consumptive coagulopathy in all patients with severe erythrocytosis. It is believed that these findings have provided a method for reducing the risk of surgery in these patients.

Studies are in progress to delineate the etiology of the consumptive coagulopathy in cyanotic congenital heart disease. The possibility that the coagulation abnormalities were caused by erythrocytosis led us to perform coagulation studies in monkeys at intervals during their stay and following their removal from a hypobaric chamber. The possibility that this might produce abnormalities was suggested by the frequent occurrence of thrombosis among persons exposed to high altitudes. The rhesus monkeys were brought to a simulated altitude of 17,500 feet and maintained at approximately 0.5 atmosphere for 28 days. Animals developed a prolonged prothrombin time and partial thromboplastin time, reflecting decreases in factor V, VIII and XI activity in the circulating blood. Thrombin times were prolonged despite normal fibrinogen levels, suggesting the presence of circulating fibrinogen degradation products. This was confirmed by immunodiffusion studies with antifibrinogen antisera. These changes did not occur until the animals had marked erythremia and could be averted by heparinization of the animals. Removal of the animals from the hypobaric chamber caused hemostasis to return to normal as the hematocrit decreased towards normal values. This latter finding suggests that the coagulopathy is more related to increased erythrocytosis and changes in blood viscosity rather than hypoxia. Further studies are needed to investigate this hypothesis.

The military requirement to station large numbers of soldiers in malarious areas has led to renewed interest in the chemoprophylaxis and treatment of malaria. Obviously, drugs which markedly alter

the metabolism and survival of the malaria parasite are likely to be toxic to man. Throughout the last two decades the therapeutic and chemoprophylactic doses of chloroquine and primaquine have been adjusted to permit maximal antimalarial effectiveness with minimal side effects to humans. This problem is complicated by the increased susceptibility of humans with certain genetic defects which make them less tolerant of certain of the antimalarial drugs. This latter problem was initially recognized by Alving because many U. S. Negroes have a mild deficiency of glucose-6-phosphate dehydrogenase in their red blood cells and the administration of primaquine caused a moderate hemolytic anemia. The current drug regimens for malaria were adjusted to permit the administration of effective doses of primaquine that would not cause sufficient hemolysis in affected Negroes to impair their normal state of health or performance. Caucasians with G-6-PD deficiency usually have a more severe enzymatic deficiency and develop a more profound anemia in response to primaquine therapy. We have attempted to quantify the response of Caucasians with G-6-PD deficiency to conventional doses of primaquine to ascertain problems that can be anticipated with continued large scale administration of antimalarial drugs and to formulate recommendations regarding the proper utilization of these individuals. A standard prophylactic antimalarial dose of primaquine causes a destruction of about twenty per cent of circulating red blood cells in Caucasians in comparison to an eight per cent hemolysis in Negroes with G-6-PD deficiency. Compensation of the anemia occurs rapidly in both groups because of the reserve capability of the bone marrow to produce excessive quantities of red blood cells. Even though a mild anemia and jaundice are usually observed in Caucasians, no serious side effects or impairment of performance occur in the normal state of health of these individuals. However, based upon clinical histories of these patients, it was ascertained that they sought hospitalization usually with an infectious disease and that the anemia was more profound following administration of primaquine during the acute or convalescent phase of the other illness than was observed in our experimental studies. This would seem to be related to an impaired reserve capability of the bone marrow during the course of many viral illnesses. More recently, patients have been evacuated from Vietnam because they developed cyanosis and fatigue in association with the ingestion of antimalarial drugs. Studies of these patients have shown that they develop significant and clinically apparent methemoglobinemia in response to conventional doses of antimalarial drugs. These patients have been shown to have a heterozygous enzymatic deficiency of NADH methemoglobin reductase. Studies with the various antimalarial drugs used for therapy and prophylaxis have shown that chloroquine, primaquine and diaminodiphenylsulfone (DDS) will increase blood levels of methemoglobinemia in affected subjects. Studies are in progress to quantify these effects.

Studies of the effects of localized irradiation to a single extremity of rats showed that doses of 1,000 or 2,000 R were followed by a disappearance of normal bone marrow with a reappearance of hemopoietic cells in the irradiated marrow several months later. However, if the radiation dose was 4,000 R or greater, a permanent marrow aplasia ensued and there was no repopulation of the marrow space with hemopoietic tissue either from adjacent nonirradiated marrow or following the intravenous transfusion of donor marrow. However, if the marrow spaces were mechanically disrupted and curetted and then normal marrow was injected into the cavity, there was regeneration of bone marrow with restoration of normal parenchymal and sinusoidal architecture. Intravenous administration of bone marrow to animals following curettage failed to produce this salutory effect.

Previous investigations in both man and animals showed persistent changes in circulating erythrocytes after splenectomy. In humans, hypertrophy of accidentally implanted splenunculi can nullify the therapeutic effects of splenectomy. In experimental animals splenic implants become functional and enlarge in response to certain stimuli and atrophy when deprived of their normal hemolytic function. To ascertain and quantify the capability of splenic implants to restore normal splenic function, we splenectomized rats and returned the excised organ to the peritoneal cavity of one-half of the animals. The tissue remained viable and was forty per cent of the weight of spleens in comparable nonsplenectomized animals. This amount of regenerated tissue prevented the morphologic erythrocytic abnormalities observed in asplenic rats, leptocytes and red blood cells with inclusion bodies did not accumulate in the peripheral blood and prevented the changes in ratio of volume to surface area usually observed in splenectomized animals. Contrariwise, abnormalities in osmotic fragility studies persisted indicating that various splenic functions require various amounts of splenic tissue.

Heme binding to albumin and to the beta globulin hemopexin was studied in plasma from normal subjects and patients with hemolytic disorders utilizing electrophoresis and immunodiffusion techniques. Deficiency of hemopexin was found in some patients with depleted haptoglobin and was related to elevation in the plasma heme pigment levels. Hemolysis unaccompanied by hemoglobinuria did not result in hemopexin deficiency. After cessation of hemolysis, hemopexin reappeared in the plasma prior to or simultaneously with haptoglobin. It was shown that hemopexin deficiency can exist with normal haptoglobin levels in patients in whom nonhemoglobin heme is released into the plasma.

Investigation of a soldier evacuated from Vietnam with a hemolytic anemia which occurred coincident with the ingestion of antimalarial prophylactic drugs demonstrated a previous undescribed

red blood cell enzymatic abnormality. Studies of the soldier's family demonstrated a similar defect which seemed to be inherited as an autosomal dominant. Unlike reported cases of pyruvic kinase deficiency, these patients had an erythrocytic enzyme activity with a normal V_{max} but a markedly elevated K_m indicating a reduction in the affinity of the enzyme for its substrate, although at high substrate levels its activity was almost normal.

The laboratory has continued collaboration with the International Committee for Standardization in Hematology. The purpose of the organization is to provide uniform methods and standards throughout the world so that results of nutritional hematologic studies from various geographic areas can be compared. Previously, this laboratory contributed significantly to the standardization of hemoglobinometry by the development of acceptable standards. Current work involves the development of an acceptable standard for serum iron and transferrin measurements and the standardization of methodology.

Summary and Conclusions.

Coagulation studies in selected viral, bacterial and parasitic diseases have demonstrated that accelerated intravascular coagulation occurs and may play an important role in both the morbidity and mortality. Similar abnormalities were found in congenital cyanotic heart disease and in animals exposed to an environment simulating 17,500 feet altitude. The coagulation abnormalities in certain of these disorders and presumably the complications of bleeding and thrombosis can be averted by heparin therapy. The adverse effects of antimalarial prophylaxis in Caucasians with G-6-PD deficiency and subjects with NADH methemoglobin reductase deficiency were described and quantified. Studies of the effects of large dose localized irradiation were performed. Permanent damage to the bone marrow was corrected by curettage of irradiated tissues and replacement with normal bone marrow. A new enzymatic abnormality of red blood cells was studied. Quantitative studies of splenic function were performed. Collaborative efforts to establish internationally accepted standards for hematology were continued.

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RESEARCH AND TECHNOLOGY RESUME

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25. (U) APPROACH- A. IN VITRO NERVE STIMULATION PREPARATION USING DIFFERENTIAL BLOCKADE METHODS. B. EVALUATION OF INTESTINAL CONTRACTILE REACTIVITY IN ANIMALS SUBJECTED TO DIARRHEAL INFECTIONS. C. VASCULAR BED PERFUSION WITH LATEX RUBBER. D. CHEMICAL ANALYSES OF INTESTINAL SPECIMENS FROM MAN AND ANIMALS TO MEASURE DISACCHARIDASE ACTIVITY LEVELS. E. ABOVE ANALYSIS USING A FECAL SAMPLE INSTEAD OF BIOPSY. F. TOTAL SMALL BOWEL PERFUSION WITH A BALANCED ELECTROLYTE SOLUTION.

(U) PROGRESS - JUL 67 THRU JUN 68 A. WORK CONTINUES REGARDING ADRENERGIC INNERVATION OF THE INTESTINE. ADRENERGIC INNERVATION OF INTESTINAL SMOOTH MUSCLE IS MEDIATED PREDOMINANTLY THROUGH BETA RECEPTORS WHICH APPEAR TO BE FUNCTIONALLY ASSOCIATED WITH AUERBACHS PLEXUS. B. SALMONELLA INFECTIONS ASSOCIATED WITH INCREASED SENSITIVITY TO ACETYLCHOLINE AND DEPRESSION OF ALPHA AND BETA ADRENERGIC RECEPTORS. C. PARA-DIMETHYLAMINOAZOBENZENE INDUCES ABNORMAL PROLIFERATION OF PORTAL CELLS, LIVER DAMAGE, TUMORS AND CIRRHOSIS WITH MARKEDLY ALTERED MICROVASCULAR PATTERNS. MICROCIRCULATION OF HUMAN INTESTINE IS BEING STUDIED AS MATERIAL BECOMES AVAILABLE. D. LACTASE LEVELS IN THAI NATIVES IS LOW, AND CANNOT BE STIMULATED BY FEEDING LACTOSE. E. WORK CONTINUES IN THE STUDY OF FECAL ENZYME ACTIVITY. F. SALT AND WATER ABSORPTION IS DECREASED IN THE ILEUM OF ANIMALS WITH SALMONELLA INFECTIONS . FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 30 JUNE 1968.

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Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 087, Gastrointestinal disease

Investigators.
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Description.

The Mission of the Department of Gastroenterology is centered on the basic investigation of the pathophysiology of diarrheal diseases. Studies in progress include intestinal water and salt transport, neural control of motility and its pharmacological control, the intestinal disaccharidases, gastric and intestinal vascular architecture. The response of the liver to chemical trauma is also under investigation.

1. Pathophysiology of Diarrheal Disease.

Description.

Several investigators in this department have embarked upon an integrated study of the pathophysiology of diarrheal disease. The areas of investigation being pursued include studies of intestinal transport of water, electrolytes and glucose in diarrheal conditions, intestinal enzyme activity in malabsorption and diarrheal conditions, intestinal smooth muscle reactivity during diarrheal conditions, and the microvasculature of the intestinal mucosa. Activities during the past year have been mostly concerned with gathering control information in the above areas plus standardizing animal models of diarrheal disease. Actual studies of intestinal pathophysiology in Salmonella typhimurium infection have been completed in several of these areas.

Progress.

A. Intestinal Smooth Muscle Reactivity and Neuropharmacology.

The receptor mechanisms involved with the adrenergic innervation of intestinal smooth muscle have not been resolved. The effects of differential receptor site blockade techniques on periarterial nerve stimulated inhibition of the intestine have been studied in this department. The results clearly indicate that the adrenergic nerve supply in the rabbit is distrubuted to beta receptor mechanisms. In

light of recent histochemical evidence that the intestinal adrenergic nerves terminate in the region of Auerbach's plexus, the study was extended to gather functional information on this possibility. A series of experiments was performed in which mecamylamine was used to achieve blockade of Auerbach's plexus. The results showed a definite attenuation of beta receptor site action with no loss of alpha activity, and a decreased nerve stimulated inhibition of rhythmic contractions. This observation indicates that sympathetic nerve inhibition of the intestine is mediated via beta receptors which are, in some way, functionally associated with the neuronal elements of Auerbach's plexus. The functional implication of the study is that the sympathetic nervous system functions to adjust the excitability of the intestinal contractile mass by buffering the excitability of the cholinergic post ganglionic parasympathetic neurons. These data have been submitted for publication.

The actions of many bacterial agents or toxins on the gastrointestinal system are strongly influenced by the functional states of the innervation and the adrenergic control mechanisms. A study is underway, in collaboration with the Department of Applied Immunology, to evaluate the effect of Salmonella typhimurium infection on the fundamental properties of intestinal smooth muscle excitability and pharmacological reactivity. In a pilot study of a group of infected rats, changes in both cholinergic and adrenergic responses were detected. The intestinal sensitivity to cholinergic stimuli was increased, whereas the inhibitory effects of alpha and beta stimulation were decreased. Since the guinea pig terminal ileum possesses excitatory alpha and inhibitory beta adrenergic receptors, as described in this laboratory last year, this animal has been adopted as our model to elucidate the effects of Salmonella infection on pharmacological responses. In an in vitro study of guinea pig ileum subjected to acute Salmonella enteritis, it was observed that there is an increased sensitivity to acetylcholine, a marked diminution of beta inhibitory responses and essentially complete loss of alpha activity. In many instances the alpha response was actually reversed to become inhibitory in nature. In addition, the ileal segments demonstrated spontaneous, large amplitude contractions which occurred rhythmically. These latter contractions may be a result of the loss of beta receptor inhibitory influence on the ganglionic cells of Auerbach's plexus. This study is being extended to characterize intestinal cholinergic and adrenergic responses in long term chronic Salmonella infections and early phases of the acute infection.

A study is being conducted in collaboration with The Department of Pharmacology, Division of Medicinal Chemistry, on a radiation protection agent, WR-2823, which has secondary action as an alpha adrenergic receptor blocking agent. WR-2823 and its thiol derivative, WR-1729, have been tested in several animal species by an in vitro analysis of the effects of the agents on intestinal and aortic strips. Tissues from animals pretreated with the agents demonstrate blockade of aortic alpha receptors and no loss of intestinal alpha activity. The addition of the compounds to an organ bath containing nontreated tissues also results in alpha blockade of the aorta with no effect on the intestine. It is only after exposure to repeated high doses of WR-1729 in the tissue chamber that there occurs attenuation of intestinal alpha receptors. The rate of onset and effectiveness of blockade with the thiol derivative is

greater than with the parent compound, suggesting that WR-2823 is metabolically dephosphorylated *in vivo* to yield the active form of the agent. This material is being prepared for publication.

Preliminary experiments are underway to establish two new protocols in this segment of the departmental mission. The first entails an *in vitro* and *in vivo* analysis of the interactions between intestinal longitudinal and circular smooth muscle layers. These procedures, when perfected, will be employed in determining the mechanisms involved in adrenergic inhibition of longitudinal and circular muscle layers; the neurological and pharmacological basis of reflex interactions of the muscle layers; and the effect of various diarrheal agents on the interactions between the layers. Early results indicate that there is an inhibitory effect on longitudinal muscle resulting from circular muscle contraction and that this response is independent of neuronal elements.

The second new protocol involves the characterization of intestinal mucosal reflex mechanisms and their associated mucosal receptor mechanisms. An intestinal perfusion procedure has been developed to test the interactions between luminal distension and the action of mucosal 5-hydroxytryptamine receptors. These studies are being done in collaboration with The Division of Biochemistry. The procedure is to be applied immediately to an evaluation of mucosal reflexes in the human. To this end, liaison has been established with The Gastroenterology Service, WRGH, and procedures for measuring human intestinal motility developed. An initial trial of dual perfusion/fluid sampling and motility recording is scheduled.

B. Microcirculation of the Gastrointestinal System.

A study of the normal microvascular architecture of the monkey (*Maccaca mullata*) small intestinal mucosa has been published. With the aid of a silicone rubber injection procedure, the following observations were made: The arterial supply to the mucosa is distributed to the undersurface of the mucosa and runs directly into the capillary plexus surrounding the crypts of Lieberkuhns. The villus blood supply is derived directly from the cryptic plexus by an extension of capillary channels that lead into the villus to form its capillary net. Venous return of the villus is accomplished through a single, centrally located venous channel that extends through the villus to join the mucosal and eventually submucosal venous return vessels. There is a second set of venous channels that extend directly from the cryptic plexus to mucosal veins. It was proposed that these secondary venous channels represent a mechanism to regulate the distribution of blood in the mucosa of the gut. In such a mechanism, regulation of the state of constriction of the secondary veins would possibly adjust the blood flow through the villus capillary net. A paper describing the technique of using silicone rubber for microvascular studies, tissue processing and microscopic photography has been published in collaboration with Mr. John E. McClain, Photomicrographic Section, Medical Audio Visual Branch.

C. Intestinal Transport of Water, Electrolytes and Glucose in Diarrheal Conditions.

In order to study the alterations in the intestinal transport of water and solute caused by infectious diarrhea, experimental systems and techniques of analysis have been devised and perfected. Polyethylene Glycol (PEG) is a commonly used nonabsorbable reference substance for measuring net water changes in intestinal absorption studies. This laboratory has modified the turbidimetric analysis of PEG by use of a vegetable gum (gum arabic) as an emulsifying agent which has resulted in a simpler, more accurate, and reproducible chemical determination. This method has been published.

Although PEG has been fairly extensively used in intestinal absorption studies, there are no data in the literature regarding what possible effect this substance may have on the absorption of water or other solutes. In collaboration with Dr. Melvin Small, Dept of Gastroenterology, D. C. General Hospital, the effect of graded amounts of PEG on D-Xylose absorption has been studied using a modified Cori technique in rats. No effects on D-Xylose absorption could be demonstrated with concentration of PEG up to 32.5%, verifying the usefulness of PEG as a nonabsorbable water marker in intestinal absorption studies. The details of this study are currently being prepared for publication. A small animal intestinal perfusion technique has been perfected and intestinal water, electrolyte, and glucose transport have been studied in jejunum, ileum, cecum and colon of rats and guinea pigs in order to find a suitable animal model in which to study diarrhea. These studies of normal intestinal transport have afforded data for the following two comminications. 1) The relationship between water and solute transport at various levels of rat intestine has been examined in detail. It was found that fluid is transported isotonically with respect to the osmotic activity of the mucosal solution from the jejunum, but the transported fluid becomes increasingly hypertonic in ileum and colon. Furthermore, net fluid transport was, in general, zero when net solute movement was zero. These findings probably reflect the decreasing membrane permeability (i.e.,effective pore radius) as one moves distally in the intestine and lends support to the theory of a passive mechanism of water transport by the gut. This report is currently in press. 2) Examination of the patterns of water, electrolyte, and glucose transport by the different segments of rat and guinea pig intestine has revealed significant species differences. The use of a glucose containing perfusion solution results in a 2-3 fold increase in water absorption and a 1 1/2 fold increase in sodium absorption from rat small bowel. By contrast, in guinea pig small intestine, a significant secretion of H_2O and $NaHCO_3$ occurs during intestinal perfusion. The guinea pig may serve as a useful model for the study of the intestinal secretory phenomena which have been suggested to occur in several pathological conditions in man. An abstract of these experiments has been published and a full report has been tentatively accepted for publication.

In conjunction with Dr. Samuel B. Formal, Department of Applied Immunology, and Dr. Ronald Maenza, Department of Experimental Pathology, Walter Reed Army Institute of Research, a model of Salmonella enterocolitis (S. typhimurium) in the white rat has been devised. An abstract describing this disease model has been published, and a full report is in preparation.

Water, electrolyte, and glucose transport were studied in the jejunum, ileum and large intestine in rats with Salmonella ileocolitis. In animals with ileocolitis, but no diarrhea, a diminution of water and solute transport was apparent at all three intestinal levels. In animals with ileocolitis and severe diarrhea, transport at the jejunal and large intestinal levels was decreased, but to a no greater extent than in the previous group. However, in these animals with diarrhea there was, in all, secretion of water and electrolytes into the ileum. Thus, ileal secretion appears to be a major physiological determinant of diarrhea in this disease model. An abstract of this study has been published and a full report is in preparation.

Future work will be concerned with elucidating the mechanism of ileal secretion in this experimental diarrheal condition. This will involve study of bidirectional fluxes of electrolytes by use of radioactive tracers, and determination of transmembrane electrical potential differences. These studies will be performed in vivo and in vitro in this and other models.

D. Intestinal Enzymes.

The adult population in Thailand exhibits an intolerance to lactose correlating with a very low level of lactose splitting enzyme in their intestinal mucosa. Feeding lactose did not stimulate an adaptive enzyme response. The lactose intolerance appears to develop at about 2 years of age following a period of milk utilization from birth.

There are apparent enviromental effects on intestinal enzyme systems. In cooperation with the U. S. Component, SEATO Medical Laboratory, Bangkok, disaccharidase activities on biopsy from the small bowel are being determined on Peace Corps workers upon their arrival in Bangkok and again after 1 to 2 years in this environment.

Certain correlations between mucosal and fecal enzyme activities have been studied. Fecal material from germ-free mice show considerable disaccharidase activities. Since there are no bacteria present in these specimens, the activities must be due to either the sloughed off mucosal epithelial cells or enzymes secreted into the intestinal lumen. Preliminary studies on conventional mice to note the effect of fecal bacteria on the fecal disaccharidase activities have given results equal to or slightly less than that of the germ-free animals. Correlation studies on the disaccharidase activities of human stools and their paired intestinal biopsy continue.

2. Experiments with Liver Damage.

Description.

As the major metabolic organ of the body, the liver is vulnerable to injuries through a wide spectrum of nutritional and metabolic disturbances, and through a wide variety of toxic and infectious diseases. These problems can become acute whenever the situation which brings about strenuous environmental conditions in a large mass of people arises. In this respect, liver injuries from nutritional, toxic and infectious causes always constitute one of the major problems in military medicine.

With the great regenerative capacity of the hepatic organ, acute liver injury heals relatively easily with removal of causative factors and, in clinical cases, with proper medical treatment. When injurious effects on the liver persist for a prolonged period of time, however, the pathogenetic mechanism which cause permanent liver damage, cirrhosis, is initiated. The main aim of this project is to study experimentally the pathogenesis of cirrhosis; that is, why and how an acute liver injury progresses to a permanent, irreversible liver damage.

Progress.

The first experimental model adopted for the project was the liver damage caused by carbon tetrachloride poisoning in rats. A substantial part of the work was already described in the last progress report. This project was completed during this report period, and the detail of the work was published in two parts in the American Journal of Pathology. In these studies, the pathogenesis of carbon tetrachloride induced liver damage, from the acute state to the cirrhotic stage, is explained, delineating four stages of the cirrhotic course.: (1) diffuse destruction of centrilobular microvasculature; (2) hepatic venous regeneration along the circulatory periphery of sinusoids; (3) lobular dissection by regenerating hepatic venous radicles with formation of portahepatic venous shunts; and (4) sclerotic change of mesenchymal tissue with formation of cirrhotic scarring.

The second experimental model used was liver damage caused by butter yellow (p-dimethylaminoazobenzene, DAB) feeding in rats. The experimental phase of this work was completed, and a paper dealing with the results is being prepared for publication. The results of this work showed that, in butter yellow feeding, the distortion of hepatic microarchitecture and the development of cirrhosis occur because of abnormal proliferation of hepatic cells in the periportal areas. Thus, the hepatic lesion in butter yellow feeding develops differently from that in carbon tetrachloride poisoning. This pathogenetic difference of experimental cirrhosis between carbon tetrachloride poisoning and butter yellow feeding seems to be particularly interesting in analyzing the pathogenicity of different types of human cirrhosis.

Studies on liver injuries caused by other hepatotoxins (phosphorus, allyl formate, etc.) and nutritional deficiencies (choline deficiency, protein deficiency, ethionine feeding, etc.) are still in early phases of development. Through the accumulation of these data, this project will assist the understanding of the pathogenetic mechanisms in which a variety of acute liver injuries progresses to chronic, irreversible liver damages.

Summary and Conclusions.

Several areas of normal and pathologic intestinal physiology have been investigated which are pertinent to the understanding of altered mechanisms involved in diarrheal diseases. They include the studies concerned with neuromuscular mechanisms of the intestine: 1) The localization of the intestinal beta adrenergic receptors in gangalia; 2) The findings suggesting that the beta rather than alpha adrengic receptors are innervated, and 3) The finding that Salmonella infections are associated with intestinal hypersensitivity to acetylcholine, and depression of alpha and beta adrengenic receptor reactivity. Studies concerned with salt and water transport in the normal rat and guinea pig; and the depression of salt and water absorption in Salmonella infections with reversal of the direction of bicarbonate passage. Concepts regarding the intestinal microvasculature have been altered significantly due to the studies described herein.

A survey of the small intestinal disaccharidase activities of normal Thais revealed low levels of lactase activity along with reduced lactose absorption. The activities of these enzyme systems were drastically reduced in monkeys subjected to shigella infections. Studies are in progress to describe the distribution of these enzyme systems in the intestinal tract of several animal species as well as an evaluation of enzyme stability during sampling, handling and storage procedures.

Studies of the pathogenesis of carbon tetrachloride intoxication of the liver demonstrated destruction of centrilobular microvasculature with hepatic venous regeneration along the circulatory periphery of the sinusoids and sclerotic changes of mesenchymal tissue. In studies in which changes were induced with p-dimethylaminoazobenzene the changes include abnormal proliferation of hepatic cells in the periportal areas.

Publications.

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4. Powell, D. W., G. R. Plotkin, R. M. Maenza, and S. B. Formal, Experimental diarrhea: Intestinal electrolyte and water transport in rat Salmonella enterocolitis. Clin. Res. 16: 290, 1968. (Abs.).

5. Powell, D. W., S. J. Malawer and G. R. Plotkin. Intestinal water and electrolyte transport in the guinea pig in vivo. Gastro. 54: 1300, 1968. (Abs.).

(U) MILITARY NURSING

3003500 CLINICAL MEDICINE

13. START DATE	14. EST. COMPL. DATE	15. FUNDING AGENCY	
03 59	NA	OTHER DA	
PRIOR FY 68	1	20	
CURRENT FY 69	1	20	

9. PERFORMANCE METHOD	C. IN-HOUSE
10. CONTRACT/GRANT	NA
b. NUMBER	NA
c. TYPE	NA
d. AMOUNT	NA
e. DATE	NA

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21. TECHNOLOGY UTILIZATION: NURSING RESEARCH
NURSING EDUCATION

22. COORDINATION: NA

23. KEYWORDS: MILITARY NURSING, MALARIA, SPONGING, BLOOD PRESSURE.

(U) TECH OBJECTIVE - TO DEVELOP RATIONALE UNDERLYING MILITARY NURSING PRACTICE BY IDENTIFYING AND TESTING PRINCIPLES OF NURSING IN MILITARY SETTING, IDENTIFYING NURSING ROLE AND FUNCTION IN CONTINUITY OF CARE OF MILITARY PATIENTS AND FAMILIES, DESCRIBING NURSING CARE OF PATIENTS WITH FALCIPARUM MALARIA AND PATIENT RESPONSES TO NURSING CARE, EXAMINING EFFECTIVENESS OF SPONGING TECHNICS TO REDUCE FEVER, EXAMINING AUTO-OXIDATION AND PHOTOXIDATION OF CHLORPROMAZINE COMPOUNDS ADMINISTERED BY NURSES, VALIDATING MANOMETER READINGS BY NURSES TAKING BLOOD PRESSURES, MEASURING ENERGY COST OF SELECTED PATIENT AND NURSE ACTIVITIES, AND SURVEYING GRADUATE ASSIGNMENTS FOLLOWING COMPLETION OF MILITARY NURSING PRACTICE AND RESEARCH COURSE.

(U) APPROACH- IDENTIFICATION OF NURSING ROLE IN CONTINUITY THROUGH ANALYSIS OF QUESTIONNAIRE SURVEY, DESCRIPTION OF HISTORY OF FALCIPARUM MALARIA NURSING CARE AND IMPLICATIONS, STUDY OF NURSING MEASURES TO REDUCE FEVER, DETERMINATION OF OXIDATIVE DETERIORATION OF LIQUID CHLORPROMAZINE IN COMMON DILUENTS UNDER VARIOUS LIGHT INTENSITIES, PASS COLLECTION OF BLOOD PRESSURE READINGS FROM FILM WITH SYNCHRONIZED SOUND TAPES OF SPHYGMOMANOMETER MERCURY EXCURSIONS, UTILIZATION OF OPEN CIRCUIT, INDIRECT CALORIMETRY TO CALCULATE ENERGY EXPENDITURE, AND SURVEY OF 201 FILES OF GRADUATES OF RESEARCH COURSE.

(U) PROGRESS - JUL 67 THRU JUN 68 FINAL ANALYSIS OF CONCEPTS OF CONTINUITY OF CARE TO SHOW DIFFERENCES IN PERCEPTIONS OF ARMY HEALTH, ADMINISTRATION, AND HOSPITAL CARE NURSES, COMPLETION OF ANALYSIS OF 50 CASE REPORTS OF PATIENTS WITH FALCIPARUM MALARIA, PRELIMINARY EVALUATION OF EXPLORATORY DATA ON METHODS FOR FEVER REDUCTION, OCCURRENCE OF LITTLE OR NO PHOTOXIDATION OF CHLORPROMAZINE AT ACID PH LEVELS REGARDLESS OF LIGHT INTENSITY AND THE OPPOSITE AT ALKALINE PH VALUES, COLLECTION OF MORE THAN 200 BLOOD PRESSURE READINGS FOR ANALYSIS OF ACCURACY OF READINGS CORRELATED WITH SELECTED PERSONAL DATA, CONCLUSION OF PILOT TESTING AND CALIBRATION OF BASIC INSTRUMENTATION FOR MEASURING ENERGY COSTS, AND IDENTIFICATION OF KEY ASSIGNMENTS AND SPECIALIZED CRITERIA OF SUCCESS FOR EACH GRADUATE. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

31. KIND OF RESOURCES APPLIED: A. PROFESSIONAL B. SUPPORT	32.	33. KIND CODE: EP	34. BUDGET CODE: 1
35. HUMAN SUBJECTS: NA		36. PARTICIPATION: NA	
37. LITERATURE SEARCHES	38. SPECIAL EQUIPMENT		
39.	40.		

DD FORM 1498 PREVIOUS EDITION OF 1 JUN 67 WHICH MAY BE USED (Supersedes DA Form 1222)

Project 3A014501 B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing

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LTC Glennadee A. Nichols, ANC
MAJ Rosemary T. McCarthy, ANC
MAJ Beverly A. K. Glor, ANC
CPT Zane E. Estes, ANC

Description.

Investigations in Military Nursing are planned to explore various aspects of nursing practice in the military. The overall aim of research under this work unit is to identify and test principles underlying nursing care and to recognize differences in military nursing from civilian practice. Nine nursing studies are reported.

Clinical manifestations and nursing care requirements were described after observation in the Republic of Vietnam of fifty patients with falciparum marlaria. This study will serve as a basis for identifying areas for future study.

When fever is present in disease, sponging to reduce fever is frequently indicated and it is the nurse's responsibility to choose an appropriate method for the desired effect. Selected techniques for fever reduction were compared in nine patients with falciparum malaria for their effect on body temperature, pulse rate, respiration rate, and blood pressure measurement.

Previous studies of the reproducibility of blood pressure have indicated a need for further exploration of similarity of readings between graduate nurses, nursing students, and nursing attendants. This phase has been completed and is being reported in the open literature.

Exploration of the validity of the recordings made by observers is under current study. Film sequences, electronically synchronized with sound of the mercury manometer, showing the effect on mercury during the blood pressure measurement process were made for normo-, hypo-, and hyper-tensive subjects. These films were displayed with a model arm, blood pressure cuff, and stethoscope to be used in mass collection of data. Each subject was given two blood pressures to record; the second reading was the only one used for analysis.

Chlorpromazine hydrochloride is a popularly used tranquilizing drug which may auto-oxidize in the presence of light. In order to define margins of safety during periods of handling prior to actual administration to the patients, a study was designed to evaluate the rapidity of auto-oxidation and the intensity of light required. This study is currently being prepared for publication.

Observation and evaluation of decubitus ulcers in patients returning from Vietnam have been made. This concludes the study concerned with identification of the pathology in this type of nursing care problem.

Continuity of care, as defined by administrative nurses, health nurses, and hospital care nurses has been analyzed. Differences in perception will be compared in relation to age of nurse, year of graduation from school of nursing, public health education, and experience.

The Army Nurse Corps sponsors a graduate level educational program in basic sciences, application to nursing practice, communication skills, and research methodology called the Military Nursing Practice and Research Course. The graduates of this program are 25 in number. The military assignments of these ANC officers, from entry on active duty until present, have been surveyed in order to observe career patterns.

Classic methods of indirect colorimetry will be used to evaluate the energy cost of selected patient and nurse activities. This study is an extension of work done by the investigator at a civilian university.

Progress.

The observations of falciparum malaria care made in Vietnam are now in press. Exploration of the original data has indicated differences in symptomatology between those patients who had no relapses and those who did. Follow-up letters to the original fifty patients and further study of greater numbers of malarial cases will be used to develop, if possible, a series of symptoms which could be used as a predictor of future relapse.

Two techniques for fever reduction were compared. One was the use of ice bags to groin and axilla, and the other was application of cold packs to the entire body. Results of this comparison show a statistically significant difference in reduction of body temperature but in no other physical parameter under study. It is anticipated that this research will be extended to include these and other fever reduction methods on a larger sample of cases.

The findings of reliability of blood pressure measurement were consistent with all other such studies; i.e., there is no difference between observers if they are using the same procedure and equipment. In the validity studies, blood pressure readings were made and recorded by more than a thousand persons attending the American Nurses' Convention this year. Of this number of subjects, about 800 readings were made by registered nurses and are currently being coded for automatic data processing and analysis.

The study of auto-oxidation of chlorpromazine hydrochloride showed only tap water caused oxidation to any appreciable extent and that the prime factor was the neutrality or alkalinity of the diluent.

Five orthopedic patients with decubitus ulcers who were injured in Vietnam were observed during this fiscal year. Their response to care will be added to the complete census of decubitus ulcer patients and will be reported in the open literature.

The perception of administrative, health, and hospital care nurses in relation to continuity of care has been analyzed as follows:

Differences in Concepts of Continuity by Three Groups of Army Nurse Corps Officers

Selected Concepts	Army Health Nurses Percent	Nurse Administrators Percent	Hospital Care Nurses Percent
Total Patient Care	35	32	32
Team Approach to Care	41	31	28
Uninterrupted Care	34	31	35
Prevention	20	34	46
Functions of Nursing	27	50	23
Family Involvement	49	34	17
Military Concern	33	44	23

Thirty-one male and female Army nurses have begun the Military Nursing Practice and Research Course. One class was curtailed due to exigencies of the war in Vietnam. One student withdrew from the course before completion.

Table I shows the educational advances of the remaining 23 graduates since completing the course:

Table I Degrees of Graduates of MIP&R Course

	Upon Entering MIP&R Course Degree	BS	MS	Total
Since Graduation	BS	7	-	7
	MS	4*	13	17
	DR	0	1*	1
TOTAL		11	14	25

*Now in full time schooling for this degree.

Table II shows the number of key assignments which required individual consideration by the assignment branch, such as instructor, career counselor, chief nurse, and research assignments.

Table II Key Assignments of Graduates of the MIP&R Course

	Degree at Survey	BS		MS***	
Age at Survey		35*	35**	35	35
Key Assignments	Prior to Course	2	0	0	2
	Since Course	0	1	0	0
	Both Prior & Since	0	1	0	14
	Never	3	1	0	1
TOTAL		5	3	0	17

* All with less than 10 years of service.
** All with 10 or more years of service.
***Individuals spread over many years of service.

Instrumentation for the energy cost study has been obtained and calibrated. Subjects and activities are being selected.

Summary and Conclusions.

The nine studies being conducted related directly or indirectly to the nursing care needs of the military patient. The nursing observations of the care of patients with falciparum malaria will be a guide to patient care expectations. Fever reducing techniques, when more data are obtained, will provide guidelines for all levels of medical service. Reliability and validity evaluations of blood pressure measurements will provide information leading to more accurate and reproducible readings in patient care areas. Auto-oxidation has been found to be of a lesser factor in the administration of chlorpromazine hydrochloride than heretofore considered. A summary of decubitus ulcer nursing care is anticipated for the future. Health nurses, administrator nurses, and hospital care nurses have similar perceptions of continiuty of care which suggest that further study might prove helpful. More time is needed to evaluate the effect of the Military Nursing Practice and Research Course in the career development of Army Nurse Corps Officers. Energy cost studies will provide knowledge of energy needs of patients during different activities.

Publications.

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6. Nichols, Glennadee A. "Sterile Shelf-Life Prolonged by Use of Plastic Outer-Wrap." Hospital Topics, D-63-64, February 1968.

7. Ibid "Measurements of Oral Temperature in Children." Journal of Pediatrics, 72: 253-255, February 1968.

8. Nichols, Glennadee A. and Verhonick, Phyllis J. "Placement Times for Oral Thermometers: A Nursing Study Replication." Nursing Research, 17: 159-161, March-April 1968.

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 047464	CSGRD-193

4. PERIOD OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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10. CURRENT NUMBER/CODE
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10A. PREVIOUS NO./CODE

11. TITLE
(U) BODY FLUID AND SOLUTE AND RENAL HOMEOSTASIS

12. SCIENTIFIC OR TECH. AREA		13. START DATE	14. CRIT. COMPLETION	15. FUNDING AGENCY
012900 PHYSIOLOGY	006900 CLINICAL MEDICINE 010200 STRESS PHYSIOLOGY	09 54	NA	OTHER DA

16. RESOURCES EST.	a. PROFESSIONAL MAN YEARS	b. FUNDS (THOUSANDS)
FISCAL YR 69	2	70
FISCAL YR 69	2	70

16. RESEARCH METHOD	17. CONTRACT/GRANT	a. DATE	b. NUMBER	c. TYPE	d. AMOUNT
C. IN-HOUSE		NA	NA	NA	NA

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20. TECHNOLOGY UTILIZATION
NA

22. COORDINATION
NA

23. KEYWORDS
PERITONEAL DIALYSIS, ACID-BASE.

24.
(U) TECH OBJECTIVE - TO INVESTIGATE THE RENAL AND RELATED MECHANISMS FOR MAINTAINING BODY FLUID AND ELECTROLYTE HOMEOSTASIS IN RESPONSE TO DISEASE, INJURY, AND ENVIRONMENTAL STRESS, TO ELUCIDATE THE PATHOGENESIS OF ACUTE RENAL FAILURE, TO DEVELOP AND IMPROVE METHODS FOR PREVENTION AND TREATMENT OF ALTERED FLUID AND SOLUTE HOMEOSTASIS AND ACUTE AND CHRONIC RENAL FAILURE.

(U) APPROACH- CONVENTIONAL CLEARANCE TECHNIQUES, EXTERNALLY MONITORED ISOTOPE TECHNIQUES, ISOTOPE DILUTION, EXPERIMENTAL MODELS OF ACUTE RENAL FAILURE, IN VIVO RENAL TUBULE MICROPUNCTURE, IN VITRO RENAL TUBULE MICROPERFUSION, MEMBRANE TRANSPORT, LIGHT AND ELECTRON MICROSCOPY.

(U) PROGRESS - JUL 67 THRU JUN 68 STUDIES DEMONSTRATING THE LACK OF A ROLE FOR THE RENAL CAPSULE IN MODULATING THE NATRIURETIC RESPONSE TO VOLUME EXPANSION WERE COMPLETED. STUDIES CONTINUED RELATING THE MAGNITUDE OF NATRIURETIC RESPONSE FOLLOWING INFUSIONS TO THE MAGNITUDE OF CHANGE IN BODY FLUID COMPARTMENTS. NATRIURESIS BEST CORRELATED WITH CHANGE IN INTERSTITIAL FLUID VOLUME, BUT RESPONSE WAS MODULATED BY CHANGES IN PLASMA SODIUM CONCENTRATION. EXTERNALLY MONITORED INFUSION METHODS FOR ESTIMATING RENAL BLOOD FLOW AND GLOMERULAR FILTRATION RATE WERE DEVELOPED AND FOUND TO BE SUFFICIENTLY PROMISING TO UNDERTAKE PRELIMINARY STUDIES IN PATIENTS. EXTENSIVE STUDIES INVESTIGATING POSSIBLE SUITABLE TAGGED BLOOD FLOW MARKERS OTHER THAN PAH WERE COMPLETED. STUDIES IN THE METHEMOGLOBIN FERROCYANIDE MODEL OF ACUTE RENAL FAILURE ESTABLISHED THE MICROSCOPIC PICTURE OF THE LESION AND QUANTIFIED THE EXTENT AND COURSE OF CAST FORMATION AND DISAPPEARANCE OF VISIBLE GLOMERULI FROM THE CORTEX. MICROPUNCTURE STUDIES OF INTRATUBULE PRESSURE AFTER INDUCTION OF ACUTE RENAL FAILURE SHOWED A DECREASE IN PRESSURE, COMPARABLE CHANGES LATE AFTER URETERAL OBSTRUCTION SUGGESTED THE POSSIBILITY OF AN INTRARENAL PRESSURE-REGULATED FEEDBACK MECHANISM MODULATING RENAL VASCULAR RESPONSES AND/OR TUBULE RESORPTION. ELECTRON MICROSCOPY STUDIES OF IN VIVO MICROPUNCTURE PERFUSED TUBULES WERE UNDERTAKEN, NORMAL HISTOLOGY WAS DEFINED, HISTOCHEMICAL STUDIES TO LOCALIZE SODIUM TRANSPORT SITE AND EVALUATE EFFECT OF TRANSPORT BLOCKING AGENTS BEGUN. COMPLEX EQUIPMENT FOR PERFORMING IN VITRO TRANSPORT STUDIES IN ISOLATED PERFUSED RENAL TUBULES HAS ACQUIRED. INITIAL EXPERIENCE WITH MICRODISSECTION OF TUBULE SEGMENTS AND MOUNTING ON MICROPIPETTES GAINED. STUDIES OF HYDROGEN ION SECRETION BY THIS PREPARATION BEGUN. STUDIES OF URINE ACIDIFICATION IN MICE SHOWED A DIRECT RELATIONSHIP BETWEEN FLOW RATE AND PH, AMMONIUM AND BICARBONATE EXCRETION, WITHOUT CHANGE IN NET ACID, PHOSPHATE, CREATININE, ORGANIC ACID AND FREE AMINO ACID EXCRETION. STUDIES OF KINETICS OF FLUID AND SOLUTE FLOW ACROSS ARTIFICIAL DIALYTIC ACID PERITONEAL MEMBRANES WERE UNDERTAKEN, THEORETICAL CONSIDERATION REGARDING BULK FLOW, SOLVENT DRAG, AND ULTRAFILTRATION ARE GIVING BODY INITIAL DATA OBTAINED FROM PATIENTS.

25. CLASSIFICATION REQUIRED	26.	27. OSD CODE	28. BUDGET CODE
		AR	1

29. SPON OR OBJECTIVE	30. PARTICIPATION
14354	NA

31. KEYWORDS AGENCY	32. SPECIAL EQUIPMENT
33. KEYWORDS CONTINUED	34.

DA FORM 1498 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 23 identical to DA Form 1023)

Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 089, Body Fluid and Solute and Renal Homeostasis

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	CPT Edward Regal, MC**
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	John Gagnon
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Description.

Human and animal studies have been directed at evaluating the homeostatic mechanisms involved in the maintenance of body fluid and electrolyte homeostasis under normal physiologic conditions and in response to disease and injury stress. Renal function and adaptive mechanisms and interrelationships with other body homeostatic mechanisms have been emphasized because of the central role the kidney plays in overall fluid and electrolyte homeostasis, and the relative ease of study of this organ by a variety of techniques. Where feasible, light microscopic and ultrastructure changes have been correlated with physiologic alteration. Specific studies have been undertaken to evaluate: 1) control and influences of body fluid volume and toxicity; 2) sodium and water handling by normal and diseased kidneys; 3) measurement of renal function in normal and disease and injury states; 4) acid-base homeostasis including renal mechanisms; 5) effect of heat-exercise stress on body homeostasis; 6) pathophysiology and prevention of acute renal failure; 7) mechanisms of fluid and solute flow across dialytic and other membranes.

*Fellows
**Assigned WRGH

Progress.

1. The control and influence of body fluid volume and toxicity:

Previous studies have suggested that the reabsorption of sodium by the renal tubule is influenced by extra-renal factors other than changing levels of known hormones or by changes in glomerular filtration rate. The relation of body fluid compartment alterations to sodium excretion has been investigated in this regard. Solutions of varying colloid and osmolar composition have been infused in dogs to give constant amounts of sodium chloride, and the measured changes in body fluid compartments correlated with the extent of natriuresis. Control studies in which no volume expansion has been interposed between repeat studies of body fluid spaces have indicated the feasibility and reliability of the isotope dilution methods used for repeated acute studies. Infusion of hypooncotic saline solutions as hypo-, iso-, or hypertonic solutions demonstrated natriuresis to correlate best with the degree of interstitial fluid volume expansion. Hypo- and hypernatremia were found to modify significantly the degree of natriuresis. Studies have recently been completed in which the effect of isooncotic saline solutions infused in a similar manner on body fluid spaces and resultant natriuresis were assessed. More marked increases in all body fluid compartments occurred in the normal saline group and natriuresis was at least as marked as in the hypooncotic isotonic group. Studies are currently being completed in which hyperoncotic solutions are infused in an effort to dissociate the changes in plasma volume and interstitial fluid volume in a further attempt to distinguish the effect of changes in these fluid volumes on natriuresis.

Studies of the effect of the renal capsule on the natriuretic response to saline infusion have been completed. Both acute and chronic decapsulation of the kidney were found to have no effect on the response of the kidney to volume expansion. This may be due to lack of effect of the capsule on intrarenal pressure or failure of any such effect to alter the renal response to ECF expansion.

Preliminary studies have demonstrated the feasibility of studying the effect of left atrial stretching on proximal tubule sodium reabsorption by micropuncture techniques. Transthoracic placement of ligatures into the left atrium has been accomplished while maintaining physiologic stability in the dog. Gross clearances have demonstrated no change in net natriuresis of urine flow by this maneuver. Changes in proximal readsorption without change in net natriuresis may yet be demonstrable. Micropuncture studies have been deferred pending interpretation of results from other laboratories suggesting that total denervation of the heart does not alter the natriuretic response to volume expansion.

2. Sodium and water handling by normal and diseased kidneys:

Study of sodium-conversing capacity and concentrating and diluting ability of patients with chronic renal disease was concluded with the

addition of several patients to the study. Patients with chronic glomerulonephritis, chronic pyelonephritis, and polycystic disease were studied on normal and low sodium intakes. Metabolic balance studies demonstrated a delay in sodium-conserving capacity and sub-maximal response to salt deprivation. Aldosterone excretory rates were within the normal range. On salt restriction, net free water clearance regularly resulted in all patients; maximum achievable urinary osmolality always decreased but dilating ability improved as indicated by lower minimum achievable urine osmolality. Studies using aldosterone-blocking drugs and exogenous supramaximal adrenal mineralocorticoids suggest that these effects on concentrating and diluting ability may be mediated by changing levels of aldosterone.

Preliminary studies of the intermediary mechanisms involved in sodium rejection by the proximal tubule following volume expansion have been continued by micropuncture methods. Previous studies concluding a humoral response from data showing decreased proximal tubule sodium reabsorption after infusion of plasma from saline loaded animals may have been confounded by ECF expansion of the test animal by the volume of infusate. Conflicting results regarding the effect of dialysate of saline loaded plasma on split droplets half disappearance time have been obtained by several laboratories. Because of the central importance of these findings to understanding of the body's ability to excrete a salt load, two studies have been undertaken: 1) the effect of intravenous infusion of minimal amounts of saline loaded plasma on proximal sodium reabsorption and the response after stopping such infusions will be studied in the same tubule segments by micropuncture. Preliminary studies show no trend effect of prolonged repeated collections from the same tubule segments on inulin TF/P ratios. 2) Necessary photomicrographic equipment for split droplet studies is being procured to test the effect of dialysate of saline loaded plasma on reabsorptive half-time.

Necessary manipulator equipment has been constructed to allow study of dissected individual tubule segments from rabbit or flounder by microperfusion and microcollection techniques. Such techniques will extend the capability of free flow micropuncture to analysis of the function (and ultrastructure) of single nephron segments under exquisitely controlled environmental conditions.

The relation of urinary concentrating ability to the morphology of Henle's loop was compared in the Rhesus monkey and man to determine the functional contribution of the thin ascending segment in producing a concentrated urine. Studies showed the maximum urinary concentrating ability of the monkey to be slightly greater than that of man. Yet, the length of the monkey medulla which contains the water impermeable portions of the loop of Henle were shorter than in man, due to a very short inner medullary zone containing thin ascending segments. Virtually all of the ascending limb of Henle's loop in the monkey was found to be composed of the more complex cuboidal epithelium characteristic by

light and electron microscopy of the thick segment, while in man half of the ascending limb is lined by squamous epithelium. These observations coupled with findings of a definite contribution of the ascending thin segment to concentrating ability in other species suggest that cellular composition of the ascending limb rather than its overall length may be of primary importance in determining urinary concentrating ability.

The techniques of renal tubule micropuncture and electron microscopy have been combined to study the functional and morphologic aspects of sodium transport and its control in the rat kidney. Potassium pyroantimonate was perfused through individual nephrons on the kidney surface as a histochemical marker of areas of sodium concentration within the tubule cell. Ouabain was perfused through tubules to determine the effect of this inhibitor of active sodium transport on sodium localization within and on ultrastructure of the tubule cell. Tubules were fixed by perfusion with fixative in vivo, and dissected individually for electron microscopy. Preliminary studies have established the normal ultrastructure of vitally-fixed tubules, the distribution of sodium deposition within the tubule cell, and suggest that ouabain may modify both the basic ultrastructure of the cell and the distribution of sodium within the cell. Studies are in progress to define further the effects of ouabain and other metabolic inhibitors on cell sodium transport and cell histology as well as to explore the functional and structural consequences of alterations in osmolality, volume expansion, and induction of acute renal failure.

3. Measurement of renal function in normal and disease and injury states:

Potential sources of error inherent in the conventional methods used to determine effective renal blood flow (ERBF) in man has led to the development of an externally monitored isotopic (I^{131} iodohippurate) technique for instantaneous measurements of ERBF independently of urinary collections in the dog. The advantages of this method, which balance the rates of infusion and renal excretion by monitoring the plasma activity externally, are that 1) it obviates the need for bladder catheterization; 2) it detects an unsteady state; 3) it simplifies the methodology and, 4) it provides an ERBF measurement within a matter of minutes after withdrawal of a blood sample.

Application of this infusion method for the simultaneous measurements of glomerular filtration rate, utilizing I^{125} iothalamate (IV-I^{125}) and effective renal blood flow (IV-I^{131}) is presently being investigated. Preliminary studies suggest that IV-I^{125} is similar to the urinary clearance of either I^{125} iothalamate (UV-I^{125}) or inulin (UV-Inulin) and that the presence of iothalamate does not interfere with the clearance of I^{131}-OIH.

The application of radioisotopes for the evaluation of renal function has led to the widespread utilization of I^{131} ortho iodohippurate (I^{131}-OIH) for the measurement of effective renal blood flow. Recent reports suggest that the simultaneous clearances of p-aminohippurate (PAH) and I^{131}-OIH are not equal. These findings are in agreement with recent observations made in this laboratory. By measuring the tubular secretion of these two substances in a sequential manner in the dog, it has been possible to show that when I^{131}-OIH is infused separately it is secreted at the same rate as PAH, but it is depressed in the presence of PAH. This suggests that the discrepancy is due to preferential tubular secretion of PAH and not protein binding, free iodine or insufficient carrier iodohippurate as has generally been suggested.

The study of separate renal function in man has been of importance in evaluating hypertensive individuals for the functional significance of radiologically demonstrable unilateral renal artery lesions. A possible use of furosemide in extending the usual separate renal functional tests to a prediction of reversibility of the lesion and its functional consequences has been suggested since the drug has been shown to eliminate medullary urea and sodium gradients in the kidney. Individual kidney function was studied in dogs with unilateral renal artery stenosis without hypertension and after intravenous furosemide. Results show proportional increases in urine flow rates bilaterally and equal urine dilution bilaterally, suggesting that an intact loop of Henle and urea gradient are necessary for concentration of the urine with renal artery stenosis.

A simple method for split bladder preparation in the dog suitable for repeated study of individual/renal function over many months was devised. The trigonal area of the bladder was sutured to the abdominal wall and repeat catheterization of the ureters performed as desired for repeat renal function studies extending over periods up to six months. Post-operative care was minimal, leakage did not occur at the time of study, and complications were rare in animals kept in drainage cages. The method was contrasted in these regards with the significant disadvantages of other reported methods of split bladder preparation and prosthesis insertion used for chronic studies.

The split bladder preparation was utilized to advantage in a study of the effects of classic suture closure with adhesive closure by polymerizing monomers of large nephrotomy incisions in dogs. Renal function and response to saline loading were studied in animals before and three months after nephrotomy and function in the incised kidney compared with the contralateral manipulated control kidney subjected to equal ischemic time. GFR and ERPF decreased in nephrotomy kidneys in both groups of animals. Although there were subtle statistically significant differences is sodium handling between the suture and adhesive groups, no significant advantage of one method over the other in the repair of nephrotomy was suggested by function studies. The ease of repair and absence of complications with the adhesive technique suggest further exploration of this method clinically.

4. Acid-base homeostasis, including renal mechanisms:

Studies of urine acidification in humans both with and without renal abnormalities were continued. Utilizing a modified short ammonium chloride test, 17 patients with recurrent calcium containing renal calculi were studied and one case of "Partial Renal Tubular Acidosis" was uncovered. This individual was indistinguishable from the remainder of the group by both history and laboratory studies, with the exception of his response to oral ammonium chloride. In addition, two other patients with classic "Renal Tubular Acidosis" were studied and an acidification defect was defined in a patient with hypergammaglobulinemia. The effects of a water diuresis on urine acidification were evaluated utilizing paired studies in six patients without renal abnormalities. Water diuresis resulted in a rise in urine pH, and an increase in ammonium and bicarbonate excretion without any diminution in net acid excretion. The change in urine pH and net acid composition was interpreted as being the result of the relative unresponsiveness of urine free base ammonia concentration to changes in urine flow rate. A number of patients both with and without renal abnormalities, who had been demonstrated to have normal acidification at normal flow rates, did not decrease urine pH to less than 5.30 during water diuresis. It is therefore clear that urine flow rate must be taken into account in assessing tests of urine acidification. The effects of potassium depletion on urine acidification were studied utilizing a similar type paired design. Preliminary results indicate that sufficient degrees of potassium depletion can result in a rise in urine pH without any decrease in net acid excretion or change in urine flow rate. These studies are being continued in an attempt to more clearly define the mechanism responsible for this change in pH.

Continued effort has been expended to utilize the technique of studying perfused isolated segments of rabbit tubule in vitro (Burg, M., J. Grantham, M. Abramow, and J. Orloff. AJP 210:1293, 1966) for assessing renal tubule mechanisms for hydrogen ion excretion. With the helpful advice of Drs. Burg and Grantham and the fine workmanship of the WRAIR Instrument Division, the necessary apparatus has been completely assembled, and slow progress has been made in learning to construct suitable pipettes and to dissect rabbit tubules for monitoring and perfusion. CPT Tannen will work full time with Dr. Burg for two months using the apparatus assembled at WRAIR to accelerate the implementation of this technique and to work out any flaws in the perfusion equipment.

5. Effect of heat-exercise stress on body homeostasis:

Reports from Brooke Army Medical Center (BAMC) and Walter Reed Army Medical Center have indicated that approximately 10% of acute renal failure seen at these centers has been associated with heat exposure and strenuous physical exertion. In order to gain information about the physiological alterations that may precede or lead to the development of renal injury attendant to heat illness or strenuous physical exercise, a field study was undertaken at BAMC during the summer of 1967. Blood and urine studies were done on 91 healthy volunteers from the Medical Training Center, BAMC, Fort

Sam Houston, Texas, before, immediately after, and 12 hours following exercise. The Combat Proficiency Test (CPT) was, in general, the exercise of study. The individuals were grouped according to status of acclimatization and degree of physical conditioning. During the period of study, there were no cases of heat exhaustion or heat stroke. All subjects developed proteinuria following exercise. Hemoglobinuria and myoglobinuria were not seen following the Combat Proficiency Test. Four of forty-two individuals developed microscopic hematuria after the 14 mile cross-country march without evidence of subsequent ill effects. Serum lactic acid dehydrogenase increased slightly after exercise with a small elevation of isoenzyme 5. There was no difference between the unacclimatized and acclimatized physically conditioned groups. Renal concentrating and diluting ability was studied sequentially in a group of 12 volunteers from the beginning to completion of basic training. Renal concentrating ability remained normal throughout acclimatization and physical training, and no quantitative comment could be made on diluting ability since the time allotted for completion of the protocol did not allow for the development of sufficient hypotonicity. Uric acid clearance decreased after exercise, and postexercise hyperuricemia persisted for at least 12 hours.

6. Pathophysiology and prevention of acute renal failure:

Studies of the methemoglobin-ferrocyanide model of acute renal failure were continued. Additional studies of prevention of the lesion using high doses of the diuretic agents ethacrynic acid and furosemide and vasoactive agents diazoxide and hydrolazine were performed in an effort to elucidate factors critical to prevention. Two groups of rats protected from developing the lesion by administration of ethacrynic acid or furosemide were compared grossly and histologically since in the rat diuresis results from furosemide but nor ethacrynic acid administration despite apparently similar nephron sites of action in other species. Results of these studies are pending completion of chemical determinations at this time. A group of rats deprived of sodium for four weeks was found to develop a more severe lesion than that seen after the usual five day sodium deprivation. Juxtaglomerular granulation was increased. The relation of the renin-angiotensin system to production of the lesion is not clear. The role of varying urine flow rates in modifying the lesion was explored in rats with hereditary diabetes insipidus. The acute renal failure lesion could not be produced definitely in rats with diabetes insipidus in contrast to that seen in control animals of the same strain.

The microangiographic study of vascular changes during early induction and throughout the course of acute failure was accomplished in order to study the pathogenesis of oliguria and uremia in the experimental model. It was found that vascular alteration was minimal, and occurred at a time of great architectural changes brought about by cast formation, interstitial renal edema, and tubular dilatation.

In another study, clearances of inulin, PAH, iothalamate I^{125}, diodrast I^{131}, and hippuran I^{131} were performed in the model. Operative preparation of the animal was perfected, and several different analytical techniques investigated before devising the protocol for the clearance studies. Preliminary results indicate a rather marked initial decrease in both glomerular filtration and effective renal plasma flow without a drop in arterial blood pressure. The laboratory is in the process of performing clearances at later times during the course of failure, and in performing micro-osmometry in order to test tubular function by means of osmolar clearances.

Further micropuncture studies of intratubule pressure following induction of the methemoglobin-ferrocyamide lesion were completed. A significant decrease in intratubule pressure was found after 36 hours dehydration in sodium-deprived rats. No further change in pressure was found after induction of the lesion in studies within the first hour of injection before and after cast appearance, and 6, 24 and 48 hours later. The effect of complete unilateral ureter obstruction on intratubule pressure was studied to ascertain what effect obstruction might be expected to have on pressure. Early after obstruction, the expected rise in intratuble pressure was found. However, 24 and 48 hours after unilateral ureter ligation, pressures in the obstructed kidney were reduced to levels seen after dehydration or methemoglobin injection and were increased slightly above normal in contralateral control kidneys. The possibility that intrarenal feedback mechanisms involving distal intratubule pressure may exist deserves further exploration.

7. Mechanisms of fluid and solute flow across dialytic and other membranes:

Using a surgically implanted, chronically indwelling peritoneal catheter, a technique has been developed to do chronic or intermittent peritoneal dialysis in monkeys. The dialysis is run and regulated by a recently developed machine which seems quite reliable. Preliminary studies are underway to examine characteristics of peritoneal transport using this model. Eventually, such dialysis will be incorporated into the monkey-uremia project to modify or define the type and extent of retained uremic solutes.

Peritoneal transport kinetics during clinical dialysis have been studied in over twenty different dialyses in a variety of patients. Analyses of the passive transport of ten solutes of a variety of molecular weights, charge and steric configuration during diffusive transport and during ultrafiltration with hypertonic dialysis solutions have been undertaken. Initial results indicate that these techniques can be utilized to yield information in the following areas: 1) Permeability characteristics of the human peritoneum and its variation; 2) Factors which enhance peritoneal permeability and dialysis efficiency; 3) Advantages and risks of utilizing ultrafiltration to remove solutes; 4) Variation and control of electrolytes transport during ultrafiltration;

5) Carbohydrate and insulin metabolism during exposure to hypertonic glucose; 6) Insulin transport across the peritoneum; 7) Peritoneal handling of larger solutes such as transferring and a specific Beta globulin of M.W. 10,000.

For purposes of analysis, a two compartment model and basic principles of diffusion and ultrafiltration have been utilized. Further studies during the large number of clinically available peritoneal dialyses are planned. The studies involving passive peritoneal transport across other capillary beds and other physiological membranes.

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PROJECT 3A014501B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 03
Psychiatry

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
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C. IN-HOUSE	a. DATE NA; b. NUMBER NA; c. TYPE NA; d. AMOUNT NA	PRIOR FY 68	11	220
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19. GOVT. LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
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21. TECHNOLOGY UTILIZATION	22. COORDINATION
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23. KEYWORDS: BEHAVIOR, AVOIDANCE, CONDITIONING, MOTIVATION, OPERANT BEHAVIOR, PERFORMANCE DECREMENT, REINFORCEMENT LEARNING, STRESS, TIME PERCEPTION.

24.

(U) TECH OBJECTIVE - 1/ TO DEVELOP COMPLEX BEHAVIORAL REPERTOIRES WITH SPECIAL RELEVANCE TO THE NEUROPSYCHIATRIC AREA AND TO ANALYZE EXPERIMENTALLY THE BASIC VARIABLES RESPONSIBLE FOR THE BEHAVIOR. 2/ TO DEVELOP BEHAVIORAL METHODS FOR PRODUCING STRESS AND FATIGUE. 3/ TO INVESTIGATE THE EFFECTS OF STRESS-INDUCING PROCEDURES ON COMPLEX BEHAVIORAL REPERTOIRES.

(U) APPROACH- THE APPROACH EMPHASIZES THE APPLICATION OF MODERN BEHAVIORAL TECHNOLOGY AND THE TECHNIQUES OF THE EXPERIMENTAL ANALYSIS OF OPERANT BEHAVIOR.

25.

(U) PROGRESS - JUL 67 THRU JUN 68 USING A COMPLEX REPERTOIRE OF REPEATED DISCRIMINATION REVERSALS, IT HAS BEEN FOUND THAT SEVERAL STRESSFUL AVERSIVE CONTROL PROCEDURES WILL REDUCE THE AMOUNT OF BEHAVIOR BUT WILL NOT DISRUPT THE ACCURACY OF THE PERFORMANCE. IN A STUDY OF PROLONGED STRESS AND VIGILANCE, THE MONKEY SUBJECTS MADE CORRECT SIGNAL DETECTIONS IN PROPORTION TO THE FREQUENCY OF THE SIGNALS. IF THE SIGNALS WERE INFREQUENT, A HIGH INTENSITY SIGNAL WAS REQUIRED TO MAINTAIN A VIGILANT PERFORMANCE. WORK IS PROGRESSING RAPIDLY IN AN EXPERIMENT SHOWING THAT THE WARM-UP EARLY IN A STRESSFUL AVOIDANCE SESSION IS A PRECISE FUNCTION OF THE INTERSESSION INTERVAL. THE PERFORMANCE OF CHIMPANZEES ON A COMPLEX TIMING REPERTOIRE WAS FOUND TO DETERIORATE SHARPLY WHEN STIMULUS FEEDBACK ON ACCURACY WAS REMOVED. EMOTIONAL REACTIONS WERE ALSO OBSERVED. AN INTERDISCIPLINARY EXTENSION OF BEHAVIORAL TECHNOLOGY TO THE WARD MANAGEMENT OF CHARACTER AND BEHAVIOR DISORDERS HAS BEEN SUCCESSFULLY CARRIED OUT. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

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DD FORM 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 24 Continue to NASA Form 1122)

Project 3A014501B71R, RESEARCH IN BIOMEDICAL SCIENCES

Task 03, Psychiatry

Work Unit 025, Analysis of behavior and of mediating mechanisms: Experimental psychological factors

Investigators.
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Associate: Donald G. Conrad, M.A.; CPT Philip N. Hineline, MSC; William Hodos, Ph.D.; Norman A. Krasnegor, M.A.; CPT Frank J. Sodetz, MSC; and PFC Wendon W. Henton

Description.

Three types of projects are included in the research program: 1) the experimental analysis of basic variables and the development of complex behavioral repertoires; 2) the study of stress-inducing procedures by the effects upon both complex behavioral repertoires and physiological processes; and 3) interdisciplinary applications of behavioral principles to certain medical, psychiatric, and military problems. The first group of projects includes studies of a prolonged vigilance task, warm-up in avoidance, avoidance sessions as aversive events, reinforcement by the delay of shock, transient conditioned suppression, escape and avoidance in pigeons, complex timing repertoires in chimpanzees, repeated acquisition of behavioral chains, social interactions among monkeys, and adjusting avoidance. The second group of projects involves the control of blood pressure and aggression by behavioral procedures, the behavioral effects of septal ablation, the effects of drugs upon several behavioral and stress-inducing baselines, and the effects of aversive control upon discrimination reversals. In the third group of projects, research has focused upon avoidance behavior and hemorrhagic shock, cardiovascular function and motor behavior, and some experiments within a psychiatric ward for delinquent soldiers.

Progress.

1. The experimental analysis of basic variables and the development of complex repertoires.

   a. Effects of signal frequency and shock probability on a prolonged vigilance task. The present research investigated variables that contribute to the maintenance of visual detection threshold in a prolonged vigilance task. Rhesus monkeys held in restraining chairs and housed in light-tight Foringer monkey booths were trained to respond within 10 seconds in the presence of a dim light to avoid a brief electrical shock. Once this avoidance response was well trained, the following

contingencies were established. A response in the presence of the light avoided the shock and decreased, by a fixed amount, the light intensity programmed for the next trial. A failure to respond in the presence of the light resulted in a brief electrical shock and increased, by a fixed amount, the light intensity programmed for the next trial. This staircase psychophysical method allowed us to track the visual detection threshold of a monkey over time. Sessions lasted seven hours and were run five days per week. To prevent development of high rates of responding, 50% of all intertrial interval (ITI) responses were punished. The average number of signals programmed per hour was systematically decreased over sessions by increasing the average length of the ITI. During a session, the detection threshold remained constant when the average ITI was 1.5, 3.0, or 6.0 minutes. When the average ITI was increased to 8.5 or 15.0 minutes a marked increase in detection threshold was observed. A similar within-session threshold increase occurred when the average ITI was held constant and the probability of a shock for missing a signal was systematically varied. These data suggest that a decrease in shock probability may account for the observed intra-session detection threshold increase.

b. Avoidance sessions as aversive events. Animals in avoidance experiments are usually observed only during the avoidance sessions, leaving us ignorant of effects the avoidance conditioning may have on pre-session or post-session behavior. To examine possible effects of the stressful avoidance conditioning on pre-session and post-session behavior, 1-hour sessions of food-reinforced responding were inserted before and after avoidance sessions. The avoidance reduced the rates of food-reinforced responding late in each food session. This reduction is probably similar to conditioned suppression (also known as "Conditioned Emotional Response") which is commonly produced by a warning stimulus followed by a brief aversive event (a shock). Here, the conditioned suppression occurred on an expanded time scale. The warning stimulus was food reinforcement for an hour, and the whole avoidance session served as the aversive event.

c. Warm-up in avoidance. When rats are conditioned to avoid electric shock, they often take many shocks at the beginning of each daily experimental session even though they may avoid all shocks late in each session. This change in performance during each session is known as the warm-up effect. Previous investigators have suggested that the warm-up reflects a build-up of motivation within experimental sessions; we have demonstrated that on the contrary it is a transient suppression of responding, for the phenomenon remains even when the animal's response is maintained with food reward simultaneous with the avoidance contingencies. An experimental program is under way to determine to what extent this suppression resembles other forms of suppression. In one experiment the repeated daily growth of warm-up suppression is being examined as a function of the time between experimental sessions.

Preliminary work done elsewhere indicated that the warm-up suppression passes through a maximum at about 4 hours between experimental sessions. The present work has thus far failed to confirm this; the magnitude of the warm-up increases sharply within the first hour after a preceding session, and appears to level off at intersession times of 2 or more hours. If the function passes through a maximum, this must occur at a much smaller intersession time than was previously thought. Additional subjects are now being run to delineate the growth of the warm-up.

d. Reinforcement by delay of unavoidable shock. An animal performing successfully on a conventional avoidance schedule is accomplishing at least two things: each response decreases the overall frequency of electric shock, and reduces instantaneously the probability of a shock. In conventional avoidance procedures it is impossible to assess the relative importance of these two features, either of which could reinforce the avoidance response. A procedure was devised that held the overall shock rate constant yet permitted the animals to reduce the instantaneous probability of shock. Preliminary work with this procedure indicated that rats will respond to reduce the instantaneous shock probability provided that this does not increase the overall shock rate. But the original procedure did not eliminate a third source of reinforcement based on removal of external conditioned aversive stimuli. A modification of that procedure has been made, eliminating the undesired source of reinforcement. Present work with the modified procedure is, so far, confirming the preliminary results. This work with rats is being extended by varying both the amount of shock delay and the overall shock rate. In addition, this procedure will soon be applied to monkeys. It is hypothesized that the monkey will be more sensitive to long-term response consequences than is the rat.

e. Transience of conditioned suppression. It has been proposed that the warm-up in avoidance conditioning is a kind of suppression similar to the Conditioned Emotional Response (CER). The CER is observed when stimulus-shock pairings are superimposed on food-reward situations. If they are indeed similar, the CER suppression should be transient within sessions, as is the warm-up, if many stimulus-shock pairings were presented in each session. A series of experiments is being carried out to look for this "adaptation" of the CER within individual sessions. If it is found that suppression decreases with successive stimulus-shock presentations during each session, we shall look for systematic changes in this transient suppression when the time between sessions is varied.

f. Escape and avoidance in pigeons. Pigeons have been used extensively as subjects in studies of positive reinforcement and of punishment of positively reinforced responding, but they have not often been used in studies of escape behavior. This may be because it is so difficult to produce or maintain a pigeon's key pecking by removal of

aversive stimuli. Captain Hineline of WRAIR, and Dr. Howard Rachlin of Harvard University, have devised a procedure for minimizing most of the difficulties in conditioning pigeons to peck a key to escape from electric shock. Work is under way to perfect the procedures necessary for conditioning the pigeon to make a transition from escape responding to avoidance responding. This experimental analysis in a species that does not easily learn to respond under behavioral stress may well lead to development of procedures for conditioning other species which avoid more easily, but which could still perform considerably more effectively.

g. Complex timing repertoires in the chimpanzee. Three chimpanzees received extensive training to press two pushbuttons in a program of complex requirements to obtain their daily rations of food. After pushing the first pushbutton, the chimp was required to wait at least 60 seconds but not more than 90 seconds, in order to obtain its food. However, the food was delivered only after the chimp had made 500 responses on the second pushbutton. After an appropriate delay, the second pushbutton produced a green light which remained on until food delivery. After delays which were too short or too long, the second pushbutton produced a red light, and 500 responses were required to relight the first pushbutton. These conditions generated efficient timing behavior. Subsequent withholding of the red and green lights demonstrated that they maintained the timing performance by mediating the delays in ultimate food reinforcement. Without the red and green lights, the timing behavior deteriorated, the distributions of timing response intervals flattened, and peaks shifted to short intervals. Efficient timing performance was then reestablished by reducing the 500 response requirement to two responses. The fixed number of responses requirement is being increased gradually to the original 500 responses. To date the data indicate that reinforcement delays produced by up to 50 responses can be sustained without the mediating stimuli to maintain efficient timing performance.

h. A basic source of errors in the repeated acquisition of behavioral chains. Monkeys were thoroughly stabilized on a procedure where they learned a new sequence of lever presses each session. This experiment investigated the repetition of previously reinforced behavior as a basic source of acquisition errors. Sequences were carefully selected so that a given sequence had 0, 2, or 4 responses in common with the previous sequence. The results showed both in total errors and in the particular levers pressed incorrectly, that the monkeys' errors could be largely accounted for by induction from previously reinforced behavior.

i. Reinforcement interaction between pairs of monkeys. This research investigated the question of whether one monkey would choose to reinforce another monkey. A pair of monkeys was placed in adjacent lever-pressing chambers. Two levers were available. When a monkey pressed the first lever, he delivered a pellet of food to himself. When he pressed the second lever, he delivered food to himself and to the neighboring monkey. After lengthening the intertrial interval and validating the basic technique against different amounts of reinforcement, the following results were found. Out of four monkeys, one reliably pressed the lever which also fed the neighboring monkey, one equally reliably pressed the lever which fed only himself, and two clearly showed no preference. While one monkey was apparently behaving altruistically, neither past history nor the sight of another monkey receiving food was sufficient to reinforce the majority of the monkeys.

2. The study of stress-inducing procedures by the effects upon complex behavioral repertoires and physiological processes.

a. Effects of drugs on a stress-inducing baseline. Recently we have developed a new baseline that will help assess the effects of exogenously administered hormones on behavior. In the present study, a rhesus monkey is required to make 30 lever presses in the presence of a blue stimulus light (S1) to avoid the onset of a green stimulus light (S2). The animal has a maximum of 30 seconds to fulfill the above requirement. If the monkey fulfills the requirement, the blue light is terminated and a 60 second time out is in effect. If he fails the requirement, the green light comes on. In the presence of this stimulus, the monkey must respond 60 times within thirty seconds to avoid the onset of a red light. If the monkey makes the required number of responses in the time allotted, the green light is turned off and a 30 second time out is put into effect. Should he fail the response requirement in Ss, a red light is turned on for 3 seconds and the animal receives 3 unavoidable shocks. At the termination of the red light, a 25 second time out is put into effect. At the completion of this time out the cycle is restarted. The major dependent variables are the ratio of S1/S2 and the number of avoidances. To test the sensitivity of this baseline, drugs with known effects upon avoidance behavior were administered (IM) to the subject. Preliminary data suggests that stimulants increase the number of avoidances in the presence of S1 and decrease the number of shocks. Depressant drugs on the other hand increase the number of avoidances in the presence of S2 and lead to an increased number of shocks.

b. Control of blood pressure by positive reinforcement. A project is now being developed to operantly control the blood pressure level of rhesus monkeys. To vitiate the unconditioned effects of a food pellet upon the recorded blood pressure response, a two component multiple schedule of positive reinforcement will be employed. In one component a decrease in blood pressure for X seconds will produce a stimulus change in which bar-press responses are reinforced by a food pellet. The magnitude and duration of the required blood pressure decrease will be gradually shifted to produce sustained lowering of

blood pressure. Following the establishment of stimulus control of the lowering of blood pressure, in the second component of the multiple schedule, an elevation in blood pressure will produce the positive reinforcement.

c. The effects of conditioned suppression upon blood pressure and aggression. Blood pressure and aggression in rhesus monkeys will be recorded during conditioned suppression. Previous studies have indicated that the magnitude of both responses are increased during the conditioned suppression stimulus. In the present study, the magnitude and duration of each response will be determined as a function of the intensity of the unavoidable shock terminating the suppression stimulus. Also, the effect of an accessible versus inaccessible aggression target upon the blood pressure, and the effect upon both responses of punishing the aggression response, will be examined. In two concurrent studies, biting behavior in rats is being recorded as a function of frequency of reinforcement on variable interval schedules, and as a function of shock intensity during conditioned suppression.

d. Septal ablation and warm-up in avoidance. Under conditions which cause normal rats to suppress responding, rats with lesions in the septal region of the forebrain continue to respond. This effect may be due to the failure of the septal rat to react appropriately to concurrent or interfering contingencies. Because this deficit is not motivational, septal ablation should provide useful information on the warm-up effect in avoidance. When rats are conditioned to avoid electric shock, they often take many shocks at the beginning of each daily experimental session even though they may avoid all shocks late in the session. This change in intrasession performance is known as the warm-up effect. Some investigators have suggested that warm-up reflects a build-up of motivation within an experimental session. Recent evidence has demonstrated that warm-up is a transient suppression of responding. If this is the case, then septal ablation should eliminate the warm-up, producing a constant level of performance throughout sessions. Preoperative baselines for avoidance behavior, including the warm-up, have been established in a number of rats. These animals will be operated shortly and their septal nuclei bilaterally ablated. Following a postoperative recovery period, they will be re-run to compare their preoperative and postoperative performances. In addition to providing evidence on the transient suppression view of warm-up, the present study may also permit a more precise understanding of septal function.

e. Septal ablation and concurrent contingencies. The present study was designed to illustrate that, following septal ablation, the organism fails to adjust its performance to two or more concurrent contingencies. Three rats have been trained to press a lever for food. Then the food reinforcement was discontinued and replaced by a Sidman avoidance schedule. The next step will be to combine the food and avoidance contingencies. Previous work has shown that the food procedure

will not appreciably improve the avoidance of shock, even though the same response both avoids shock and produces food. When behavior is stable on the concurrent food and avoidance procedure, lesions will be made in the septal region of the forebrain. Then we will assess the effects of the lesions on the food contingency, the avoidance contingency, and on the combined concurrent contingencies. Our working hypothesis is that the combined food and avoidance contingencies will not interact following septal ablation and that the animals will perform as though responding to the food contingency alone.

f. Sidman avoidance following septal or hippocampal ablation. Ablation of either the septal nuclei or the hippocampus changes the performance of organisms responding to various procedures. To date, no data has been published regarding the effects of destruction of these regions on performance of Sidman avoidance. A study has been designed to answer three questions. 1) Can the septal or hippocampal rat perform Sidman avoidance? 2) Does the performance of either the septal or hippocampal rat differ in any way from that of the normal rat? 3) Does the performance of the septal rat differ from that of the hippocampal rat? Six septal rats are now recovering from surgery. They will be run on Sidman avoidance using several response shock and shock-shock intervals. A group of six normal controls have already been observed. Six hippocampal animals will be operated shortly and observed as apparatus becomes available.

g. Septal ablation and response disinhibition. When conditions that suppress responding in the intact animal are presented to rats with septal lesions, they show little or no suppression. This common observation has led to a "response disinhibition" concept of septal function. An alternative hypothesis suggests that the failure of the septal rat to suppress responding is due not to any general inability to inhibit responses, but rather to a specific inability to compensate for an additional concurrent contingency. A variant of the Sidman avoidance procedure offers the necessary conditions for testing this hypothesis. If the length of the response-shock interval is reduced to one-half or less of the shock-shock interval, intact rats cease responding. They, in effect, inhibit a well-established lever-pressing response. The working hypothesis of the present study is that septal rats will also inhibit this response because only one contingency is involved. Preliminary training and surgery is now being carried out with 18 rats.

h. Comparison of methylphenidate and pemoline-magnesium hydroxide on several complex behavioral performances. Several studies have reported that pemoline and magnesium (PMH) enhanced learning and retention, although other studies have failed to do so. The present research examined this question in animal subjects. The experimental approach was 1) to use several complex behavioral techniques and 2) to compare PMH with a known CNS stimulant (methylphenidate) which would simulate the stimulant actions of PMH but not the alleged enhancing actions.

Two procedures (repeated stimulus reversals and repeated acquisition of behavioral chains) used monkeys as subjects, while the third procedure (cumulative avoidance with multiple warning stimuli) used rats. The general results of both monkey experiments involving complex relearning repertoires were similar. No dose of either drug consistently decreased the number of errors made by the monkeys in learning either a new behavioral chain or a new stimulus discrimination. However, at the higher dosage levels (10 mg/kg P.O. of PMH and 16 mg/kg P.O. of methylphenidate), the behavior was disrupted in that the monkeys made more errors and/or stopped performing. The rat avoidance experiment disclosed the rate-increasing effect of both drugs with low to intermediate doses. However, the precision of a stimulus discrimination of shock proximity was not improved at any dose of either drug. High doses of both drugs disrupted the discrimination. These results did not reveal any enhancement of performance by PMH, and therefore did not confirm the enhancement effects reported in certain studies in the literature.

i. The effects of aversive control upon discrimination reversals. Random shock and conditioned emotional response (CER) procedures have been studied for their effects upon the repeated reversals of complex stimulus discriminations. Monkeys were trained to respond to a triangle for food reinforcement and to ignore a circle; a few minutes later the monkeys had to respond to the circle and to ignore the triangle in order to get food, and so on for other stimuli. During a series of such reversals, two aversive control procedures were programmed in an effort to disrupt the monkeys' performance. The results showed that either random shocks or the CER could sharply reduce the rate at which the monkeys would work on the stimulus problem. However, the accuracy of the performance (in terms of errors per correct response) showed little, if any, disruption.

3. Interdisciplinary applications of the experimental analysis of behavior.

a. Avoidance behavior and hemorrhagic shock. Behavioral stress has been studied in conjunction with physiological changes that occur during hemorrhagic shock. Monkeys were first implanted with indwelling electrodes, permitting recording of physiological changes that occur when an animal at rest is subjected to gradual but extensive blood losses. When these physiological changes had been assessed and characterized, the hemorrhagic shock procedure was carried out on animals which, instead of being at rest, were performing on an avoidance conditioning procedure. The monkeys were presented with warning stimuli which they could turn off to avoid electric shocks. The avoidance conditioning had a marked effect on the course of hemorrhagic shock; these animals maintained their blood pressure at near-normal levels considerably longer than did the animals at rest. Control procedures are planned to discover whether the avoidance animals will continue to maintain their blood pressure longer when the electric shock is omitted on avoidance trials where there is no response. Another control to be run is that of delivering periodic shocks to animals not on the avoidance procedure. The basic combination of

procedures is being used further to see whether blood losses affect color vision. Pilots in unpressurized airplanes and other individuals in anoxic conditions have reported that colors disappear before blackout occurs. Also, studies on dark adaptation during anoxia show that the photopic and scotopic visual systems are differentially affected. In an experiment now in progress, monkeys are presented with visual and auditory warning stimuli which they can turn off to avoid shock. One visual stimulus is of a wavelength to which only the photopic visual system is sensitive; the other visual stimulus is of a wavelength to which both photopic and scotopic visual systems are sensitive. We are looking for a differential effect of blood loss on photopic and scotopic vision, which would result in differential latencies of responses to the red and blue stimuli. Preliminary results have revealed no systematic differences between responding to the two visual stimuli as blood losses occur. To obtain the anticipated effect, it may be necessary to run the experiment under dark adaptation.

b. Cardiovascular function and motor behavior. A set of recent experiments has suggested that, in humans, the cardiovascular system may modify motor behavior. Specifically, reaction time in humans seems to be related to the amount of sinus arrythmia, even when the arrythmia is produced by external stimulation. We are attempting to replicate these findings, using monkeys prepared for external pacing of the heart and trained to release a lever quickly at the presentation of an auditory tone. To date,we have established stable response baselines in two monkeys, and have paced them at fixed rates. There is some indication that external pacing, even at a fixed rate, has a slight effect on reaction time. We are assessing this further before moving on to externally paced sinus arrythmia. We are also prepared to present the auditory tone during controlled phases of the cardiac cycle, when the arrythmia experiment has been completed. If reliable effects are found, we shall attempt to isolate receptors and neural pathways mediating these effects, and also to examine effects of cardiac function upon more complex behavior.

c. Some experiments within a psychiatric ward for delinquent soldiers. A psychiatric ward for delinquent soldiers was the setting for several experiments exploring the effects of certain behavioral procedures. The soldiers were awarded points when they engaged in socially desirable behaviors. They could then exchange the points for reinforcements, such as coffee, pool room privileges, week-end passes, etc. Within the context of this "point" economy, the following procedures were examined: 1) manipulation of points as reinforcement for specified behavior; 2) shaping a high performance level with differential amounts of reinforcement; 3) demonstration of "model" behavior by a ward officer; 4) punishment by a point fine to control undesired behavior; 5) use of a chaining-type reinforcement contingency to increase desired behavior; and 6) differential reinforcement of the individual versus the group to increase the frequency of a verbal performance. The following results were found: 1) Using the behavior of running a half-mile course, we found that soldier participation would be increased by

awarding larger amount of reinforcement (points) for running. 2) The speed of running was greatly increased when the running speed required for a larger point award was successively increased from week to week. 3) When the ward's psychiatric resident made the run with the men (thereby serving as a behavioral "model"), the soldiers' participation in the run was unchanged. 4) An effort was made to increase attendance at the unit meeting by punishing with a 10 point fine the conflicting behavior, namely, sleeping in bed. The effect was to sharply reduce attendance at programmed activities including the unit meeting. 5) In contrast, a chaining-type positive reinforcement contingency was quite successful in increasing attendance at the unit meeting. The contingency was simply that a soldier must attend the meeting in order to receive points for several other activities. 6) To increase the frequency of verbal reports at a group meeting, a large number of points were given to the group (the audience) when a soldier presented a verbal report. At a later time, the same number of points was allocated to the individual speakers. The more effective procedure with infrequent speakers was the individual reinforcement.

Summary and Conclusions.

1. The experimental analysis of basic behavioral variables has resulted in a number of new findings in the areas of avoidance, vigilance, conditioned emotional response, complex repertoires of behavior, and social interaction. These studies have furnished basic scientific information and have also laid the groundwork for future research on the disruption of complex behavior by stress.

2. Complex behavioral repertoires have been used in the study of stress, selected brain lesions, and drugs. These studies have revealed widespread effects upon blood pressure, aggression, warm-up in avoidance, and several complex behaviors.

3. Interdisciplinary research has been done in certain medical and psychiatric areas where behavior can provide useful measures or where behavioral technology can be usefully applied. Progress has been made in the areas of hemorrhagic shock, cardiovascular functioning, and psychiatric ward procedures.

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24.

(U) TECH OBJECTIVE - THIS RESEARCH IS CONCERNED WITH THE PERCEPTUAL DETERMINANTS OF BEHAVIOR AS THESE ARE REFLECTED IN THE RELATIONSHIP BETWEEN SENSORY INPUT AND NEUROPHYSIOLOGICAL FUNCTION. EMPHASIS UPON THE VISUAL SYSTEM AS A MODEL FOR SUCH PSYCHOPHYSIOLOGICAL INTERACTIONS PROVIDES THE BASIS FOR ANALYZING CENTRAL AND PERIPHERAL MECHANISMS INVOLVED IN DIFFERENTIAL SENSITIVITY TO THE ENVIRONMENT AND ATTENTIONAL FACTORS IN PERCEPTUAL PERFORMANCE.

25 (U) APPROACH- PSYCHOPHYSICAL METHODS AND ELECTROPHYSIOLOGICAL RECORDING TECHNIQUES ARE UTILIZED TO CORRELATE SENSORY INPUT FROM THE ENVIRONMENT WITH BEHAVIORAL AND NEUROPHYSIOLOGICAL OUTPUT IN THE ANALYSIS OF MEDIATING MECHANISMS IN PERCEPTUAL PERFORMANCE. MICROELECTRODE AND GROSS ELECTRODE ELECTROENCEPHALOGRAPHY, ELECTRORETINOGRAPHY, AND BEHAVIOR CONTROL PROCEDURES ARE INTERGRATED THROUGH THE APPLICATION OF COMPUTER PROGRAMMING TO DATA ANALYSIS.

(U) PROGRESS - APR 67 THRU JUN 68 THE EFFECT OF INFORMATION PROCESSING AND RELEVANCE OF INDIVIDUAL STIMULI IN A VISUAL SERIES ON AMPLITUDE OF CORTICAL EVOKED RESPONSES AND ON EEG HAVE BEEN ANALYZED. SIMILAR STUDIES HAVE BEEN CONDUCTED WITH AUDITORY STIMULI. COMPARISONS BETWEEN DOMINANT AND SUBORDINATE HEMISPHERES ARE BEING MADE. SPECTRAL SENSITIVITY OF SINGLE UNITS IN PRIMITIVE EYES HAS BEEN MEASURED AND HAS REVEALED SURPRISINGLY LOW THRESHOLDS IN THE ULTRAVIOLET RANGE. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Task 03, Psychiatry

Work Unit 026, Analysis of behavior and of mediating mechanisms: Psychophysical and electrophysiological data correlation

Investigators.

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Description.

The purpose of this work unit is to investigate relations between the physical and psychological input of stimulation, both historical and contextual, and the organism output of neurophysiological functioning and behavior. It is at this interface between physiology and behavior where some of our most baffling problems occur and currently where some of our most significant advances are being made. In the end, it is the behavior of the individual that is of concern, but well functioning behavior is supported and in detail mediated by physiological mechanisms and their integrative action. The elucidation of these mechanisms is fundamental to solving a wide range of problems that exist. The principal methods are those of psychophysics, biophysics, sensory stimulation, experimental psychology, electrophysiological recording, and statistical analysis. The work is divided into two categories: (1) correlative behavioral and electrophysiological investigations, and (2) studies of central and sensory mechanisms.

Progress.

1. Correlative behavioral and electrophysiological investigations.

a. Visual evoked responses to meaningful stimuli. The effects of task relevance and stimulus parameters on averaged evoked responses are being studied. It has been found that the evoked response is influenced not only by the physical parameters of the stimuli, but also by psychological parameters. That is, not only do the light intensity, wavelength, duration and stimulus shape affect the brain response, but also the meaningfulness of the stimuli, where the meaningfulness is established by task relevance. Controls showed that the meaningfulness effect does not depend on relative energy in the stimuli, changes in pupil size or accommodation, monocular viewing,

eye movements, or amount of alpha EEG activity. In the general experimental design, control stimuli are a regular part of each trial. Since they occur in temporal proximity with the relevant stimuli, they rule out the possible role of any longer term changes. One of the problems is separating expectancy and post-perceptual processes. This has been investigated by randomizing not only the informational content of the visual stimuli, but also the presentation order of relevant and irrelevant stimuli within each trial. This has required a very large amount of computer processing time, since the EEG activity must be sorted out in order to obtain the average evoked potentials for each class of stimulus in each of the various orders. The data analyzed thus far have shown marked differences that may be associated with expectancy and with the nature of the post-perceptual processing. This work will proceed considerably faster when the new computer satellite system is installed. Using the same experimental design, cerebral hemisphere dominance effects are being investigated.

b. Brain activity and assessment of intelligence. Several investigators have recently suggested that certain aspects of the averaged evoked potential have a significant relation to intelligence. This suggests the use of the averaged evoked potential as a culture free, non-verbal, non-motor test of intelligence. This far-reaching possibility is being subjected to experimental test. Several special purpose, solid-state pieces of equipment have been designed, fabricated, and evaluated and preliminary tests obtained.

c. Chromatic mechanisms in primitive photoreceptors. Electrical recording from Limulus median ocelli has shown two spectral mechanisms, one in the green part of the visible spectrum (near 530 nm) and the other in the near-ultraviolet (near 360 nm). Several lines of evidence concur in this conclusion: analysis of response waveforms (rise times and fall times), slopes of response-energy functions, spectral sensitivity functions, and chromatic adaptation. Similar analyses on electrophysiological responses from the lateral eyes fail to find evidence for more than one spectral mechanism, whose spectral sensitivity agrees with the difference spectrum of a photopigment extracted from lateral eyes. Especially striking is the extremely high sensitivity to UV. A paper was published this year. The contribution of these spectral mechanisms to behavior was investigated by studying phototatic behavior in a field study. One pair of eyes was occluded and circus movements produced by unilateral occlusion of the other type of eye. Both lateral eyes and median ocelli were effective in controlling the phototatic behavior under full spectrum stimulation. However, only near-ultraviolet, and not long-wavelength visible, light was effective with the median ocelli. These data agree with the electrophysiological analyses and further suggest the possibility of wavelength discrimination at a primitive level. These studies investigate some of the fundamental mechanisms in photoreception and its relation to visual behavior.

d. Task relevance and auditory evoked responses. The influence on auditory averaged evoked potentials (AEP) of information processing during problem solving was studied. An experimental design was used which presented two relevant and two irrelevant auditory stimuli on each trial where the task was to determine which of the two relevant stimuli was of lower frequency. Two classes of stimuli were used: narrow band tones and broad band noises. Either class of stimuli could be designated relevant in order to control for physical differences in the stimuli. AEP were computed from the subject's EEG while processing this auditory information. Separate AEPs for each of the four stimuli in a trial were computed. The AEPs tended to have two prominent positive peaks with latencies of approximately 150 and 300 msec. The amplitudes of these peaks were measured and the influence of task relevance, stimulus class, and presentation order within a trial were evaluated statistically by analyses of variance with factorial designs. Although the tones and noises were physically and qualitatively quite different, their AEPs were similar. However, the task relevance of the stimuli had marked effects on the AEPs, the magnitude depending greatly on the order of the stimuli within a trial. Both positive peaks were larger when the stimuli were relevant than when the identical stimuli were irrelevant, with peak 2 showing larger effects than peak 1. Peak 2 was especially elevated for the second relevant stimulus. This was related to differences in post-perceptual processing, since the information in the first relevant stimulus had only to be "stored," while the problem could be solved with the second relevant stimulus. Thus, AEPs may be used to study brain processes that are of a higher order than simple attention, uncertainty (information theory), or stimulus gating. A paper has been submitted for clearance for publication.

e. Behavioral spectral sensitivity. Spectral sensitivity determinations have been made so that quantitative comparison can be made between these behavioral data and electrophysiological and photochemical action spectra from the same organism in an elucidation of basic mechanisms underlying the visual system. The behavioral testing situation for Rana Catesbiana involves a forced-choice, paired-comparisons preference procedure with test lights of controlled wavelength and energy. From these choice data, a family of functions with wavelength as the parameter relate percent choice to light energy. A criterion of equal choice (50%) was applied to this family of functions in order to obtain a spectral sensitivity function. The spectral sensitivity function obtained after dark adaptation had a maximum at 540 nm and was much narrower than the usual electrophysiological or photochemical curve. It appears that several spectral mechanisms contribute to this behavior. This was confirmed by shifting the curve with long-wavelength chromatic adaptation. Regression models are being applied to determine the connection coefficients, assuming the action of spectral mechanisms uncovered by electrophysiological techniques.

f. Alpha EEG activity and eye orientation. Recent work has 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 had far-reaching implications in the evaluation of EEG indices of brain function. This hypothesis was tested by measuring the amount of alpha activity 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. 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 quite 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. A paper for publication is in the final stages of preparation.

g. EEG audiometry. The use of EEG audiometry as an aid in the diagnosis of deafness and hearing deficits in the young was studied. Clicks and pure tone stimuli were used. Follow-up of infants with rubella syndrome diagnosed previously as deaf using EEG audiometry is being done. Other young children referred by Walter Reed Army Hospital and by Children's Hospital of the D.C. are being evaluated independently by the audiologist and by EEG audiometry. Our experience with EEG audiometry was reported and discussed by two of our investigators at a two-day workshop organized by the Army Audiology & Speech Center as part of the Basic Science Course for Military Otolaryngology Residents and sponsored by the Armed Forces Institute of Pathology and the D.C. Medical Society, Neurological and Neurosurgical Meeting.

h. Studies of normal development of click and flash evoked EEG responses. Longitudinal and cross sectional study of averaged evoked electroencephalographic responses of the normal human infant to sensory stimuli has been continued this year. Sets of stimuli were presented and the responses recorded from scalp electrodes were electronically averaged using a digital computer. A recording on magnetic tape was made so that additional analyses could be done. The Bayley Infant Scale of Motor and Mental Development was administered to the infants in the EEG study group and each infant received a neurological examination. Cross sectional data on a sample of 150 normal infants under one year of age are currently being analyzed. Light and click evoked response characteristics are being correlated with age, state of consciousness, stimulus intensity, rate, order of presentation, and number of stimuli presented, as well as with the results of the behavioral testings.

i. Evoked responses in mentally retarded infants. Some infants who are mentally retarded show evoked responses which differ from those of normal infants. Our greatest experience has been with a subject population with mongolism. The most striking variation is in the evoked response amplitudes recorded from an electrode near the vertex to clicks presented at a slow rate, but the visual evoked responses may also show a similar variation. A report of these findings has been published. A series of approximately 200 recordings on normal children under one year indicated that, under the recording conditions used, the amplitudes of the averaged click evoked response are in the range of 5-50 microvolts. Amplitudes in the abnormal group are often 50-105 microvolts. Infants in the large amplitude group have included those with mongolism, congenital rubella, congenital hypothyroidism, and one patient with idiopathic microcephaly with severe developmental retardation. In the last named patient, the EEG findings were the first indication of abnormality. Accurate prognosis as to the eventual mental development of infants who are neurologically suspect at birth is difficult. Our studies have thus far indicated that the sensory evoked responses may aid in prognosis.

j. Mongolism- 5HTP Project. The metabolism of serotonin in mongoloid infants is also being studied in collaboration with Dr. Richmond Paine and Dr. Mary Bazelon of the Department of Neurology. The major result to date is an improvement of muscle tone in the drug-tested infants. Several of the drug-treated mongoloids have also shown striking EEG evoked response changes which are currently being evaluated. The first group of infants is being followed longitudinally. A double blind experimental group was started in May, 1967. These children will be followed until they are 3 years old. The effect of serotonin - 5HTP on EEG sleep patterns of mongoloids is also being evaluated. Five sleep and evoked response studies were performed on a mongoloid infant whose parents inadvertently gave their child high doses of 5HTP. No immediate grave effects were apparent but this infant is being followed closely. A report of this case has been accepted for publication in Brain Research.

k. Binocular rivalry and evoked responses. When dissimilar stimuli are presented to the two eyes, the observer tends to alternate between seeing the two stimuli. This constitutes experimental binocular rivalry which has many of the properties of the clinical syndrome. An experiment was devised to study this visual suppression using the averaged evoked response as an objective index. To identify the evoked potentials, each eye was stimulated by flickering light at a different frequency. Building on the preliminary experiments of last year, a more efficient kind of visual stimulation was developed which involved counterphase sine-wave modulated alternating checkerboard stimulation. Positive results were obtained which showed that the brain response to the momentarily non-dominant (image suppressed) eye was slower and usually of smaller magnitude.

2. Studies of central and sensory mechanisms.

a. Origin of dark noise in human foveal vision and signal detection. Signal detection in human vision assumes that dark noise exists and results in the occurrence of false positive responses, i.e., light judged by an observer to be present when no light is present. The origin of the dark noise is unknown. Experimental evidence has been obtained which indicates that at least some of the dark noise relevant to psychophysical measurements in the visual system is introduced at a stage of information processing preceding light adaptation, and therefore originates in the eye itself. When brief flashes of nonchromatic light were presented to the fovea at intensities sufficiently low that not all were seen, the hues of the detected flashes varied. In a previous experiment, it was shown that the hues of the detected flashes were matched by a steady monochromatic light of moderate intensity at either 625 nm or 525 nm. The matching wavelengths required did not depend on the wavelength of the flash; only the relative numbers of red and green observations varied with the flash wavelength. Using carefully controlled test flashes in a randomized procedure with various levels of red light adaptation, it was found that the ratio of red to green detected flashes was decreased by adaptation to the red light at all wavelengths where the ratio was measurable. If dark noise is introduced at a stage of visual information processing which precedes adaptation, the gain change would be expected to operate on the dark noise as well as the incident light. This hypothesis was supported by finding a decrease in the ratio of red to green false positives after adaptation to a red light.

b. Glaucoma mechanisms: ERG and optic nerve potential as function of intraocular pressure. It has been previously shown that the small vessel structure of the papilla and lamina cribrosa in man is the ciliary circulation and that this circulation has a lower intravascular blood pressure than the inner retinal circulation. We have previously shown that elevated intraocular pressure compromises the small vessel structure of the papilla and lamina cribrosa and it has been hypothesized that this creates a functional disturbance. Optic nerve potential recording with control of the intraocular pressure furnishes information about the effects of pressure on eye function. Animal surgery and stereotaxic apparatus was constructed to make possible intracranial optic nerve recording. Thus far 14 experimental animals (cats) have been used. Besides establishing a reliable technique, it has been possible to obtain data relating the retinal artery pressure and the intraocular pressure to optic nerve function. It is clear that the effects are not linear, but it may be possible on the basis of the data to write a mathematical description of the physiological processes involved.

c. Activation energy of photoreceptor by electrical recording from single cells. The retinula cell of the lateral eye of Limulus exhibits (spontaneous) discrete slow potentials in the dark when monitored by an intracellular micropipette. These discrete events constitute a physiological source of photoreceptor dark noise. Possibly they arise from the breakdown of visual pigment in the photoreceptor due to thermal energy. To test this hypothesis the activation energy of the spontaneous discrete events is being measured by determining the effect of temperature on their rate of occurrence. Previous experiments performed on this project have indicated that the activation energy for the breakdown of visual pigment in situ can be estimated from the change of relative spectral sensitivity with temperature. In these experiments the rate of spontaneous events and the spectral curves are being measured for the same single photoreceptor at several different temperatures in the range 5-35°C. An extremely accurate method of determining spectral sensitivity has been developed for this experiment involving two light paths.

d. Quantum statistical analysis of light induced EMF's in photoreceptor. When very dim flashes are presented to Limulus lateral eye the frequency of discrete slow potentials is increased for a short time interval following the flash. Preliminary experiments suggest that the induced events result from single photon absorptions on a one to one basis. In order to gain more insight into the statistical properties of the discrete events it has been arranged to present flashes of variable duration to the eye in such a way as to keep the number of photons delivered per flash constant regardless of the flash duration. The critical time for the production of discrete events and the latency distribution of the discrete events is now being studied. Suitable theory to relate these metrics to the quantum coincidence required for the production of a discrete event has been developed and tested using monte carlo methods.

e. Electroretinogram and occipital responses: developmental study. Previous work on the development of retinal and central visual function in newborn human infants has been extended by simultaneously recording averaged electroretinogram and occipital responses to a luminance series of orange and white light flashes. The data suggest that photopic as well as scotopic function is present at or shortly after birth. These findings are consonant with recent behavioral observations of pattern and color vision in the young infant.

f. Dark adaptation and night vision under hemoglobin saturation. The effect of a hemoglobin saturation of 85% oxygen (equivalent altitude of approximately 15,000 feet) on the proficiency of night vision and dark adaptation is being studied. This is a continuation of a study begun at the University of Chicago in which it was found that anoxia affected peripheral retinal thresholds to a greater extent than central areas. A laboratory for human electrophysiology has been set up and, to the benefit of their diagnosis and treatment, retinal profiles have been

done on WRGH eye patients. The results of these studies have been forwarded to the attending ophthalmologists. These results will be used as baseline records for comparison with the data obtained from experimental subjects.

g. Effect of light damage on the retina. This study was designed to correlate changes in visual threshold measured by psychophysical methods with both the electrophysiology and the histopathology of the retina. A special technique employing curare and a respirator has been set up for rodent electroretinography analysis. A method has been established for intravital fixation of the eyes. The microtome and staining equipment of the WRAIR Veterinary Pathology Laboratory have been made available and eye sectioning has been started. Initial studies have indicated that clearly defined retinal changes are present in animals which have elevated visual thresholds as measured by psychophysical means. Currently, apparatus is being constructed to improve the control of the light damage, and the information already obtained appears highly promising.

h. Congenital ocular albinism: histological and electrophysiological experimental study. The histology facilities of the WRAIR Veterinary Pathology Laboratory are also being used to obtain sections of eyes from a series of cats with congenital nerve deafness. This study is in cooperation with Cesar Fernandez, professor of Physiology and Otolarynology at the University of Chicago. These animals have an abnormal absence of both choroidal pigmentation and tapetum lucidum and initial studies indicate that the pathology is similar to that seen in congenital ocular albinism of man. In an effort to understand the adult pathology, the normal embryology of the retina and choroid is being analyzed in a series of developing kitten eyes. The electrophysiology of the pathological adults is also being studied in an effort to elucidate functional changes in vision second to retinal pigmentation anomalies.

i. Vascular conductivity of the ocular papilla. It is anticipated that calorimetric methods will make possible the analysis of the vascular conductivity of the papilla relative to the intraocular pressure. Construction of a thermocouple amplifier has been started for this study of the vasculature of the optic nerve.

j. Organization of single motor units in human muscle. It has been shown that the single motor unit as defined by Sherrington is the smallest functional unit in a voluntary muscle, and that isometric tension is graded both by altering the output of single units and by varying the number of units active. This study is to qualify the relationship between the number of active motor units, their firing frequencies, and gross tension output in order to elucidate the mechanism by which a muscle produces and controls tension output. The required equipment including oscilloscopes, tension-measuring devices, and recording electrodes is being assembled. Also a four-channel

amplifier system capable of gains from 100 to 50,000 with a noise level of less than 10 microvolts has been designed and fabricated.

k. Visual changes produced by anti-malarial drugs. A multifaceted program for studying ocular toxicity of drugs, especially antimalarial drugs such as analogs of chloroquine, was pursued. As chloroquine given in large dosages can produce irreversible visual damage, the ocular effects resulting from anti-malarial drug dosages were studied. Radioautography was used to determine the relative localization of chloroquine accumulation in the eye. This technique was designed to skirt the problem that chloroquine is highly soluble in almost all polar and nonpolar solvents. Additionally, an animal testing technique was under development for use in testing ocular toxicity of drugs. This technique utilized an effect of constant illumination of the retina which results in destruction of rods, cones, and pigment epithelium to accelerate the rate of destruction. The effects of the drug then are superimposed upon this light-induced destruction. The ERG was used as a functional test of damage.

Summary and Conclusions:

This work unit has been concerned with the neurophysiological mechanisms mediating between physical and psychological stimulation and behavior. The research has been divided into two categories:
(1) Correlations between behavioral and physiological functioning; and
(2) Studies of central and sensory mechanisms.

In the first group of studies, it was found that the cortical evoked response was influenced not only by the physical parameters of the stimuli, but also by psychological parameters. Evoked response differences have been associated with expectancy and with the nature of the post-perceptual processing. The use of the averaged evoked potential as a culture free test of intelligence is being investigated. Correlations have been found between electrophysiological and behavioral measures of chromatic mechanisms in primitive photoreceptors and these mechanisms are being pursued at the cellular level. In a problem solving situation auditory evoked potentials were differentially related to storage and problem solving stimuli. Comparisons of spectral sensitivity at the electrophysiological and behavioral level for a color-detecting vertebrate eye have been obtained and integrative models are being assessed. A recent hypothesis linking alpha EEG activity with eye orientation has been rejected in a carefully controlled study. The use of EEG audiometry as an aid in the diagnosis of deafness and hearing deficits in difficult cases was studied with promising results. Studies are being conducted on the normal development of click and flash evoked EEG responses. Averaged evoked response differences associated with mentally retarded infants have been found.

In a continuing study of the metabolism of serotonin in mongoloid infants, an improvement of muscle tone and striking EEG changes in the drug-tested infants have been found. In a study of binocular rivalry a correlate of visual suppression was found in a temporally coded evoked response.

In the second group of studies, the origin of some of the dark noise in human foveal vision in signal detection has been found to precede light adaptation and therefore originates in the eye itself. In an experimental study of glaucoma, the ERG and optic nerve potentials are being studied as a function of intraocular pressure. The activation energy for photoreceptors is being investigated by electrical recording from single cells. The quantum statistics of light induced EMF's in single photoreceptors are being analyzed. The development of photopic and scotopic function was assessed by electroretinogram and occipital response techniques. Effects of hemoglobin saturation on the proficiency of night vision and dark adaptation are being studied in connection with retinal profiles for eye patients. An experimental study is underway to assess the effect of light damage on retinal changes and visual thresholds determined by psychophysical methods. An experimental analog of congenital ocular albinism is being followed histologically and electrophysiologically. Calorimetric methods to study the vasculature of the optic nerve are being developed. An investigation of the organization of single motor units in human muscle is underway. Studies were conducted on the effects of antimalarial drugs on visual and ocular functioning.

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PROJECT 3A025601A806
MILITARY PREVENTIVE MEDICINE

Task 01
Military Preventive Medicine

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA CA6457	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT	WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	U	U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A806 00 030

10b. PRIOR NUMBER CODE:

11. TITLE: (U) GLOBAL HEALTH DATA

12. SCIENTIFIC OR TECH. AREA: 005100 DOCUMENTATION 4 1 007800 HYGIENE AND SANIT 007000 GEOGRAPHY

13. START DATE: 07 63

14. CRIT. COMPL. DATE: NA

15. FUNDING AGENCY: OTHER DA

16. PROCURE. METHOD: C. IN-HOUSE

17. CONTRACT/GRANT: a. NUMBER NA; b. TYPE NA; c. DATE NA; d. AMOUNT NA

18. RESOURCES EST.

	a. PROFESSIONAL MAN-YEARS	b. FUNDS (in thousands)
PRIOR FY 68	1	35
CURRENT FY 69	1	35

19. GOVT. LAB/INSTALLATION/ACTIVITY

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TEL: 202-576-3551

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INVESTIGATORS PRINCIPAL: VIVONA, COL S.

ASSOCIATE: FRED, A. C.

TEL: 202-576-3553 TYPE: DA

21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

INFECTIOUS DISEASES, DISEASES OF ANIMALS, DISEASES OF NUTRITION AND METABOLISM, PUBLIC HEALTH, EPIDEMIOLOGY, SANITARY ENGINEERING, CLIMATE, GEOGRAPHY, PREVENTIVE MEDICINE.

24.

(U) TECH OBJECTIVE - HEALTH DATA REPORTS ARE PREPARED FOR THE USE OF ARMY MEDICAL SERVICE OFFICERS AND CONTAIN UNCLASSIFIED INFORMATION REGARDING THE HEALTH AND SANITARY CONDITIONS LIKELY TO BE ENCOUNTERED IN FOREIGN COUNTRIES TO WHICH THEY ARE DEPLOYED.

(U) APPROACH- HEALTH DATA REPORTS ARE UNCLASSIFIED REPORTS OF HEALTH AND SANITARY CONDITIONS IN FOREIGN COUNTRIES FOR THE USE OF ARMY MEDICAL SERVICE OFFICERS. THEY DESCRIBE THE GEOGRAPHY, CLIMATE, RELIGION, LIVING CONDITIONS, ANIMALS AND PLANTS OF MEDICAL IMPORTANCE, WATER SUPPLY, METHODS OF WASTE AND SEWAGE DISPOSAL, DISEASES PRESENT, MEDICAL FACILITIES, ETC., OF EACH COUNTRY REPORTED ON.

(U) PROGRESS - JUL 67 THRU JUN 68 HEALTH DATA REPORTS HAVE BEEN COMPLETED AND PUBLISHED ON 44 COUNTRIES. SOME OF THESE HAVE BEEN REVISED AND BROUGHT UP TO DATE. DURING THE YEAR THE FOLLOWING REPORTS WERE PUBLISHED - ISAREL, LEBANON, EGYPT, SYRIA, JORDAN, IVORY COAST, PAKISTAN, TANZANIA, LIBYA. REPORTS ON UGANDA, NORTH KOREA, AND BRAZIL ARE IN PROGRESS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JUL 1967 - 30 JUN 1968.

26.

TEXT NOT REPRODUCIBLE

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
[ ] a. COMSEC OR COMSEC RELATED [X] b. NOT RELATED		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
14350-21-	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (in thousands)	36.

DD FORM 1490m JAN 65 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 inclusive) to NASA Form 1122)

Project 3A025601A806, Military Preventive Medicine

Task 01, Military Preventive Medicine

Work Unit 030, Global Health Data

Investigators.
Principal: COL Stefano Vivona, MC
Associate: Ann C. Fred, M.D.

Description.

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

Progress.

At the end of FY 1968, Health Data Reports had been completed and published on 43 countries. Six of these, Sierra Leone, Pakistan, Tanzania, Lebanon, Egypt and the Ivory Coast were published during the past year. Reports on Libya, Uganda, Israel, Jordan, Syria, Ethiopia and Afghanistan are in varying stages of completion.

Project 3A025601A806 Military Preventive Medicine

Task 01, Military Preventive Medicine

Work Unit 031, Optimum Allocation of Sanitary Engineering Manpower Resources

Investigator: MAJ Wladimir Gulevich, MSC

Description.

Considerable amount of effort has been traditionally expended by preventive medicine personnel in conducting routine inspections of the operations having public health significance. As yet, no rational evaluation of the efficacy of such surveillance visits has been made, nor have methods been developed to permit such an evaluation on a quantitative basis. It is the purpose of this task to address itself to developing a statistical study to be conducted under actual operational conditions in locations where preventive medicine units are assigned.

Progress.

The feasibility study phase of the task was initiated in August 1967 and was completed in May 1968. The results of that phase indicate that it is possible to describe the operational behavior of the person being inspected and the influence exerted by the inspector in terms of a stochastic (statistical) model. The developed methods were found to be sufficiently sensitive to detect the inspector's influence upon operation outcome if it indeed exists. A proposal is being prepared to conduct a field study which will enable the investigator to gather pertinent data under actual operating conditions.

PROJECT 3A025601A811
MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 02
Tropical and Subtropical Military Medical Research

RESEARCH AND TECHNOLOGY RESUME

1. DATE OF RESUME	3. KIND OF RESUME	4. SECURITY	5. DOWNGRADING	6. REL. AND LIMITATION	7. LEVEL OF SUMMARY
01 07 68	D. CHANGE 01 01 67	U U	NA	GA	A. WORK UNIT

2. GOVT. ACCESSION | 3. AGENCY ACCESSION: DA 094435 | REPORT CONTROL SYM: CSCR-1

8. CURRENT NUMBER CODE: 62196011 3A025601A811 00 301

9. PRIOR NUMBER CODE:

10. TITLE: (U) MILITARY MEDICAL RESEARCH PROGRAM, SEATO (THAILAND)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. ESTIMATED COMPL. DATE	15. FUNDING AGENCY
002500 CLINICAL MEDICINE	08 63	NA	OTHER DA

16. PROCUREMENT METHOD	17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. FUNDS (THOUSANDS)
	a. NUMBER	b. DATE	a. PROFESSIONAL MAN-YEARS	b. MAN-YEARS	c. FUNDS (THOUSANDS)
C. IN-HOUSE	NA	NA			
	b. TYPE: NA	d. AMOUNT: NA	FY 68: 68	C	250
			CURRENT FY: 69	C	250

20. GOVT. LAB/INSTALLATION/ACTIVITY
NAME: WALTER REED ARMY INST OF RES
ADDRESS: WASHINGTON D C 20012

21. PERFORMING ORGANIZATION
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INVESTIGATORS PRINCIPAL: BECK, COL M. B.
ASSOCIATE
TEL: NA TYPE: DA

23. TECHNOLOGY UTILIZATION	24. COORDINATION
MEDICINE	NA

25. KEYWORDS
METABOLISM, DISEASE.

26.

(U) TECH OBJECTIVE - TO CONDUCT THE STUDIES REQUIRED TO IMPROVE MEDICAL CAPABILITIES TO SUPPORT LIMITED WAR GROUND COMBAT IN SOUTHEAST ASIA.

(U) APPROACH- A METABOLIC UNIT WHICH WILL PERMIT SOPHISTICATED MEDICAL PROCEDURES TO BE CONDUCTED ON PATIENTS IS NEARING COMPLETION.

(U) PROGRESS - JUL 67 THRU JUN 68 STUDIES PREVIOUSLY REPORTED HAVE BEEN CONTINUED AND EXTENDED. AS MUCH OF THE INFORMATION SOUGHT UNDER THIS PROGRAM HAS BEEN OBTAINED IN VIETNAM AND ELSEWHERE, THE SCOPE OF THIS PROJECT IS CURRENTLY UNDER RE-EVALUATION. FOR TECHNICAL REPORTS, SEE ANNUAL PROGRESS REPORT, SEATO MEDICAL RESEARCH PROJECT, BANGKOK, THAILAND.

27. CONTINUING EFFORT: ☐ a. YES ☒ b. NO | 28. | 29. DOD CODE: AR | 30. BUDGET CODE: 1

31. KWDS OR DESCR.: AR 70-5 | 32. PARTICIPATION: NA

33. REFERENCING AGENCY | 34. SPECIAL EQUIPMENT

35. PAGES OF TEXT | 36.

DD FORM 1498 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED UNTIL 5 TO 28 CONVERTED AMSA Form 1022

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. POST CONTROL SYMBOL
		DA CA6458	CSCRD-103

5. DATE OF RESUME	6. NATURE OF RESUME	7. SECURITY	8. REGRADING	9. RELEASE LIMITATION	10. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	U U	NA	GA	A. WORK UNIT

11. PROJECT NUMBER CODE: 62156011 3A025601A811 00 302

12. PRIOR NUMBER CODE: 62156011 3A025601A811 01 113

13. TITLE: (U) MILITARY MEDICAL RESEARCH PROGRAM, SEASIA (THAILAND)

14. SCIENTIFIC OR TECH. AREA	15. START DATE	16. CNTR. COMPL. DATE	17. FUNDING AGENCY
010100 MICROBIOLOGY	08 63	NA	OTHER DA

18. PROJECT METHOD	19. CONTRACT/GRANT	20. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (in thousands)
C. IN-HOUSE	a. NUMBER NA; b. TYPE NA; c. DATE NA; d. AMOUNT NA	PRIOR FY 68	21	750
		CURRENT FY 69	21	750

21. GOVT. LAB/INSTALLATION/ACTIVITY	22. PERFORMING ORGANIZATION
NAME: WALTER REED ARMY INST OF RES	NAME: WALTER REED ARMY INST OF RES
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RESP. INDIV.: MERONEY, COL W. C.	INVESTIGATORS PRINCIPAL: BECK, COL M. R.
TEL.: 202-576-3351	ASSOCIATE TEL.: NA TYPE: DA

23. TECHNOLOGY UTILIZATION	24. COORDINATION
MEDICINE	NA

25. KEYWORDS

VIRUS DISEASES, DIARRHEA, PARASITIC DISEASES.

26.

(U) TECH OBJECTIVE - TO CONDUCT THE STUDIES REQUIRED TO IMPROVE MEDICAL CAPABILITIES TO SUPPORT LIMITED WAR GROUND COMBAT IN SOUTHEAST ASIA.

(U) APPROACH- A BALANCED LABORATORY STAFF IS MAINTAINED IN BANGKOK AUGMENTED, AS NECESSARY, BY TDY PERSONNEL.

(U) PROGRESS - FEB 67 THRU JAN 68 LABORATORY STUDIES OF DENGUE HEMORRHAGIC FEVER CONTINUE. THESE HAVE INCLUDED A DESCRIPTION OF AN OUTBREAK OF HEMORRHAGIC FEVER IN AN INSULAR SETTING AND SUCCESSFUL PILOT EFFORTS AT VECTOR CONTROL. A LARGE SCALE AEDES AEGYPTI CONTROL PROGRAM IS CURRENTLY UNDERWAY, THE GOAL OF WHICH IS TO ELIMINATE HEMORRHAGIC FEVER FROM THE ISLAND. VIROLOGIC CAPABILITY HAS BEEN EXPANDED TO INCLUDE STUDY OF RESPIRATORY VIRUSES. A RUBELLA EPIDEMIC IN BANGKOK WAS DOCUMENTED AND IS THE SUBJECT OF A CONTINUING STUDY. SURVEILLANCE STUDIES OF DIARRHEAL DISEASES, INCLUDING CHOLERA, CONTINUE. A SEARCH FOR ANIMAL MODELS TO STUDY THE PATHOGENISIS OF FILARIASIS IS IN PROGRESS. A SEARCH FOR ENDEMIC SYLVATIC PLAGUE IN RODENTS IN THAILAND HAS BEGUN. STUDIES OF UNDIFFERENTIATED FEVERS IN US TROOPS IN VIETNAM CONTINUE. THE GEOGRAPHIC DISTRIBUTION OF ENDEMIC FOCI OF SCRUB TYPHUS IN THAILAND IS BEING MAPPED. STUDIES OF RABIES HAVE INCRIMINATED A VARIETY OF RODENTS, INCLUDING MEMBERS OF THE GENERA RATTUS, SUNCINUS AND BANDICOTTA, AS NATURALLY OCCURRING INFECTED ANIMALS. FOR TECHNICAL REPORTS, SEE ANNUAL PROGRESS REPORT, SEATO MEDICAL RESEARCH PROJECT, BANGKOK, THAILAND.

27.

NOT REPRODUCIBLE

28. COMMUNICATION SECURITY	29.	30. OBJ CODE	31. BUDGET CODE
[illegible]		AR	1

32. MISSION OBJECTIVE	33. PARTICIPATION
AR 705-5	NA

34. SPECIAL INDIVIDUAL	35. SPECIAL EQUIPMENT

36.

DD FORM 1493m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 25 identical to NASA Form 1122)

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. GOVT. CONTROL NO.
			DA 056442	[illegible]

5. DATE OF RESUME	6. KIND OF RESUME	7. SECURITY	8. REGRADING	9. RELEASE LIMITATION	10. LEVEL OF RESUME
01 07 68	B. CHANGE 01 07 67	U U	NA	CA	A. WORK UNIT

11. CURRENT NUMBER CODE	10B. PRIOR NUMBER CODE
62156011 3A025601A011 00 303	62156011 3A025601A011 02 116

11. TITLE (U) MILITARY MEDICAL RESEARCH PROGRAM, SEASIA(MALAYSIA)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 MICROBIOLOGY 003500 CLINICAL MEDICINE	08 63	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (IN THOUSANDS)
C. IN-HOUSE	a. NUMBER NA b. DATE NA c. TYPE NA d. AMOUNT NA	ESTIMATE 68	1	10
		CURRENT FY 69	1	10

19. GOVT. LAB INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
NAME WALTER REED ARMY INST OF RES	NAME WALTER REED ARMY INST OF RES
ADDRESS WASHINGTON D C 20012	ADDRESS HEADQUARTERS TRAIR WASHINGTON D C 20012
RESP. INDIV. MERONEY, COL W. H.	INVESTIGATORS PRINCIPAL TIGERTT, COL W. D.
TEL. 202-576-3551	ASSOCIATE RAPMUND, LTC G.
	TEL. 202-576-3551 TYPE DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
MEDICINE	NA

23. KEYWORDS

MALARIA, CHLOROQUINE.

24.

(U) TECH OBJECTIVE - TO EVALUATE THE EXTENT OF MALARIA AND OF DRUG - REFACTORY MALARIA IN VARIOUS PARTS OF MALAYSIA.

(U) APPROACH- STANDARD SURVEY TECHNIQUES AND STANDARD W H O DRUG TEST PROGRAM WILL BE USED IN COOPERATION WITH MALAYSIAN WORKERS

(U) PROGRESS - JUL 67 THRU JUN 68 APART FROM THE PREVIOUSLY REPORTED SINGLE MAJOR FOCUS OF FORUS REFRACTORY FALCIPARUM MALARIA IN NORTHEAST MALAYA OR THAT BORDER, NO OTHER LOCATIONS HAVE BEEN UNCOVERED. SPORADIC PRESUMPTIVE CASES IN JOHORE ARE NOW UNDER ACTIVE INVESTIGATION. FOR TECHNICAL REPORTS, SEE ANNUAL PROGRESS REPORT, SEATO MEDICAL RESEARCH LABORATORY, CLINICAL RESEARCH CENTER, BANGKOK, THAILAND, 1 JULY 1967 - 30 JUNE 1968.

25.

TEXT NOT REPRODUCED

26. COMMUNICATIONS SECURITY	27.	28. GSD CODE	29. BUDGET CODE
[illegible]		AR	1

30. MISSION OBJECTIVE	32. PARTICIPATION
AR 705-5	NA

31. LEAD INSTALLATION	33. SPECIAL EQUIPMENT

34. FUNDS	35.

1392m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 22 inclusive) to NASA Form 1122

RESEARCH AND TECHNOLOGY RESUMS

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION DA CA6649	4. REPORT CONTROL SYMBOL CSG-RCS		
01 07 67	6. CHANGE 01 07 67	5. SECURITY U U	7. DOWNGRADING NA	8. RELEASE LIMITATION CA	9. LEVEL OF SUMMARY A. WORK UNIT

10. KEY WORDS (KWIC) 3A762716A811 00 304

10. TITLE (C) MILITARY MEDICAL RESEARCH PROGRAM, SEASIA (U)

11. PRIMARY GFTCA 3E762716A811 MICROBIOLOGY	003500 CLINICAL MEDICINE	13. START DATE 09 63	14. ORIG. COMPLET DATE NA	15. FUNDING AGENCY OTHER DA
12. CONTRACT/GRANT a. NUMBER NA b. TYPE NA c. DATE NA d. AMOUNT NA		16. RESOURCES ESTIMATED FOR NEXT FY	PROFESSIONAL MAN-YEARS	FUNDING (In Thousands)
		68	3	300
		CURRENT FY 69	3	300

17. GOVT LAB/INSTALLATION ACTIVITY
NAME: WALTER REED ARMY INST OF RES
ADDRESS: WASHINGTON D C 20012

18. PERFORMING ORGANIZATION
NAME: WALTER REED ARMY INST OF RES
ADDRESS: MED RES TEAM VIETNAM
WASHINGTON D C 20012

19. GROUP INDIV: MERONEY, COL W. H.
202-576-3551

INVESTIGATORS PRINCIPAL: JONES, LTC L.
ASSOCIATE
TEL: NA TYPE: DA

21. TECHNOLOGY UTILIZATION: PEDIATRIC SURGERY

22. COORDINATION: NA

23. KEYWORDS
PLAGUE, TRAUMA.

24.
(U) TECH OBJECTIVE - TO CONDUCT THE STUDIES REQUIRED TO IMPROVE MEDICAL CAPABILITIES TO SUPPORT LIMITED WAR GROUND COMBAT IN SOUTHEAST ASIA.

(U) APPROACH- A SMALL STAFF IS MAINTAINED IN SAIGON AUGMENTED BY TDY PERSONNEL. FIELD UNITS ARE ESTABLISHED AS REQUIRED. MANY STUDIES ARE DONE IN COOPERATION WITH THE PASTEUR INSTITUTE OF SAIGON.

(U) PROGRESS - JUL 67 THRU JUN 69 STUDIES OF INFECTIOUS DISEASES, PRIMARILY MALARIA, PLAGUE AND ENTERIC INFECTIONS HAVE CONTINUED. STUDIES ON TRAUMA ARE INCLUDED UNDER 3A025601A821C1121. FOR TECHNICAL REPORTS, SEE ANNUAL PROGRESS REPORT, USA MEDICAL RESEARCH TEAM (MRAIR) VIETNAM AND INSTITUTE PASTEUR OF VIETNAM.

TEXT NOT REPRODUCIBLE

25.

27. COMMUNICATIONS SECURITY [ ] b. NOT RELEASABLE TO FOREIGN NATIONALS [X] b. NOT RELEASABLE	28.	29. OSD CODE AR	30. BUDGET CODE 1
31. MISSION OBJECTIVE AR 705-5		32. PARTICIPATION NA	
34. LOCATING AGENCY	35. SPECIAL EQUIPMENT		
36. KEYWORDS (Optional)	37.		

DD FORM 1 JAN 64 1190m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 23 identical to NASA Form 1122)

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYM.
		DA 049662	CSO

4. FY OF SUBMISSION	5. KIND OF RESUME	6. SECURITY	7. DOWNGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	U U	NA	6A	A. WORK UNIT

10. PROGRAM ELEMENT/PROJECT/TASK NUMBER AND WORK UNIT NUMBER
62106811 3A062960A811 00 105

11. TITLE (U) MILITARY MEDICAL RESEARCH PROGRAM, SEASIA (MAIRTZOONOSES)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
21C10G MICROBIOLOGY 005900 ENVIRONMENTAL SC	04 63	NA	OTHER DA

16. PROCUREMENT METHOD	17. CONTRACT/GRANT			18. RESOURCES EST.	19. MAN-YEARS	20. FUNDING (THOUSANDS)
	a. NUMBER	b. DATE				
C. IN-HOUSE	c. TYPE NA	d. AMOUNT NA		FY 68	9	300
				FY 69	9	300

21. GOVT. LAB/INSTALLATION/ACTIVITY

NAME: WALTER REED ARMY INST OF RES
ADDRESS: WASHINGTON D C 20012

PERSON INDIV. PERONEY, COL W. P.
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22. PERFORMING ORGANIZATION

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WASHINGTON D C 20012

INVESTIGATORS PRINCIPAL: ASBILL, COL S. G.
ASSOCIATE: ALEXANDER, A. D. PH.D.
TEL: 202-576-5376 TYPE: DA

23. TECHNOLOGY UTILIZATION: NA

24. COORDINATION: NA

25. KEYWORDS
ZOONOSES, LEPTOSPIROSIS, MELIOIDOSIS, EPIDEMIOLOGY, GENETICS, SEROLOGY.

26.

(U) TECH OBJECTIVE - TO DEFINE ZOONOSES THAT HAVE POTENTIAL MILITARY SIGNIFICANCE, TO DETERMINE PREVALENCE, SOURCES AND MODES OF INFECTION, AND TO DEVISE MEASURES FOR DIAGNOSIS, PREVENTION AND CONTROL. STUDIES ARE COORDINATED WITH FIELD UNITS, EMPHASIZING LEPTOSPIROSIS AND MELIOIDOSIS AND INCLUDE- IDENTIFICATION AND STUDIES OF ISOLATES OBTAINED IN EPIDEMIOLOGICAL INVESTIGATIONS, CHEMO AND VACCINE PROPHYLAXIS FOR LEPTOSPIROSIS, DEVELOPMENT OF LABORATORY DIAGNOSTIC TECHNICS FOR MELIOIDOSIS, SURVEILLANCE OF THESE DISEASES IN S. E. ASIA.

27.

(U) APPROACH- CONVENTIONAL CULTURAL AND SEROLOGICAL TECHNICS ARE USED IN EPIDEMIOLOGICAL STUDIES. GENETIC TOOL UTILIZED FOR STUDY OF ANTIBIOTIC RESISTANCE IN MELIOIDOSIS. HAMSTERS AND DOGS ARE USED TO EVALUATE VACCINES AND DRUGS. MAJOR PROBLEMS- MAINTENANCE OF STRAIN VIRULENCE FOR EXPERIMENTAL INFECTIONS, -WET-TAIL- IN HAMSTERS, AND LACK OF SAFE OF ANIMAL FACILITIES FOR MELIOIDOSIS STUDIES. PROGRAMMED LIQUID NITROGEN FREEZING TECHNICS ARE BEING STUDIED FOR LONG TERM PRESERVATION OF LEPTOSPIRAL STRAINS.

(U) PROGRESS - JUL 67 THRU JUN 68 SEROLOGICAL STUDIES ON LEPTOSPIRAL ISOLATES PROVIDED ADDITIONAL INFORMATION ON OCCURRENCE OF SPECIFIC SEROTYPES IN MALAYSIA, THAILAND AND VIETNAM. SCREENING DRUG PROCEDURES FOR LEPTOSPIROSIS WERE IMPROVED. NITROGEN FREEZING EXPERIMENTS FOR STORAGE OF LEPTOSPIRA WERE INITIATED. DRUG TESTING FOR LEPTOSPIROSIS WAS CONTINUED. GENETIC STUDIES HAVE AFFIRMED TAXONOMIC RELATIONSHIP BETWEEN A. MALLEI, P. PSEUDOMALLEI AND P. MULTIVORANS. ANTIBIOSIS AMONG MELIOIDOSIS STRAINS DEMONSTRATED AND DEFINED. ANTIBIOTIC SENSITIVITIES OF MELIOIDOSIS STRAINS CONTINUED. OBSERVATIONS ON PREVALENCE OF MELIOIDOSIS ANTIBODIES IN INDIGENOUS S. E. ASIA POPULATIONS EXTENDED AND RELATED TO ECOLOGICAL FACTORS. INCREASED MELIOIDOSIS CASES IN U.S. MILITARY PERSONNEL IN S. E. ASIA HAVE BEEN DETECTED. LABORATORY DIAGNOSIS INCLUDING SEROLOGICAL TECHNICS FOR MELIOIDOSIS HAVE BEEN FURTHER IMPROVED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

27. COMMUNICATIONS SECURITY	28.	29. DOD CODE	30. BUDGET CODE
a. COVERS CLASSIFIED [ ] b. NOT RELATED [X]		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
AR 705-5	NA

33. PROJECTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS [illegible]	36.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 25 identical to NASA Form 1122)

Project 3A025601A811, MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 02, Tropical and Subtropical Military Medical Research

Work Unit 305, Tropical and Subtropical Military Medical Research Program, SEA, WRAIR - Zoonoses

Investigators.
Principal: COL S. G. Asbill, VC; A. D. Alexander, Ph.D.; M. Rogul, Ph.D.; CPT S. Green, MSC
Associate: A. Warner, Jr.; S. Schwarting, M.S.; R. D'Agostino, SP5; J. Brendle, SP4

Description.

Major objectives are to evaluate real or potential military significance of selected zoonoses, to characterize etiologic agents, to define epidemiological factors, and to establish methods of diagnosis, treatment, and control.

Progress.

1. Antileptospiral Drug Screening.

An in vivo procedure to screen drugs for antileptospiral activity was developed previously (Annual Report 1966-67). The test system was a predictable 5-7 day fatal disease in 22-25 day hamsters infected with canicola or bataviae. Infection was initiated by i.p. inoculation of a known number of organisms in supernatant fluid from a 10% suspension of infected hamster liver. Test drugs in a peanut oil base were administered s.c. and per os in a single dose 48 hours postinfection. Three doses, 640, 160, 40 mg/Kg of drugs were tested, each in five hamsters. Determination of drug activity was based on survival of infection or significant increase in mean survival time.

This test procedure was used to screen 190 compounds selected and submitted by the Department of Medicinal Chemistry, WRAIR. Minocycline (87781A) was active at all dose levels; all animals treated were cured. Nine other compounds had equivocable activity manifest by sporadic survival of treated animals. These compounds will be rechecked.

2. Leptospirosis Surveillance.

Single or paired sera from individuals of a marine company, 3rd Division (Khe Sann) and a company of 199th Light Infantry Brigade (Long Binh) under surveillance for leptospirosis for periods of 7 and 13 weeks respectively were submitted by the U. S. Army Medical Research

Team (WRAIR), Vietnam, for confirmatory leptospirosis serological tests. The two companies' sera were examined by conventional microscopic-agglutination, slide, and hemolytic test (HL) procedures. HL titers of 1:160 or greater were seen in sera from 5 of 52 marines. There was only one rise in titer, the remainder were stationary titers. Two of the reactors were positive by microscopic-agglutination and/or slide agglutination tests; one soldier had microscopic-agglutination test titer of 1:100, but was negative with 2 other procedures. There were, therefore, a total of 6 reactors in this group. In only one case could the reactions (by HL test only) be related to the surveillance period. Other titers may have been incited by previous exposures.

Nine of second serum samples from 90 subjects of the 199th Infantry Company had HL titers of 1:160. Three of these had partial or questionable microscopic-agglutination test titers. All were negative with the slide test. In this series, as in the Khe Sanh Marine series, microscopic-agglutination and slide test findings failed to establish the occurrence of infections during the surveillance periods.

Single convalescent sera from 31 and paired acute and convalescent sera from 27 leptospirosis cases in military personnel in Vietnam were submitted by the 9th U. S. Army Medical Laboratory for additional tests with standard agglutination technics. The laboratory diagnoses at the 9th U. S. Army Medical Laboratory were established on the basis of hemolytic tests. Microscopic-agglutination test results affirmed HL test findings in 53 of the 58 patients. In the series containing paired sera, significant titer rises were demonstrated in 24 patients. One patient had a high stationary titer. Agglutinin titers ranged from 1:100 to 1:25,600. The distribution of titers in convalescent specimens was as follows: 1:100 - 12, 1:400 - 12, 1:1600 - 23, 1:6400 - 7, 1:25,600 - 1. The distribution of predominant antibody reactions for this series of cases, as well as for 29 patients tested in the previous year, is shown in Table 1.

Table 1 - Distribution of Predominant Antibodies in Leptospirosis Cases Occurring in Troops in Vietnam

Predominant Reactions to Test Antigens	No. of Cases		
	FY '67	FY '68	Total
hebdomadis group	7	5	12
pyrogenes group	4	3	7
icterohaemorrhagiae	4	5	9
bataviae	3	5	8
javanica group	1	5	6
grippotyphosa	2	3	5
autumnalis-pomona	2	5	7
canicola	1	3	4
australis		3	3
hyos		2	2
butembo	1		1
multiple*	4	14	18
Total	29	53	82

* Multiple reaction to same titer to 2 or more antigens.

The diversity of agglutinin response in patients was again apparent in the serums submitted from the 9th U. S. Army Medical Laboratory and provided evidence that infections were incurred by a large variety of serotypes. Notwithstanding the different antibody response, one of the antigens used in the microscopic-agglutination test -- a biflexa strain, Patoc -- elicited presence of antibodies in all but 5 of 53 patients positive in microscopic-agglutination tests. Serum from one of the cases positive on HL gave a positive reaction with patoc but not with other agglutinating antigens.

The sera from 57 of 58 cases were also tested with 4 sets of pooled microscopic-agglutinating antigens which are commercially available. Plate test and microscopic-agglutination test findings in 53 patients were consistent. Sera from 50 patients were positive on both tests; 3 patients were negative on both tests. In 3 cases plate tests were negative, whereas, microscopic-agglutination tests were positive. On the other hand, sera from two of the cases negative in latter procedure gave positive reactions with the plate tests. These findings would support the use of the plate test antigens for diagnosis of leptospirosis cases in operational area laboratories.

Two strains isolated from soldiers by the 9th U. S. Army Medical Laboratory were identified to be members of the pyrogenes and hebdomadis groups. One of the strains was isolated from a patient with classic renal-hepatic signs. This patient was subsequently evacuated to WRGH, where a second isolate was obtained from the urine approximately 3 months after disease onset, during the convalescent period. The strain was recovered by direct cultivation of a mid-stream urine sample in 5-Fluorouracil leptospiral media. Parallel attempts to recover leptospirosis by hamster inoculation methods were unsuccessful.

Laboratory diagnosis of leptospirosis has also been established from 2 cases seen in CONUS Army hospitals in recent returnees or evacuees from Vietnam.

During the period of this report 50 leptospiral isolates obtained during the course of epidemiological studies in Malaysia were submitted for identification. These isolates were related to types previously found in Malaysia.

One of the representative strains of isolates from Malaysia with distinct serological properties was definitively identified to be a new serotype in the autumnalis group.

Twelve isolates obtained from human cases in Malaysia by the Virus Laboratory, IMR, Kuala Lumpur, were identified by serogroup as follows: hebdomadis - 2, icterohemorrhagiae - 2, pyrogenes - 3, canicola - 1, autumnalis - 3, and pomona - 1

3. Melioidosis.

a. Serological studies. Support of melioidosis laboratory diagnostic services of the Armed Forces Medical Laboratories in S. E. Asia as well as in CONUS was continued. These services included preparation, standardization, and submission of serological diagnostic reagents; development of improved procedures for primary isolation of cultures; provision of Standing Operating Procedures for the use of these reagents and for isolation and identification of the etiologic agent; and conduct

of bacteriological and serological procedures on specimens submitted for laboratory diagnosis or for confirmation of findings in Armed Forces laboratories. In addition, tests were done on "follow-up" samples from previously proved cases.

During the period of this report, serological or cultural, or both types of specimens from approximately 510 subjects were tested. Laboratory confirmation of melioidosis was established for 40 melioidosis cases in U. S. Armed Forces personnel in Southeast Asia. The diagnosis in 18 cases was confirmed bacteriologically. Laboratory diagnosis in the other 22 cases was established serologically by the demonstration in single, paired, or serial serum samples of significant antibody titers in both hemagglutination (1:40 or greater) and complement-fixation tests (1:8 or greater). The serological diagnosis of 16 other patients whose sera were reactive by one test only or reactive at low titers in both tests were considered to be equivocable.

A total of 89 such cases have now been affirmed or established by this laboratory since February 1965. Check tests are now in progress on serum samples from approximately 70 cases that were diagnosed at the 9th U. S. Army Medical Laboratory in Vietnam.

Approximately 115 cultures in the stock collection of P. pseudomallei were freeze-dried.

Significant serological reactions were demonstrated in 5 laboratory personnel working with Pseudomonas pseudomallei -- 3 at a U. S. Naval Medical Laboratory in Vietnam, 1 at Parke Davis, and 1 at WRAIR. There was bacteriological or clinical evidence of infection in WRAIR and Parke Davis reactors. The Navy subjects have clinical signs of illness and are being studied further.

b. Selective media for P. pseudomallei. Brain heart infusion agar containing 3% glycerol, 10 U of penicillin and 100 U of polymyxin per ml has been recommended as a selective media for the isolation of P. pseudomallei. This medium was reevaluated for possible inhibitory growth affects on P. pseudomallei strains. Tests were conducted with seven strains selected on the basis of varying antibiotic susceptibilities. Each of the organisms was suspended to a standard density of approximately 10^6 organisms per ml. Ten-fold dilutions were prepared and 3 terminal dilutions estimated to contain 1 to 100 organisms were plated in triplicate in both test antibiotic media and in optimum growth media. There was no remarkable difference in counts in the two media.

The same test procedure was used to evaluate the potential usefulness of a commercially available antibiotic additive VCN Inhibitor (vancomycin 3.0 ug/ml, colistin 7.5 ug/ml, nystatin 12.5 U/ml) used for selective isolation for Meningococcus sp. Nutrient agar medium containing VCN did not inhibit the growth of P. pseudomallei.

c. Genetic studies. Studies on the nucleic acid characteristics and intra-strain antagonism of P. pseudomallei were continued. The objectives of these studies were to resolve questions on the epidemiology of melioidosis and the ecology of P. pseudomallei.

Previous attempts to hybridize nucleic acids of Pseudomonas pseudomallei and Actinobacillus mallei together and with other organisms were described in WRAIR Annual Research Progress Report 1967. Nucleic acids were attached to membrane filters and hybridized according to the method of Denhardt (1966). The most specific temperature of incubation appeared to be 72°C. Under these conditions, genetic relatedness was not quantitatively reproducible. The amount of single stranded deoxyribonucleic acid (DNA) was monitored by radioactivity and phosphorus determinations. It was found that DNA was eluted from the filters in unpredictable amounts during overnight incubation. It was presumed that the high heat of incubation was eluting the DNA from the membrane filters. New conditions were imposed to lower the temperature of renaturation by combining the Denhardt method with that of Legualt-Demare (1967) who used the denaturant dimethyl sulfoxide (DMSO). The same method of DNA retention was used as before, but the filters and radioactive DNA were incubated in 2 SSC (SSC = 0.15 M NaCl - 0.015 M sodium citrate) containing 30% DMSO. The optimal renaturation temperature was determined by incubation at five degree increments of temperature between 45 and 65° C. It was found that the most specific binding occurred at 60°C.

Again the results were not reproducible. By applying radioactive DNA to the filters and performing phosphate determinations, it became evident that DNA was still eluting from the filters in unpredictable amounts during overnight incubation. Therefore, the most logical method of computing homologies was to determine the amount of radioactive DNA hybridizing with the DNA attached to the filters, comparing DNA annealed to DNA retained on the filter. Even this was not satisfactory.

In Table 2, the results of hybridization tests with millipore filters (MPF) using radioactive DNA from P. pseudomallei 4845 and phenotypically similar organisms were compared. Micrococcus lysodeikticus was used as a genetically nonrelated control because its overall DNA base composition is similar to P. pseudomallei.

It was evident that three groups of organisms did have some DNA homology when the results were compared to P. pseudomallei annealing to M. lysodeikticus DNA. It is also obvious that the non-specific aggregation in 2 SSC + 30% DMSO is greatly diminished as compared to 2 SSC alone. At best, these results could only be used as a screening test to demonstrate relatedness. The most reactive DNA came from P. pseudomallei, as well as A. mallei and P. multivorans.

The agar column technique of McCarthy and Bolton (1963) was then used to quantify the nucleic acid homologies of these organisms. This technique afforded an opportunity of examining the heat elution profile of the hybridized nucleic acid. To this end native DNA from P. pseudomallei was dissolved in solvents of different salt concentrations with and without DMSO. The solutions were heated gradually and changes in optical density were recorded.

Table 2. Determinations of Nucleic Acid Homologies on MPF*

Strain	% Relatedness to P. pseudomallei 4845+			
	Preincubation Medium		Preincubation Medium + 30% DMSO	
P. pseudomallei 4845	100	100	100	100
P. pseudomallei 1691			122	123
P. pseudomallei 295	79.1	91.9	120	347
A. mallei 3873				132
A. mallei 4			170	
P. aeruginosa	13.9	6.5	0.7	0.6
P. multivorans 382	40.5	51.6	19.7	18.4
P. fluorescens A	5.6	7.1	0.1	0.1
P. fluorescens B			0.4	0.05
P. acidovorans	7.0	6.3	1.2	0.8
P. putida	4.1		0.5	1.0
C. janthinum			1.3	0.2
C. violaceum			2.5	1.7
C. amethystinum			1.8	1.7
C. lividum			2.3	2.8
A lignieresii	0.8	1.2	0.4	0.3
A. equuli	1.6	1.0		
M. lysodeikticus	9.3	19.9	0.4	1.3

* Annealing in preincubation medium (PM) was performed at 72C; PM + 30% DMSO at 60C overnight. Ratio of radioactive DNA to DNA originally retained on filter is 1:50.

+ Relatedness is derived by determining dpm per µg DNA retained on filter after incubation and dividing by dpm per µg of homologous reaction.

Table 3. Range of P. pseudomallei DNA Denaturation Temperatures as Determined by Hyperchromicity in Various Solvents

Solvent	Temperature of	
	Initial Hyperchromicity	Final Hyperchromicity
	C	C
0.01 SSC	56	77
0.1 SSC	72	88
0.01 SSC + 30% DMSO	51	65
0.1 SSC + 30% DMSO	60	76

The ranges between the temperatures of initial and final rise of hyperchromicity are analogous to the temperatures of initial and complete denaturation of DNA. Because the agar column matrix melts at 75°C, we chose 0.01 SSC + 30% DMSO as our eluant for the hybridized DNA's. The temperature spread was 14°C and the approximate mid-point of thermal denaturation was 58°C.

Having determined the denaturation conditions, attention was directed to the optimal renaturation conditions of DNA. The most specific temperature of renaturation in 2 SSC is believed to occur approximately 25°C below the Tm determined in 1 SSC. Nucleic acids with 70% guanine + cytosine would theoretically best renature at 74°C (Marmur and Doty, 1961). To test this hypothesis, renaturations were carried out in 2 SSC and 2 SSC containing 30% DMSO at various temperatures. The technique is similar to that of McCarthy and Bolton except that the hybridized DNA in agar was transferred to a heat regulated chromatography column and washed with 200 ml of incubation fluid at incubation temperature to remove non-specifically bound DNA. A final wash of 10 ml was used to monitor leakage. The temperature was dropped to 44°C and elution of the specific hybridized material was done in 10 ml washes of 0.01 SSC containing 30% DMSO and raising the temperature every 10 minutes in 2°C increments up to 74°C.

Table 4 is concerned with the homologous hybridization of P. pseudomallei 4845. This table presents the percent of annealing and elution midpoint (Em) of the hybrids in 2 SSC and 2 SSC + 30% DMSO at various renaturation temperatures.

The elution midpoint is the temperature at which 50% of the hybridized radioactive DNA is eluted. It is analogous to the Tm. In 2 SSC the Em's did not even approximate the Tm of the native DNA. Secondly, at 60°and 65°C M. lysodeikticus DNA would appear to have a 35% and 17% genetic homology when compared to P. pseudomallei normalized to 100%. Considering other taxonomic criteria, we cannot accept this as genetic homology. In 2 SSC containing 30% DMSO, the Em approximates the Tm of native DNA over a wide range of incubation temperatures and the amount of non-specific binding of M. lysodeikticus was consistently very low. The renaturation solvent of choice was 2 SSC + 30% DMSO. 67°C was chosen as the most specific and restrictive temperature of renaturation and 0.01 SSC + 30% DMSO was the eluant. These conditions were used to determine the genetic relatedness of P. pseudomallei, A. mallei, and P. multivorans.

Table 4. Percent Annealing and Em of Hybrids in Different Solvents at Various Renaturation Temperatures.

Renaturation Temperature	Percent Annealing of P. pseudomallei DNA+ 2 SSC			2 SSC + 30% DMSO		
	P. pseudomallei	Em* Temp.	M. lysodeikticus	P. pseudomallei	Em Temp.	M. lysodeikticus
C	%	C	%	%	C	%
60	55.9	44.5	19.9	70.3	59	3.1
65	50.1	48.8	8.5	54.5	59.2	2.2
70	41.0	50.2	2.1	41.8	58	2.2
75	32.7	52.9	0.7	18.5	56.8	2.5

+ Ratio of radioactive DNA to DNA in agar is 1:50.

* Elution median temperature is only for P. pseudomallei DNA in agar. Em is obtained in 0.01 SSC containing 30% DMSO.

Table 5. Quantitation of Nucleic Acid Homologies in Agar Columns

	Per Cent Relatedness to:*	
	P. pseudomallei 4845	A. mallei 3873
P. pseudomallei 4845	Homologous	83.8 ± 2.1
P. pseudomallei 1691	93.4 ± 6.2	83.1 ± 3.3
A. mallei 3873	78.4 ± 8.8	Homologous
A. mallei 4	79.3 ± 3.7	86.3 ± 1.8
P. multivorans 249	18.9 ± 0.6	27.2 ± 2.3
P. multivorans 85	11.9 ± 1.4	19.6
P. multivorans 59	7.5 ± 1.9	10.7 ± 3.5
M. lysodeikticus	1.9 ± 0.4	1.4 ± 0.1

* Relatedness is expressed as percentage of heterologous binding when homologous binding is normalized to 100%. Results are presented as the mean and standard deviation of at least two determinations.

Radioactive DNA from P. pseudomallei 4845 and A. mallei 3873 were both used as references for nucleic acid homologies. As anticipated from the membrane filter experiments (Table 1) the DNA from P. pseudomallei and A. mallei were highly reactive with each other (Table 5). P. multivorans strains had DNA homologies of 7 to 19% with P. pseudomallei 4845 and 10 to 27% with A. mallei 3873. Calculations of the Em elicited almost the same temperatures for P. pseudomallei strains and A. mallei strains even in reciprocal hybridizations. The Em of P. multivorans strains when hybridized with P. pseudomallei and A. mallei was consistently 3 to 4°C below the reference strains.

These results are consistent with the physiological and serological data previously accumulated by other workers in substantiating the genetic relatedness of Pseudomonas pseudomallei and Actinobacillus mallei. In addition, we have corroborated Stanier's opinion (1966) that P. multivorans is somewhat genetically related to both of these organisms.

In our hands, nucleic acid homologies on MPF could not be used as a quantitative test for genetic relatedness but could be used as a qualitative screening test.

The hybridization of nucleic acids of high guanine and cytosine (GC) content presents special problems. The temperatures of denaturation and renaturation are proportional to the increase in GC of the DNA. In order to overcome this problem DMSO was added to lower the temperatures of denaturation and renaturation and still maintain specificity.

d. Intra-strain growth inhibition of P. pseudomallei. The ability of strains of P. pseudomallei to inhibit one another has been previously reported in WRAIR Annual Report 1966-67. The strains could be divided into two groups. One group was inhibitory to all strains. The other group was not inhibitory. Inhibition was correlated with the ability to produce alkaline conditions in agar media.

Fluid was expressed from agar cultures of inhibitor strains. The pH was found to be approximately pH 8.6. Wahba agar media was made up and buffered to pH 8.6 with Tris-HCl. All P. pseudomallei strains were capable of growing at this pH; thus, ruling out pH as an isolated inhibitory phenomenon.

Agar cultures of inhibitors were chloroformed and scraped. The plates were then treated as follows: series 1 - no treatment; series 2 - flooded with Tris buffer pH 8.6, drained and dried; series 3 - flooded with 0.2% pronase in 0.1M $CaCl_2$ pH 8.6 (Tris buffer soln), drained and dried; series 4 - flooded with 0.2% trypsin in pH 8.6 Tris buffer soln, drained and dried. P. pseudomallei strains were streaked at right angles to the original culture streaks. Pronase was found to reverse inhibition almost completely. Trypsin was less effective. These results suggested that the inhibitor may have been a protein or peptide.

Wahba agar media was made up with 0.6% Difco agar and another set with 0.3% agarose. Fluid was expressed from inhibitor cultures of both media. As yet, the expressed fluids from either media have not been shown to inhibit agar cultures of the organisms. It is assumed that this is either a matter of an inability to concentrate the inhibitory substance or that the inhibitory substance was retained by the agar and agarose matrix. The collapsed agar was placed in petri dishes and overlayed with a fresh agar preparation. This combination did demonstrate inhibition. Therefore, we do know that enough inhibitor is retained in the matrix to be demonstrable. However, when the collapsed agar was placed in bored out portions of fresh agar media, it did not inhibit the growth of subsequent bacteria in its immediate vicinity. This indicates that inhibition is concentration dependent.

During the course of this study it was noticed that some of the strains which were originally designated as inhibitors had lost their inhibitory properties on repeated subculture and cloning. The non-inhibitors never reverted to inhibitors. Finally, inhibition and alkalinity were correlated with smooth phase organisms. Noninhibition and acid production in Wahba agar was associated with rough looking colonies. The distinction between rough and smooth was most apparent on Difco brain heart infusion agar containing 3.0% glycerol. The correlation between rough dissociants and acid substance(s) production and smooth with alkaline has previously been noted by Nicholl (1932). We have not found this correlation in broth cultures.

This antagonism is reminiscent of the work of W. Braun (Bacterial Genetics, 2nd ed., 1965). He found that smooth cultures of Brucella abortus secreted large amounts of alanine which was toxic to smooth and rough strains. The rough strains were more resistant than the smooth, but still susceptible to inhibition. None of our results negate this possibility. The reversal of inhibition by proteolytic enzymes may possibly be due to the detoxifying ability of the protein properties of the enzymes rather than their specific enzymatic character.

We are presently investigating expressed fluids from cultures grown on protein-free media. An attempt is being made to determine a difference in amino acid secretions of inhibitory and non-inhibitory cultures.

Summary and Conclusions.

1. Antileptospiral Drug Screening.

A previously-developed in vivo drug screening system was used to test 190 compounds selected by the Department of Medicinal Chemistry, WRAIR. One compound, minocycline, had antileptospiral activity at all test dose levels.

2. Leptospirosis Surveillance.

Serological tests were done on subjects of 2 companies in Vietnam under surveillance for leptospirosis. Occurrence of infections during the surveillance period could not be affirmed by standard reference tests for leptospirosis. Reactions in conventional agglutination tests were seen in approximately 2% of 142 subjects. These antibodies may have reflected previous exposures to infection.

Serological studies employing microscopic and macroscopic agglutination tests were done on single, paired, or serial serum samples from 58 cases of leptospirosis diagnosed at the 9th U. S. Army Medical Laboratory. Tests affirmed 9th U. S. Army Medical Laboratory findings in 55 of 58 patients. The diversity of agglutinin responses in patients provided evidence that infections were incurred by a large variety of serotypes. The close correlation of macroscopic test findings with other procedures served to support the use of these tests in operational area laboratories. The serological studies provided additional evidence of the usefulness of a biflexa strain antigen (in microscopic-agglutination tests) for detecting antibodies provoked by diverse types.

Culture typing tests were done on 50 strains isolated in Malaysia to support epidemiological studies by USAMRU in Kuala Lumpur. The strains were related to types previously found in this country. A strain submitted previously was identified as a new serotype.

3. Melioidosis.

a. Serological studies. Armed Forces laboratories in Southeast Asia as well as CONUS were supported by provision of serological diagnostic reagents and by conduct of bacteriological and serological tests on specimens for laboratory diagnosis. Forty melioidosis cases in U. S. Armed Forces personnel in Southeast Asia were confirmed or established by laboratory tests. There were 18 other possible cases. To date a total of 89 cases have been established by laboratory tests at WRAIR. Sera from an additional 70 cases diagnosed at the 9th U. S. Army Medical Laboratory are being checked.

Collection of P. pseudomallei stock cultures of approximately 115 strains from various sources have been freeze-dried.

Serological evidence of melioidosis have been elicited in 5 laboratory personnel from 3 different laboratories handling P. pseudomallei.

b. Selective media for P. pseudomallei. A VCN medium (vancomycin 3.0 ug/ml, colistin 7.5 ug/ml, nystatin 12.5 U/ml) used for Meningococcus sp. could also be used for primary isolation of P. pseudomallei. A 3% glycerol brain heart infusion medium containing 10 U of penicillin and 100 U of polymyxin per ml was reevaluated and found to have no growth inhibitory properties for P. pseudomallei.

c. Genetic studies. The genetic relatedness of P. pseudomallei to phenotypically similar species and intra-species strain antagonism was studied. Nucleic acid homologies demonstrated a high degree of genetic homology between P. pseudomallei and A. mallei. P. multivorans strains were also found to be related to both of these organisms in the range of 7 to 27% genetic homology.

The specificity of nucleic acid hybridizations of DNA containing large amounts of guanine and cytosine was investigated. It was found that DMSO was needed to lower the temperature of renaturation and increase the specificities of this test.

d. Intra-Strain Growth Inhibition of P. pseudomallei. The use of bacteriocins as a tool in the epidemiology of P. pseudomallei does not seem feasible. The strains studied could be separated into two groups. One group inhibited the growth of all strains. The other group did not cause any inhibition. Inhibition was associated with smooth type colonies which caused the production of alkaline conditions in Wahba agar. Non-inhibitors were rough variants which produced acid conditions in agar media.

Publications.

1. M. Rogul, J. Brendle, D. K. Haapala, and A. D. Alexander. DNA homologies among Pseudomonas pseudomallei, Actinobacillus mallei and phenotypically similar organisms. Bacteriological Proceedings, 1968.

2. M. Rogul, S. R. Schwarting, and A. D. Alexander. Inhibition of Pseudomonas pseudomallei by strains of the same species. Bacteriological Proceedings, 1968.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY						
1. DATE OF SUMMARY: 01 67	2. TYPE OF SUMMARY: C. CHANGE	C. 67	3. SECURITY: U	U	4. WORK UNIT:	5. [illegible]

6. CURRENT NUMBER CODE: 6215601A 3A022001A841 00 [illegible]

7. TITLE: (U) PROPHYLACTIC USE OF GAMMA GLOBULIN TO PREVENT INFECTIOUS HEPATITIS

12. SCIENTIFIC OR TECH AREA	13. START DATE	14. CMPL. DATE	15. FUNDING AGENCY
003500 CLINICAL MEDICINE	01 67	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT				18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
	a. NUMBER	b. DATE					
C. IN-HOUSE	NA	NA	c. TYPE NA	d. AMOUNT NA	PRIOR FY 68	1	400
					CURRENT FY 69	1	80

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
MEDICAL RESEARCH	NA

23. KEYWORDS: HEPATITIS, GAMMA GLOBULIN, PASSIVE IMMUNIZATION.

24.

(U) TECH OBJECTIVE - TO ASCERTAIN IF THE PROPHYLACTIC ADMINISTRATION OF GAMMA GLOBULIN TO TROOPS IS EFFECTIVE IN REDUCING THE INCIDENCE OF CLINICAL HEPATITIS, TO DETERMINE THE PERIOD OF IMMUNITY AND THE DOSE REQUIRED.

(U) APPROACH- ALL PCS MALE U. S. ARMY PERSONNEL ASSIGNED TO EUSA RECEIVE AN INTRAMUSCULAR INJECTION AT THE AERIAL PORT OF ENTRY AND AGAIN FIVE MONTHS LATER. THESE INJECTIONS CONTAIN EITHER 2, 5, OR 10 ML. OF 16 PERCENT GAMMA GLOBULIN OR PLACEBO MATERIAL. THE INOCULATIONS ARE GIVEN IN A DOUBLE BLINDED MANNER BASED UPON THE LAST DIGIT OF THE SOLDIERS SERIAL NUMBER. DATA WILL BE ANALYZED BASED UPON THE INCIDENCE OF DOCUMENTED VIRAL HEPATITIS IN SOLDIERS RECEIVING EACH OF THESE MATERIALS.

(U) PROGRESS - JUL 67 THRU JUN 68 A WRAIR COMPOSITE TEAM WAS ASSIGNED TO KOREA DURING MAY 1967. THIS TEAM HAS ESTABLISHED AN INOCULATION AND RECORD CENTER AT THE AERIAL PORT OF DEBARKATION FOR ALL U. S. ARMY PERSONNEL ARRIVING IN KOREA. SOLDIERS ASSIGNED TO KOREA RECEIVE BIOLOGIC MATERIALS UPON ARRIVAL AND AGAIN FIVE MONTHS LATER. SCRUTINY OF ALL ADMISSIONS TO HOSPITALS IS MAINTAINED TO INSURE PROPER DOCUMENTATION OF HEPATITIS IN EACH STUDY PATIENT. FIFTY THOUSAND SOLDIERS HAVE RECEIVED INITIAL INOCULATIONS. COMPLIANCE WITH THE SECOND INJECTION AT FIVE MONTHS IS 68 PERCENT. SURVEYS OF HEPATITIS PATIENTS AND RANDOMLY SELECTED WELL SOLDIERS WERE INITIATED TO ASCERTAIN IF U. S. SOLDIERS WERE IMMUNE TO HEPATITIS CONTRACTED IN KOREA. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

TEXT NOT REPRODUCIBLE

27. COMMUNICATIONS SECURITY	28.	29. DOD CODE	30. BUDGET CODE
☐ a. COMSEC INFO ☑ b. NOT RELATED		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. DOD FORCES	36.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 28 identical to NASA Form 1127)

Project 3A025601A811 MILITARY MEDICAL RESEARCH PROGRAM S. E. ASIA

Task 02, Tropical and Subtropical Military Medical Research

Work Unit 308, Prophylactic use of gamma globulin to prevent infectious hepatitis

Investigators.
Principal: LTC Marcel E. Conrad, MC
Associate: CPT Bertram F. Felsher, MC, CPT George M. Bernier, MC, Joseph D. Boggs, M.D., Hans F. Smetana, M.D., and MSG 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.

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.

Progress

During 1964 a program was initiated by the U. S. Army to immunize all military personnel stationed in the Far East and Southeast Asia with gamma globulin to reduce the incidence of infectious hepatitis. Soldiers were injected with 10 ml of 16 per cent human serum gamma globulin shortly after arrival overseas and again five months later. It was hoped that this would produce passive immunity against hepatitis and that active immunity would develop through a subclinical infection. The escalation of the conflict in Vietnam increased the number of troops stationed in Asia and markedly reduced the amount of gamma globulin available in national stockpiles. During 1965, the recommended dose of gamma globulin was reduced to 5 ml twice during a one year tour in Asia and during 1966 the recommendation was made that the reduced dose be administered only to soldiers assigned to units with a continued

high incidence of hepatitis. Examination of the incidence of hepatitis reported from both Korea and Vietnam showed a marked and sustained reduction in the incidence of hepatitis from 1964-7 when compared to previous intervals. However, this reduction in incidence occurred several months before the initiation of the gamma globulin prophylactic program and could not be attributed to it. In addition, the incidence remained low following reduction in both the dose of gamma globulin and the number of troops included in the immunization program. This information and the relatively limited availability of gamma globulin made it desirable to ascertain the effectiveness of gamma globulin administration in a controlled study.

During May 1967 a field study was initiated in Korea in which soldiers assigned PCS to EUSA were given various doses of gamma globulin or placebo upon arrival and again five months later. Soldiers receive either: (1) 10 ml of 16 per cent human serum gamma globulin (20 per cent); (2) 5 ml of gamma globulin and 5 ml of an albumin-sucrose-potassium glutamate solution (20 per cent); (3) 2 ml of gamma globulin and 8 ml of control material (20 per cent) or (4) 10 ml of the placebo injection (40 per cent). All materials must be characterized for antibodies against known bacteria and viruses and for fragmentation and content of various gamma globulin components. Selection of soldiers for each injection is made based upon the last integer of their military serial number. The various materials used for injection are bottled in 10 ml containers marked with the ten integers, 0 through 9. Two integers are used for each material containing the various quantities of gamma globulin; (1), (2) and (3) above and four integers are utilized for control material. Each soldier receives the contents of a bottle labeled with a number matching the last number in his serial number upon arrival in the aerial port of debarkation in Korea and again in medical military dispensaries throughout Korea five months later. All cases of suspected hepatitis are evacuated to one of two military hospitals in Korea where the diagnosis is evaluated by both clinical and laboratory studies. Each documented case of hepatitis is verified as a study patient by maintenance of central immunization files at the aerial port of debarkation. It is believed that between 50,000 and 100,000 man years of study will be required to document the value or limitations of gamma globulin in the prevention of infectious hepatitis. During the period May 1967 through May 1968, 50,000 soldiers have received their initial immunizations. Icteric hepatitis is occurring in soldiers receiving materials labeled with all ten integers. The possibility that gamma globulin provides limited protection for less than five months is being considered in the study. However, insufficient information is available at present to make conclusions regarding this possibility. Lastly, it is possible that the etiologic agent of hepatitis in Korea is different from that in the U. S. This would make U. S. obtained gamma globulin ineffective to prevent hepatitis incurred by U. S. military

forces in Korea. The epidemiologic background of each soldier with hepatitis is under investigation to ascertain if selected portions of the U. S. population are more susceptible to hepatitis. Preliminary data indicate that most soldiers with hepatitis list rural home addresses. This might indicate that urban U. S. populations are relatively immune to hepatitis contracted in Korea; presumably because they were exposed to this "viral illness" at an earlier period of life. Lifelong geographical histories are being obtained from each patient and from a randomly selected population of well soldiers to ascertain if this can be documented. Establishment of an "immune" group among U. S. soldiers would permit more catholic interpretation of observations.

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 (3) produce the disease in animals to obviate the need for human studies. During this fiscal year serum was provided by Dr. Krugman from children with hepatitis at the Willowbrook School on Long Island. Oral administration of small aliquots of serum produced clinical hepatitis in adult volunteers; whereas randomly selected volunteers who received control material did not develop any evidence of hepatitis. The specimens collected in this successful study will be prepared for distribution to various laboratories within the next few months. It is hoped that the isolation of identical virus in several laboratories and the fulfillment of Koch's postulates with these isolates will permit identification of the causative agent of hepatitis and ultimately provide a vaccine to prevent the disease.

Summary and Conclusions

A double blinded field study was initiated in Korea by a WRAIR team to evaluate the usefulness of gamma globulin in the prevention of infectious hepatitis. This team has completed the first of three years of anticipated operation. The occurrence of hepatitis among volunteers has provided materials for distribution to laboratories attempting to isolate the causative agent of hepatitis.

Publications.

None.

PROJECT 3A025601A816
MILITARY MEDICAL MATERIEL

Task 01
Military Medical Materiel

RESEARCH AND TECHNOLOGY RESUME

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(U) MILITARY MEDICAL MATERIEL

13. SCIENTIFIC OR TECH AREA: 0000 MEDICAL AND HOSPI	13. START DATE: 06 62	14. CRIT. COMPL. DATE: NA	15. FUNDING AGENCY: OTHER DA
16. PROCURE METHOD: C. IN-HOUSE	17. CONTRACT/GRANT: NA; b. NUMBER: NA; c. TYPE: NA; d. DATE: NA; e. AMOUNT: NA	18. RESOURCES EST.: PRIOR FY 68; CURRENT FY 69	a. PROFESSIONAL MAN-YEARS: 3; 3 — b. FUNDS (Thousands): 100; 100

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21. TECHNOLOGY UTILIZATION: CLINICAL MEDICINE
TOXICOLOGY

22. COORDINATION: NA

INSTRUMENT, EQUIPMENT + SUPPLIES, CHEMISTRY, ANALYTICAL, SPECTROPHOTOMETRY, ELECTROPHORESIS, TOXICOLOGY, WATER PURIFICATIONS, GAS CHROMATOGRAPHY.

(U) TECH OBJECTIVE - TO DEVELOP EQUIPMENT AND PROCEDURES TO SUPPORT THE ARMY IN THE FIELD WHICH WILL REDUCE LOGISTICAL REQUIREMENTS AND IMPROVE EFFICIENCY. GREATER SPECIFICITY IN IDENTIFICATIONS AND REDUCTION IN ANALYTICAL TIME ARE SPECIFIC GOALS FOR TOXICOLOGIC ANALYSIS.

(U) APPROACH- THE STATISTICAL EVALUATION OF AVAILABLE PROCEDURES, WILL PROVIDE A BASIS FOR ACCEPTANCE OF PROCEDURES OR TO INDICATE FURTHER DEVELOPMENTS. EQUIPMENT PROTOTYPES WILL BE DEVELOPED TO MEET THE SPECIFIC REQUIREMENTS FOR SUPPORT OF MILITARY PROGRAMS.

(U) PROGRESS - JUL 67 THRU JUN 68 EVALUATION OF THE UNITEST SYSTEM IS COMPLETED. CAPABILITY INCLUDES GLUCOSE, CHOLESTEROL, PROTEINS, HEMOGLOBIN, URIC ACID, UREA NITROGEN AND ALKALINE PHOSPHATASE. THIS UNIT PERFORMS WELL AND MAY BE OF SIGNIFICANT VALUE FOR MANY-SMALL OR EMERGENCY APPLICATIONS. CONTRACTS HAVE PROVIDED FOR A SPECIAL FORCES WATER DISTILLATION UNIT AND FOR A SERIES OF DRY-PACK INTRAVENOUS SOLUTIONS. AN EVALUATION HAS SHOWN THAT IT IS ENTIRELY FEASIBLE TO PREPARE PURE WATER FOR INTRAVENOUS USE UNDER FIELD CONDITIONS. LABORATORY AUTOMATION OF GAS CHROMATOGRAPHY EQUIPMENT HAS PROVIDED A HIGHLY VERSATILE PROGRAM OF ANALYSIS FOR MULTIPLE COMPONENT SAMPLES FOR TOXICOLOGY AND CLINICAL EVALUATIONS. THE ULTRAMICRO SYSTEM FOR CLINICAL CHEMISTRY (USING 5-20 MICROLITERS OF SERUM) HAS ALLOWED THE STUDY OF METABOLIC CHANGE IN INFANTS DURING THE FIRST WEEK OF LIFE WITH GOOD SUCCESS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

23. [illegible]	24.	25. DOD CODE: AR	26. BUDGET CODE: 1
27. [illegible]: 14102-2--A-,14124-3-		28. PARTICIPATION: NA	
29. [illegible]	30. SPECIAL COMMENT		
31. [illegible]	32.		

DD FORM 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED [illegible]

Project 3A025601A816 MILITARY MEDICAL MATERIEL

Task 01, Military Medical Materiel

Work Unit 205, Military Medical Materiel

Investigators.

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SP 4 Malcolm Guilbeau

Description.

This project is designed to evaluate new concepts of materiel development for incorporation into the medical laboratories, or for the overall benefit of the Army Medical Department. Programs include an in-house effort for instrumental and technique improvements and an evaluation service for coordinating developmental contracts within the Medical Research and Development program.

Progress.

1. Automated analytical toxicology: In the interest of Drug Metabolism and Analytical Toxicology, new procedures are developed, techniques are adapted, and equipment is modified to meet the requirements for drug detection and distribution studies. Further developments directly concerning military operations were undertaken with evaluation of prepared kits for laboratory use and development effort to provide for pure water preparation under field conditions.

a. Development of laboratory automation continues to dominate the activities of this task. One of the most significant accomplishments in this regard was the modification of the gas chromatograph to operate completely unattended. With the Barber-Colman automatic injector system, samples entered the gas chromatograph by remote control, and the data output was collected, digitized and punched on IBM cards automatically. Recycling of the system was governed by a control module and timer which could be pre-set to any desired interval. The apparatus performed so effectively that further modifications were made to accommodate a second gas chromatograph to double the capability. Upon completion of the present series of tests designed to determine the total performance characteristics of the apparatus, this equipment will be utilized in drug studies intended to support the Food and Drug investigation of the effectiveness of brand

name drugs versus the same compounds commercially available under generic names. Drug distribution studies, pertinent to military medicine, will be one of the more important aspects of this investigation.

b. Recently, a computer program, developed and used by this laboratory for ultraviolet spectra identification of compounds of toxicological interest, was modified for the IBM 360 Model-30 computer. The purpose of this endeavor was to provide the Toxicology Section of the Armed Forces Institute of Pathology with the facility for rapid search of ultraviolet spectra compiled for the identification and quantification of suspect compounds encountered in toxicological analyses. Further distribution of this program to other Army Area Medical Laboratories is intended as soon as computer facilities become available to these installations. Other computer programs will be available as well for distribution, and new programs will be constantly under development to improve the precision and accuracy of laboratory procedures and to extend capabilities.

2. Evaluation of packaged procedure assemblies:

a. A simplified method for routine blood chemistries, as supplied by Bio-Dynamics, Inc., Indianapolis, Indiana, was evaluated from the potential standpoint of use within the military hospitals. The "Unitest" system includes complete analytical assemblies for the analysis of whole blood or serum for a number of components; nine were evaluated under this study. They include: Total protein, total globulin, bilirubin, hemoglobin, blood urea nitrogen (BUN), true glucose, total cholesterol, uric acid and alkaline phosphatase.

b. Each test was evaluated by performing 100 independent analyses on serum standards (both normal and abnormal), and comparing the data with the known values, and the values obtained with the present methods used in this laboratory. Factors including the variations due to individuals (i.e., technicians), analytical kit lot numbers, different brands of serum standerds and shelf life were evaluated. Complete statistical analysis of the data is currently being prepared.

c. Results show the "Unitest" system to be an excellent series of methods for the rapid and efficient performance of routine blood chemistries. The values obtained for each series were within an acceptable range for analysis. Additional favorable factors include low cost per analysis, lack of requirement for highly skilled technical personnel, favorable shelf life and light-weight and compactness.

3. Development of portable water still and prepackaged intravenous solutions: The preparation of packaged dry chemicals for reconstitution of solutions for intravenous use has proceeded to the final phases of laboratory evaluation. Under contract by the Medical Research and Development Command, Beckman Instruments has developed a portable water distillation system under guidelines provided by a Development Objective initiated by Special Forces requirements. The distillation unit, as provided, contains the following characteristics:

a. Weight characteristics: The total weight of the assembly, including the distillation unit, autoclave and sterilization cans, and an initial supply of "dry pack" chemicals for reconstitution into intravenous solutions, is less than 30 pounds and can easily be carried as a back pack, or dropped by parachute if necessary.

b. Materials: All components are of stainless steel or of approved plastic compositions.

c. Rate of production of distilled water using a flame source at 18,000 BTU input exceeds one gallon per hour. On charcoal, the still produces water at approximately one gallon per hour. Electrical heat may also be used. A wood fire has been unsatisfactory since sufficient heat transfer is not possible with this arrangement.

d. Quality control: A battery-operated monitor using the principles of conductivity furnishes a continuous means for checking quality of the water produced by the still. The monitor has both a visual blinking light and an audio-alarm to indicate that water exceeds the proper limits of safety. In evaluation tests this monitoring system has consistently operated well and when set at a three micromho conductivity range water quality has consistently met the specifications of the United States Pharmacopoeia for water for injection. Any samples not meeting the requirements of sterility have been traced to operator error.

e. Water source: The still has demonstrated satisfactory performance from a wide variety of water sources which include sea water, raw river water from highly polluted sources, diluted sewerage treatment influents, and potable water supply. From these water sources, the distillation unit has produced USP quality water with two minor exceptions. A water source such as the sewerage which contains a high ammonia or urea content will not remove the ammonia component. This problem is currently being evaluated to determine the critical levels of these components. The second area of concern was a demonstration that waters having a high organic content could cause foaming that would contaminate the product. This concern has been essentially eliminated by incorporation of a defoaming screen assembly over the boiler component.

f. Terminal sterilization: The portable autoclave has proven to be the largest technical problem. Plastic bags cannot be sterilized directly in an autoclave without special considerations since the bags expand and burst under normal autoclaving processes. A satisfactory method has been devised however which allows processing of four one-liter units of solution. Each bag is placed in a metal container which is sufficiently sealed to prevent any air leaks and to securely enclose the bag. So long as this geometry is maintained, the bags may be terminally sterilized to add further assurance of safety. The autoclave operates on the same fuel sources as described for the still with a one-hour sterilization cycle for the bags. The autoclave and still, therefore, are operating at the same production

levels with four units of intravenous solutions being produced per hour.

g. Maintenance requirements: The components of the still and autoclave are so constructed that maintenance is very low and can be accomplished by any operator after simple instruction. Scaling of the boiler has not been a problem, even with sea water, due to the construction of this stainless steel which expands and contracts sufficiently during operation to break scale loose. This is then carried away in the waste component.

h. Utility requirements: No plumbing connections needed. The unit will operate from Lyster bags or any other water supply which will provide cooling water and feed water. These may be improvised readily under most any conditions where the still would logically be operated.

i. Solutions provided: Travenol Laboratories, under subcontract with Beckman Instruments, have provided an improved plastic bag with a series of five "dry pack" reagent assemblies. The five intravenous solutions which are reconstituted have been submitted to the Food and Drug New Drug Development Division (FDA) for approval for human use. These include the following:

NDA 16-673	5% Dextrose
NDA 16-677	0.9% Sodium Chloride
NDA 16-678	5% Dextrose in 0.9% Sodium Chloride
NDA 16-692	M/6 Sodium Lactate Injection
NDA 16-694	10% Dextrose

The preliminary clinical evaluation data for the assemblies have been provided to FDA by the manufacturer. The efficacy of the production of USP quality water will be filed with the FDA as soon as the current series of evaluations have been completed.

j. Evaluation of pre-production models: Three stills are currently being evaluated as are the five "dry pack" assemblies. Approximately two hundred pyrogen tests have been used to evaluate the products. At this preliminary stage it has been demonstrated that the distillation assembly will produce USP quality water from highly contaminated water collected from the Potomac River with very high assurance of safety. Minor problems in the prototype units have been assessed and can easily be overcome prior to final-type fabrications. It would appear that most technical problems have been overcome in developing a system for constitution of intravenous solutions under relatively primitive conditions. Training problems for user personnel are considerably simplified and a variety of water sources may be safely used.

Summary and Conclusion:

In addition to other equipment that has been modified for computer automation, the gas chromatograph provides a potential for the complete systematic approach to automated analytical toxicology. If a small computer could be obtained to operate "on line" with this system, analysis

time could be reduced to a small fraction of what is now required. Off-line computer processing by an independent data processing center has produced considerable delays which have reduced overall efficiency and offset most of the time reduction accomplished by this laboratory automation system.

Prepackaged procedural assemblies have been evaluated and have been shown to perform well for clinical chemistry procedures. These procedures are especially appropriate for the small laboratory and for night emergency applications.

A system for production of pyrogen-free, sterile distilled water has been developed by a commercial contract. Evaluation of pre-production models has demonstrated that USP quality water may be produced under primitive conditions from a variety of fuel sources and may be safely used to reconstitute "dry pack" intravenous solution assemblies. Only minor problems remain before a production unit may be manufactured. Food and Drug New Drug Development evaluations have been filed and are currently under evaluation.

Publications.

Kazyak, L., Knoblock, E. C., and Permisohn, R. C. The Determination of Combinations of Quinine, Chloroquine, and Pyrimethamine in Antimalarial Therapy. Proc. Am. Chem. Soc., 31 May 68, San Francisco, California

PROJECT 3A025601A821
COMBAT SURGERY

Task 01
Combat Surgery

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CA6466	CSGD-167

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY CLASS	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	O. CHANGE 01 07 67	U U	NA	CA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A821 01 120

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) WOUND HEALING

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. ORIG. COMPL. DATE	15. FUNDING AGENCY
002500 CLINICAL MEDICINE 012900 PHYSIOLOGY	09 64	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT			18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
	NA	a. DATE		PRIOR FY 69	5	140
C. IN-HOUSE	b. NUMBER NA c. TYPE NA	d. AMOUNT NA		CURRENT FY 69	5	140

19. GOVT. LAB/INSTALLATION/ACTIVITY

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INVESTIGATORS PRINCIPAL: NATSUMOTO, LTC T.
ASSOCIATE: MOSELEY, CPT R. V.
TEL: 202-576-3791 TYPE: DA

21. TECHNOLOGY UTILIZATION: EXPERIMENTAL SURGERY

22. COORDINATION: NA

23. KEYWORDS: WOUND HEALING, AEROSOL TISSUE ADHESIVE SPRAY, LIQUID PROPELLANT, CROSS-LINKED GELATIN COMPOUND, TOPICAL ANTIBIOTICS, CONTAMINATED CRUSH WOUND.

24.

(U) TECH OBJECTIVE - 1. TO EVALUATE THE EFFICACY OF AEROSOL TISSUE ADHESIVE SPRAY, VARIOUS HEMOSTATIC AGENTS, + TISSUE ADHESIVE IN EXPERIMENTAL SURGERY. 2. TO EVALUATE EFFICACY OF TOPICAL ANTIBOTIC SPRAY IN CONTAMINATED CRUSH WOUNDS IN EXPERIMENTAL ANIMALS.

(U) APPROACH- 1. AEROSOL TISSUE ADHESIVE SPRAY- A. SELECTION OF MONOMER, DEVELOPMENT OF SURGICAL TECHNIQUES + HISTOLOGIC STUDY COMPLETED. B. LONG-TERM TOXICITY + PHARMACOLOGIC STUDY, INFLUENCE ON WOUND HEALING, BACTERIOLOGIC STUDY OF BUTYL CYANOACRYLATE MONOMER + LIQUID PROPELLANT EVALUATED. 2. CROSS-LINKED GELATIN COMPOUND- HISTILOGIC STUDY + SURGICAL TECHNIQUE IN HEMOSTASIS OF SOLID ORGAN WITH THIS COMPOUND EVALUATED. 3. TOPICAL ANTIBIOTIC SPRAY- MORTALITY + BACTERIOLOGIC STUDY EVALUATED IN CRUSH WOUNDS OF RABBITS + GUINEA PIGS USING NEOSPORIN + OXYTETRACYCLINE TOPICAL SPRAY.

(U) PROGRESS - JUL 67 THRU JUN 68-1. AEROSOL TISSUE ADHESIVE SPRAY- STERILE INDIVIDUALLY DISPOSABLE SMALL SPRAY UNIT CONTAINING BUTYL CYANOACRYLATE MONOMER + LIQUID PROPELLANT HAS BEEN DEVELOPED IN THIS DIVISION. 2. AEROSOL TISSUE ADHESIVE SPRAY HAS BEEN TESTED + HAS BEEN FOUND THAT A. SMALL AMOUNT OF LIQUID PROPELLANT (FREON) DOES REMAIN IN THE POLYMER FILM WITHOUT INCREASING TISSUE REACTION MORE THAN THAT CAUSED BY CYANOACRYLATES. FREON REMAINING IN LIVING TISSUE MAY BE EXHALED FROM LUNG THROUGH BLOOD SUPPLY. B. CYANOACRYLATE IS NOT BACTERICIDAL OR BACTERIOSTATIC. C. CYANOACRYLATE DELAYS WOUND HEALING WHEN USED FOR BONDING OF TISSUE. D. LONG-TERM STUDY, INCLUDING TWO GENERATIONS IN RATS + MICE, INDICATES NO EVIDENCE OF TUMOR FORMATION. 2. FROM THE ABOVE FINDINGS, THE CLINICAL INDICATION OF AEROSOL TISSUE ADHESIVE IS USE FOR HEMOSTASIS OF WOUND SOLID ORGAN OR SEALING OF BLEEDING SUTURE LINE WHEN OTHERWISE FATAL. 3. CROSS-LINKED GELATIN COMPOUND REVEALED SEVERE TISSUE REACTION + REQUIRED FURTHER IMPROVEMENT IN METHOD OF APPLICATION. 4. TOPICAL ANTIBIOTIC SPRAY PREVENTS INFECTION + REDUCES MORTALITY BY LIMITING BACTERIAL COUNT ON CONTAMINATED CRUSH WOUND. SATISFACTORY RESULTS OBTAINED WHEN LARGE AMOUNT OF NEOSPORIN OR OXYTETRACYCLINE WAS SPRAYED ON THE WOUND SOON AFTER INJURY. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ A. CONTROL OBTAINED ☑ B. NOT REQUIRED		AR	1

31. PROJECT NO.	32. PARTICIPATION
1435A	NA

33. KEYWORDS ADDED	34. SPECIAL EQUIPMENT
35. REMARKS ADDED	36.

DD FORM 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 35 identical to NASA Form 1122)

Project 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 120, Wound healing

Investigators.
Principal: LTC Teruo Matsumoto, MC
CPT Thomas B. Ducker, MC; BG George J. Hayes, MC*; CPT Paul B. Lamborn, Jr., VC; CPT Henry B. Soloway, MC; MAJ Donald R. Smith, MC**; COL Ludwig G. Kempe, MC**

Description.

1. Cyanoacrylate tissue adhesives. N-alkyl-alpha-cyanoacrylate monomers are known for their ability to adhere to moist living tissue. It was thought that the success of surgical application of cyanoacrylate tissue adhesives depended on the development of new surgical techniques suitable for use with the monomers, a simple method of application, and the selection of a suitable monomer.

After intensive experimental work during the last year, butyl and isobutyl cyanoacrylate monomers have been selected as ideal monomers and aerosol tissue adhesive spray has been developed. Study was focused on the (a) toxicity of propellant, (b) long-term effect on experimental animals in which various cyanoacrylate monomers were used, (c) bacteriological characteristics of these monomers, and (d) other tissue adhesive -- cross-linked gelatin compound.

2. Topical spray of antibiotics. The principal treatment of a massive open wound in combat is debridement. However, in a military situation, debridement may be delayed for a period of several hours to days. A wound untreated for this length of time becomes infected. There is no recommended treatment except dressing for the wound during the delay period. The rationale for local use of antibiotics is that, before debridement, large amounts of vascular dead tissue cannot deliver a systemic drug to the wound surface and, in addition, very high levels of drug concentration can be attained at the wound surface. The purpose of this work was to determine the efficacy of topical antibiotics sprayed on large contaminated crush wounds and to select the best available antibiotic. During the current year the main effort was concentrated on determining (a) time factor, (b) dose factor, and (c) selection of antibiotics. In addition, the efficacy of systemic antibiotics was also evaluated.

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3. Evaluation of homologous alpha-alkyl-cyanoacrylates in skin wound closures. The intention of this project is to evaluate the use of methyl-2, n-butyl and isobutyl cyanoacrylates for closing skin wounds. Silk suturing is used as the control for comparison.

4. Peripheral nerve injury. Studies are designed to improve the repair of severed peripheral nerve and the functional result after repair.

5. Peripheral nerve injuries, nerve grafts. A two part study was carried out. Part I dealt with the best kind of graft and Part II was concerned with the maximum practical length of an irradiated graft in chimpanzee.

6. Cerebral vascular injury. A study in monkeys to evaluate methods of preserving cerebral blood flow in an injured cerebral vessel has been carried out.

Progress.

1. Cyanoacrylate tissue adhesives.

a. Toxicity of propellant. Aerosol tissue adhesive spray introduces a small amount of Freon into the body. This results from physical trapping of Freon in the polymer film and not from any chemical change in the monomer. Freon trapped in the polymer appeared to be absorbed by the blood and eliminated from the lungs.

Histological and functional examinations of each organ revealed no apparent pathology resulting from the use of Freons as propellants; findings were similar to those in which the monomer was sprayed by a nitrogen-activated spray gun.

b. Long-term effect on experimental animals in which various cyanoacrylate monomers were used. (1) No tumor formation, either gross or microscopic, was seen in a series of dogs followed up to two years. (2) Similar results were found in rats and mice, as well as in the following generation of rats born to treated animals. (3) Twenty-seven dogs still alive show no symptoms or evidence of tumor formation at this time.

c. Bacteriologic characteristics of these monomers. Bacteriologic studies indicate that methyl, isobutyl, and normal butyl, cyanoacrylate monomers have neither bacteriostatic nor bacteriocidal properties for either vegetative or spore forms of Staphylococcus epidermidis, Staphylococcus aureus, lactose-fermenting enterobacteria, Pseudomonas, Clostridia, and group D Streptococcus.

d. Other tissue adhesive -- cross-linked gelatin compound. In an experimental study in which we compared cross-linked gelatin with cyanoacrylate monomers as hemostatic agents and as tissue adhesives, we have

found that cross-linked gelatin compound effectively controlled hemorrhage from experimental liver wounds, but the method of application of this compound was complicated and time-consuming compared to that for applying cyanoacrylate by aerosol spray. The tissue reaction due to cross-linked gelatin compound were more than those found in hepatic tissue in which butyl cyanoacrylate was sprayed. The gelatin on the wound surface disappeared from the body within six months, while butyl cyanoacrylate polymer did not disappear from tissue within six months after surgery.

2. Topical spray of antibiotics.

Oxytetracycline was topically sprayed on the contaminated crush wounds of animals' thighs at various time intervals. The study indicated that maximum effect of maintaining bacterial counts at low levels and reducing the hazard of infection was obtained when the oxytetracycline was sprayed within five minutes after injury, whereas minimum benefit was noted from spraying the wounds with oxytetracycline four hours or more after injury.

When Neosporin, 15 times the recommended dose, was used, the mortality was the same as those experiments in which recommended dose of oxytetracycline (200 times the minimal inhibitory concentration) was sprayed.

3. Evaluation of homologous alpha-alkyl-cyanoacrylates in skin wound closures. The data have shown the three homologous glues to have a higher tensile strength than sutures prior to five days postoperative. The suture wounds, however, surpassed all three of the glued wounds thereafter.

4. Peripheral nerve injury. Three important contributions: (a) The proper size of a silastic cuff to fit over a repair has been worked out in detail. This study was carried out after studies last year showed the thin silastic as the best cuffing material. (b) Improving the functional result after nerve repair has been worked out by CPT Dan Donaghue in a study on muscle rehabilitation. (c) Clinical trials of the improved technique have been done in the hospital.

5. Peripheral nerve injuries, nerve grafts. Of all the grafts available, an autograft is the best. Second best is an irradiated homograft, but this graft is more readily available.

The maximum of which a high degree of success can be anticipated in irradiated nerve graft in higher primate (chimpanzee) is 4 cm. At 5 or more centimeters the graft success rate drops from over 80 per cent to 30 per cent.

6. Cerebral vascular injury. The experiment has been completed and indicated that transient, massive heparinization will help maintain cerebral cellular integrity while a vessel is repaired by microscopic techniques.

Summary and Conclusions.

1. Cyanoacrylate tissue adhesives. Further study of tissue adhesive indicated that there are no ideal tissue adhesives available at the present time. The best adhesives, at present, are n-butyl and isobutyl cyanoacrylate monomers in aerosol spray and, on some occasion, drop bottle form. These adhesives degrade slowly and therefore it is essential to continue more intensive and longer-term study, although life time study with small animals was encouraging. Presence of polymer film between bonded tissue limits the application of these adhesives in surgery. Hemostasis and sealing of wound in solid organ and suture line reinforcement with or without coagulation disorder are the areas in which these monomers are useful. These monomers should be used only as a lifesaving measure after all conventional surgical methods fail.

2. Topical spray of antibiotics. Topical applications of adequate doses of oxytetracycline or Neosporin from individually disposable spray units to large contaminated crush wounds of the thighs of rabbits reduced mortality from 64 per cent in the control group to 4 per cent in the experimental if the wounds were sprayed within five minutes after injury. Local antibiotic therapy is not advocated as a substitute for debridement of these kinds of wounds, but it does retard bacterial growth and thus prolong the "golden period" until debridement can be effected.

3. Evaluation of homologous alpha-alkyl-cyanoacrylates in skin wound closures. The histology confirms the postulation that the glues inhibited collagen bridging and thereby with time the uninhibited sutured wound becomes stronger more rapidly than the glued wound. After six to seven weeks the rapidly rising tensile strength values for both wounds level off and approximate each other.

4. Peripheral nerve injury. Advances in peripheral nerve repair have been made with a thin silastic cuff of proper dimensions.

5. Peripheral nerve injuries, nerve grafts. Properly prepared irradiated homografts of 4 or less centimeters can be anticipated to succeed in man.

6. Cerebral vascular injury. Massive heparin may be used to prevent intravascular coagulation while repairing a cerebral vessel for a period of 15 to 30 minutes. Then it is reversed by protamine, and there are no complications of bleeding in completing the craniotomy.

Publications

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 066467	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	NOT U / MOD U	NA	GA	A. DOD LIMIT

10a. CURRENT NUMBER/CODE: 62156011 3A025601A821 00 121

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) RESPONSES TO TRAUMA

12. SCIENTIFIC OR TECH AREA	13. START DATE	14. CMPL. COMPL. DATE	15. FUNDING AGENCY
003500 CLINICAL MEDICINE 012900 PHYSIOLOGY	09 63	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT			18. RESOURCES EST	a. PROFESSIONAL MAN-YEARS	b. FUNDS (IN THOUSANDS)
	a. NUMBER	c. DATE				
C. IN-HOUSE	NA			PRIOR FY 68	3	70
	b. TYPE NA	d. AMOUNT NA		CURRENT FY 69	3	70

19. GOVT. LAB/INSTALLATION/ACTIVITY
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ASSOCIATE: SIMMONS, CPT R. L.
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21. TECHNOLOGY UTILIZATION: CLINICAL SURGERY

22. COORDINATION: NA

KEYWORDS: HEMORRHAGIC SHOCK, TOXIC SHOCK, IRREVERSIBLE SHOCK, MICROCIRCULATION, MICROANGIOGRAPHY, VITAL MICROSCOPE, ALPHA ADRENERGIC BLOCKING AGENT.

24

(U) TECH OBJECTIVE - TO IMPROVE THE METHODS OF DIAGNOSIS AND TREATMENT OF THE SEVERELY INJURED TO MINIMIZE MORBIDITY AND MORTALITY ASSOCIATED WITH COMBAT TRAUMA.

(U) APPROACH- 1. STUDIES HAVE BEEN UNDERTAKEN ON LABORATORY ANIMALS. AS AN EXTENSION OF OUR ORIGINAL FINDINGS IN THIS DIVISION THAT SHOCK OF ALL CAUSES IS DUE TO POOR CAPILLARY PERFUSION, FURTHER EVALUATION WAS CONTINUED USING MICROANGIOGRAPHY TECHNIQUE AND VITAL MICROSCOPE. 2. THE NERVOUS SYSTEM RESPONSE TO TRAUMA, CIRCULATING RESPONSES TO INCREASED INTRACRANIAL PRESSURE WERE EVALUATED. 3. IN ADDITION, STUDY OF PULMONARY PATHOLOGY IN ACUTELY TRAUMATIZED ANIMALS AND PATIENTS WAS INITIATED.

(U) PROGRESS - JUL 67 THRU JUN 68 - 1. THE SHOCK STUDIES HAVE DOCUMENTED THE CONSUMPTION OF ALL THE PLASMA CLOTTING FACTORS IN THE SHOCK STATE. OUR INITIAL RESULTS SUGGEST THAT TO OBTAIN EFFECTIVE CIRCULATING BLOOD VOLUME, AS DETERMINED BY PULMONARY ARTERY PRESSURE, CENTRAL VENOUS PRESSURE AND RADIOACTIVE BLOOD VOLUME, MUCH LARGER VOLUMES OF FLUID REPLACEMENT THAN CONVENTIONALLY DEEMED APPROPRIATE, ARE NEEDED. IN ADDITION, MECHANICAL VOLUME CYCLED RESPIRATORY SUPPORT, VASODILATION USING PHENOXYBENZAMINE, CORRECTION OF THE METABOLIC ACIDOSIS AND REVERSAL OF FREQUENTLY PRESENT CLOTTING ABNORMALITIES HAVE GIVEN STRIKINGLY GOOD RESULTS IN OUR INITIAL STUDY GROUP. 2. ROLE OF CAPILLARY PERFUSION IN HEMORRHAGIC SHOCK WAS DEMONSTRATED BY VITAL MICROSCOPIC OBSERVATION. EFFICACY OF ALPHA ADRENERGIC BLOCKING AGENT FOLLOWED BY EPINEPHRINE WAS DEMONSTRATED IN THE ABOVE OBSERVATION. WR-2823, A NEW SHORT ACTING ALPHA ADRENERGIC BLOCKING AGENT DEVELOPED AT WRAIR, WAS ALSO TESTED AND PROVED TO BE EFFECTIVE. 3. TECHNIQUES OF MICROANGIOGRAPHY HAVE BEEN ESTABLISHED AND EVALUATION OF PULMONARY VASCULAR PATHOLOGY FOLLOWING SEVERE TRAUMA IS BEING EVALUATED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

27. DOD COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC EVALUATION ☒ b. NOT RELATED		AO	1

31. WORK UNIT	32. PARTICIPATION
143EA	NA

33. PROCESSING AGENCY	34. SPECIAL EQUIPMENT

35. KEY WORDS (OPTIONAL)	36.

DD FORM 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 28 identical to NASA Form 1122)

Project 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 121, Responses to trauma

Investigators

Principal: COL Harold F. Hamit, MC

Associate: LTC Teruo Matsumoto, MC; CPT Thomas B. Ducker, MC; CPT Richard L. Simmons, MC; BG George J. Hayes, MC*; CPT Irwin R. Berman, MC; CPT Roger V. Moseley, MC; CPT Paul B. Lamborn, Jr., VC; H. Kenneth Sleeman, Ph.D.; CPT Arthur M. Martin, Jr., MC; CPT Henry B. Soloway, MC

Description.

1. Microcirculation of experimental animals in shock. Blood pressure is measured as one index of adequacy of circulation. Circulation of blood delivers adequate oxygen to tissue and carries away metabolic products. Adequate function depends on the volume of blood flow and rate.

The purpose of this study was to observe and analyze the effect of various drugs on microcirculation of the mesentery, bowel, and conjunctiva in dog and guinea pig, monkey, and baboon before and after (a) hemorrhagic shock and (b) malaria infection and to observe and analyze the microcirculation in (a) wounded liver and kidney and (b) crush wounds in soft tissue.

2. Spinal cord injuries. A comparative evaluation of treatments for acute spinal cord injury by which we used a designed experimental model to deliver a 275 gm-cm force to the spinal cord. Standard laminectomy, local cord hypothermia, intramuscular decadron, and intrathecal depomedral. All treatment was started three hours after injury and neurologic functional recovery was compared.

3. Craniocerebral injuries. Studies have been carried out to describe the cardiovascular hemodynamic response to increased intracranial pressure.

4. Studies were designed to obtain information pertaining to perfusion of splanchnic, somatic, and pulmonary capillary beds after increased intracranial pressure.

5. Since thoracic duct lymph is primarily an effusate of hepatosplanchnic tissues, consumption of oxygen and production of carbon dioxide and lactate as reflected in thoracic duct lymph have been employed as indirect parameters of splanchnic perfusion. Lysosomal enzyme levels in

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thoracic duct lymph have also been determined as measures of splanchnic cellular dysfunction.

6. A new instrument has been designed based on the Furniss clamp principle which is capable of producing rapid, precise suture plication of the inferior vena cava. Studies were performed to determine the capability of the instrument in producing effective plication without stasis or thrombosis at the plication site.

7. Pathology of pulmonary oxygen toxicity. Pulmonary hyaline membranes are not uncommon findings at autopsy in cases of shock or trauma. Because of the similarity of this change to that previously described after prolonged oxygen therapy, experimental studies have been undertaken to determine the possible role of oxygen in the etiology of this pulmonary change in these patients.

Progress.

1. Microcirculation of experimental animals in shock.

a. Majority of diagnostic observations had been reported in 1967.

b. Portal microcirculation and systemic microcirculation were somewhat different in response to trauma.

c. The major difference was development of aggregation, sludging and stasis in portal venule in shock.

d. The above difference was remarkable in dog and not extensive in monkey.

e. Dibenzyline (at least 5 mg/kg) dilated capillaries and increased microcirculation.

f. Epinephrine constricted capillaries and created multiple shunting between arterioles and venules.

g. Low molecular weight dextran increased diameter of capillaries and improved microcirculation.

h. WR-2823 was found to be an effective short acting alpha adrenergic blockade from the microcirculation view point.

i. Microcirculatory studies performed upon monkeys with P. knowlesi malaria showed sludging of blood elements within the microvasculature. This sludging began at 72 hours after intravenous transmission of infection and became progressively more severe with time. Aggregates of cells remained static within the microvasculature. Many capillaries separating these aggregates contained only plasma, and showed constriction of their lumina. To reverse this sludging which was thought to be

due in large part to capillary constriction, mice with malaria were given varying doses of phenoxybenzamine (Dibenzyline), an alpha adrenergic blocking agent. At doses of 30 mg/kg phenoxybenzamine treated mice showed significantly increased survival times compared to controls.

j. The response of the microcirculation and fibroblast proliferation in wounded liver, kidney and skin after hemostasis or reapproximation with sutures and cyanoacrylate tissue adhesive was studied.

The presence of cyanoacrylate polymer fragments between the reapproximated tissues definitely delayed wound healing by preventing the proliferation of the fibroblasts and microcirculatory vessels bridging the wounded surfaces.

Striking proliferation of microcirculatory vessels including capillaries, arterioles, and venules reached a maximum degree at 72 hours to one week then gradually subsided, regardless of the use of suture or tissue adhesive.

2. Spinal cord injuries. The study has been completed and a paper written.

3. Craniocerebral injuries. These studies have been completed and papers written.

4. Pulmonary venous admixture and somatic and splanchnic arteriovenous shunting follow increased intracranial pressure produced in dogs by an extradural balloon. Dibenzyline and cordotomy abolish pulmonary shunting. Adrenalectomy abolishes splanchnic shunting. Propanolol abolishes somatic shunting. Peripheral shunting is increased following cervical vagotomy and atropine.

5. Studies indicate that thoracic duct lymph is a more direct indicator of splanchnic perfusion and anaerobiosis than is arterial or venous blood. Lymph drainage affords lower levels of lysosomal enzymes in blood of shocked animals.

6. Thirty-five canine studies have been followed up to 17 weeks. Plication is rapid and precise and prevents passage of emboli 3 mm or greater in diameter. Cavagrams show intravascular streaming of dye, with only one instance of thrombosis.

7. Pathology of pulmonary oxygen toxicity. An experimental model of oxygen toxicity was developed which produced an LD_{50} in mature guinea pigs after 48 hours of 100% oxygen. This model has been utilized for two studies which are currently in progress. The first study concerns the acute and long-term effects of acute oxygen toxicity on the pathologic anatomy of the lungs. In the acute portion of the study animals are sacrificed shortly after the period of oxygen exposure. In these lungs there are extensive hyaline membranes throughout the alveolar spaces and

terminal bronchioles. There appears to be an acute necrosis of bronchiolar walls in conjunction with these membranes. In the chronic portion of the study the guinea pigs which survived an LD_{50} exposure to oxygen were killed three months later. Animals already sacrificed show mild emphysema and medial hyperplasia of pulmonary arteries in comparison to control animals. These findings are preliminary and the studies are continuing.

Summary and Conclusions.

1. Microcirculation of experimental animals in shock and malaria infection (P. knowlesi) revealed a significant change in the size of capillaries and venules, appearance of circulating blood cells (aggregation and sludging) and surrounding tissue.

Capillary proliferation in the repaired wound with cyanoacrylate monomer delayed considerably compared to that in wound repaired with sutures.

2. Spinal cord injuries. Both local cord hypothermia and intramuscular decadron improved neurological function recovery to a small, but definite, degree.

3. Craniocerebral injuries. A systematic integrated cardiovascular hemodynamic response to increased intracranial pressure has been shown to include, in order of occurrence: (a) systemic venoconstriction, (b) an inotropic effect on the heart, (c) increased cardiac output, and finally (d) increased arterioconstriction and a rise in peripheral resistance. The mechanism of pulmonary edema in this setting has also been described.

4. Evidence is provided that the hypoxemic response to increased intracranial pressure is mediated by alpha receptor stimulation; somatic and splanchnic shuntings are mediated by beta receptor stimulation. A potential role of peripheral arteriovenous shunting in predisposition toward neurogenic pulmonary edema is suggested.

5. Flow rate and composition of thoracic duct lymph vary in a predictable manner with splanchnic hypoperfusion in endotoxin and hemorrhagic shock. Thoracic duct drainage modulates levels of blood lysosomal enzymes in shock. Evidence is provided that lysosomal enzymes gain access to the circulation via the lymphatics.

6. Inferior vena caval plication with the new instrument results in rapid, precise plication with effective trapping, absence of stasis, prolonged patency, and no foreign body other than the suture itself.

7. Pathology of pulmonary oxygen toxicity. Studies with isobaric 100% oxygen have produced an experimental model in guinea pigs with an LD_{50} after exposure to 48 hours of oxygen. Preliminary studies revealed

extensive hyaline membranes lining the alveolar walls and terminal bronchioles and possible acute necrosis of bronchiolar walls in animals sacrificed shortly after exposure to oxygen. Chronic studies have indicated a possible relationship to the production of chronic pulmonary changes of mild emphysema and pulmonary artery hyperplasia.

Publications.

1. Matsumoto, T., Hardaway, R. M., and Heisterkamp, C. A.: Determination of muscle viability after trauma. Arch. Surg. 94: 794, 1967.

2. Matsumoto, T., Hardaway, R. M., and McClain, J. E.: Microcirculation in hemorrhagic shock with relationship to blood pressure. Arch. Surg. 95: 94, 1967.

3. Matsumoto, T., Hardaway, R. M., and Heisterkamp, C. A.: Determination of the viability of muscle soon after wounding. Modern Med. 25: 78, 1967.

4. Jennings, P.B., Matsumoto, T., and Hardaway, R. M.: Sublingual venography: evaluation of a new method to study cervical venous and superior vena cava blood flow. Angiology 19: 36, 1968.

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6. Ducker, T. B.: Increased intracranial pressure and pulmonary edema. I. A clinical study of eleven cases. J. Neurosurg. 28: 112, 1968.

7. Ducker, T. B., and Simmons, R. L.: Increased intracranial pressure and pulmonary edema. II. The hemodynamic response of dogs and monkeys to increased intracranial pressure. J. Neurosurg. 28: 118, 1968.

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6448	CSCD-100

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY PET	WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	U	U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE
62156011 3A025601A821 00 122

10b. PRIOR NUMBER/CODE

11. TITLE
(U) EXPERIMENTAL ANESTHESIA

12. SCIENTIFIC OR TECH. AREA		13. START DATE	14. CMPL. COMPL. DATE	15. FUNDING AGENCY
012900 PHYSIOLOGY	008800 LIFE SUPPORT	05 58	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT		18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (IN THOUSANDS)
	NA	a. DATE	PRIOR FY 69	3	45
C. IN-HOUSE	b. NUMBER NA c. TYPE	d. AMOUNT NA	CURRENT FY 69	3	45

19. GOVT. LAB/INSTALLATION/ACTIVITY

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21. TECHNOLOGY UTILIZATION
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22. COORDINATION
NA

23. KEYWORDS
ANESTHESIA,, PULSATILE, BLOOD PUMP, RESPIRATION OXYGEN TOXICITY, PULMONARY INSUFFICIENCY, RESUSCITATION MEMBRANE OXYGENATOR, HEART MONITOR.

24.

(U) TECH OBJECTIVE - TO DEVELOP EQUIPMENT FOR RESUSCITATION AND SUPPORT OF CIRCULATORY AND RESPIRATORY SYSTEMS IN CRITICALLY INJURED PATIENTS AND TO STUDY THE EFFECTS OF PROLONGED ADMINISTRATION OF HIGH CONCENTRATIONS OF OXYGEN AND MECHANICAL VENTILATION ON PULMONARY FUNCTION.

(U) APPROACH- MECHANICAL DEVICES HAVE BEEN DESIGNED AND BUILT BY THE HARRY DIAMOND LABORATORIES TO SPECIFICATIONS PARTLY SUPPLIED BY THE DEPARTMENT OF ANESTHESIA AND RESUSCITATION, WRAIR. MEDICAL EVALUATION OF THESE DEVICES IS CARRIED OUT AT WRAIR. HUMIDIFIED OXYGEN AT AMBIENT TEMPERATURE AND PRESSURE IS BEING ADMINISTERED TO IMMATURE AND MATURE GUINEA PIGS, LD 50 ARE OBTAINED AT 46-48 HRS. THE LUNGS ARE BEING EXAMINED HISTOLOGICALLY FOR HYALINE MEMBRANES AND SURFACTANT ACTIVITY. BLOOD IS WITHDRAWN FOR COAGULATION STUDIES IN AN ATTEMPT TO DEFINE THE NATURE OF THE MEMBRANE. THE LUNG HAS NOW BECOME THE CHIEF OBJECT OF STUDY IN THE SHOCK UNIT. PULMONARY FUNCTION AND RESPIRATORY PARAMETERS ARE BEING STUDIED IN DETAIL IN AN ATTEMPT TO EVALUATE THE ETIOLOGY OF PULMONARY FAILURE IN SHOCK.

(U) PROGRESS - JUL 67 THRU JUN 68 1. ARMY PULSATILE BLOOD PUMP-FURTHER STUDY OF LEFT HEART BYPASS IN DOGS COMPARING PULSATILE AND NONPULSATILE FLOW INDICATE PULSATILE FLOW TO BE SUPERIOR. 25 NEW PUMPS WITH DISPOSABLE VALVE VENTRICLE ASSEMBLY HAVE BEEN PROCURED, TESTED AND ARE READY FOR DISTRIBUTION. POSTSYSTOLIC MYOCARDIAL AUGMENTATION IS BEING PERFORMED ON DOGS WITH THE PUMP AND TESTS SHOW GOOD REDUCTION IN WORK LOADS OF THE HEART. 2. MEMBRANE OXYGENATOR-IN VITRO TESTS ARE IN PROGRESS UTILIZING A PROTYPE OF THE CAPILLARY MEMBRANE OXYGENATOR. GOOD THOUGH NOT OPTIMAL GAS EXCHANGE IS SEEN. 3. ARMY EMERGENCY RESPIRATOR FURTHER WORK IS SUSPENDED PENDING RE-DEFINITION OF CURRENT FIELD NEEDS. 4. EXTERNAL CARDIAC COMPRESSOR-FURTHER WORK IS SUSPENDED IN FAVOR OF UTILIZING COMMERCIAL HAND OPERATED DEVICES. VOLUME CYCLED RESPIRATOR-GOOD REPORTS FROM FIELD TESTING UNDER COMBAT CONDITIONS HAVE APPEARED. 5. HEART MONITOR- FINAL FEATURES ARE BEING INCORPORATED IN THE PROTOTYPE UNIT, READY FOR HUMAN TESTING AND EVALUATION IN 90 DAYS. ADJUSTABLE HIGH AND LOW RATE LIMITS AND A SILENT MODE READ-OUT OF THE EMERGENCY ALARM SYSTEM ARE BEING ADDED. 6. OXYGEN TOXICITY EXPERIMENTS IN GUINEA PIGS HAVE SHOWN A VAST DIFFERENCE BETWEEN THE IMMATURE AND MATURE GUINEA PIG. THE IMMATURE GUINEA PIG IS 2X MORE RESISTENT TO OXYGEN TOXICITY THAN THE MATURE GUINEA PIG. COAGULATION STUDIES HAVE SHOWN AN INCREASE IN CIRCULATING FIBRINOLYSINS. SURFACTANT MEASUREMENTS HAVE REVEALED A DECREASE IN FORMATION SECONDARY TO A DESTRUCTION OF THE ALVEOLAR LINING CELLS. 7. THE SHOCK UNIT IS BEING CONVERTED INTO A STUDY UNIT FOR THE MEASUREMENT OF PULMONARY FUNCTION, AND QUANTITATION OF PULMONARY DISEASE IN PATIENTS IN SHOCK, AND IN THOSE THAT ARE SEVERELY ILL PRIOR TO SURGERY.

25. COMMUNICATIONS SECURITY	26.	27. OLD CODE	28. BUDGET CODE
☐ a. CERTIFICATE REQUIRED ☒ b. NOT REQUIRED		AR	1

29. WORK UNIT OBJECTIVE	30. PARTICIPATION
14364	NA

31. RESEARCH AGENCY	32. SPECIAL EQUIPMENT
	33.

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Project 3A025601A821, COMBAT SURGERY

Task 01, Combat Surgery

Work Unit 122, Experimental anesthesia

Investigators.
Principal: LTC James A. Meyer, MC
Associate: CPT Steven R. Wyte, MC; CPT Duke Weeks, MC*; CPT Peter Sellei, MC*; CPT Robert L. Hewitt, MC; CPT Victor S. Gutierrez, MC; LTC Teruo Matsumoto, MC

Description.

1. Heart Monitor. A solid state device has been designed and constructed which will give medical personnel information concerning the presence, rate and rhythm of EKG signals from seriously ill patients. A special circuit gives an alarm which immediately informs medical attendants when cardiac arrest occurs and also discriminates between standstill and ventricular fibrillation. (The equipment under development is part of a combined effort with the Harry Diamond Laboratory.**)

2. Army Pulsatile Blood Pump. This pump which was developed by the Walter Reed Army Institute of Research-Harry Diamond Laboratory (WRAIR-HDL) Team has been evaluated and revised to better meet the needs for field application in new techniques of assisted circulation. Current in-house projects include: (A) Effects on blood ---a project to study the effects of prolonged extracorporeal bypass on blood during a bypass period and also for periods of up to one month following the bypass. This study utilizing the Army pulsatile pump and dogs was performed using a technique of left heart bypass. Another project (B) myocardial augmentation is to evaluate the ability of the Army pulsatile blood pump to perform post-systolic myocardial augmentation and counter pulsation. Dogs are used and pressure changes, as well as carotid and coronary blood flow, are measured during periods of augmentation timed electronically and synchronized to the EKG.

3. Ventilator. A volume-cycled ventilator also capable of pressure cycling has been constructed using the principles of fluid amplification. A piston driving a bellows is the basic mechanism while the control is by fluidics thereby reducing the need for moving parts.

4. Membrane Oxygenator. A capillary membrane oxygenator is under development which will be small, disposable and require a minimum of "setting up" work in the field. The unit will be pre-sterilized and modular, so that two or more units may be used in parallel depending on the size of the perfusion to be performed.

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*Walter Reed General Hospital Residents

**E. A. Wright, R&D Supervisor
J. W. Joyce
C. Lanham
C. W. Ragsdale

5. Oxygen toxicity in the guinea pig at normobaric pressure. A model for the study of oxygen toxicity has been devised. Adult and young guinea pigs were used. An LD_{50} was established for the groups. Analysis and study for changes in the lung were performed including: light and electron microscopy, succinic dehydrogenase levels, surfactant presence and samples of blood for a coagulation profile were taken.

6. Pulmonary function in the severely ill patients. With the severe trauma caused by the Vietnam conflict a large influx of seriously ill patients is being treated for extended periods of time in military hospitals. There is evidence suggesting that prolonged bed rest in these patients causes decided changes in red cell mass, plasma volume, and pulmonary compliance and resistance. A study has been started to study in detail pulmonary function, cardiac output, blood volume, and extracellular space in patients that will require prolonged bed rest and one or more major surgical procedures.

7. Cardiac arrest following succinylcholine. Several cases are now known where severely wounded patients after multiple transfusions and wound sepsis have come to surgery again and had cardiac arrest occur after the administration of succinylcholine. Preliminary studies in humans show that a sudden rise in serum potassium appears after the injection of succinylcholine. A study was performed to demonstrate this rise in potassium in dogs and then to elucidate the mechanism.

Progress.

1. Heart Monitor. Animals undergoing digitalis intoxication, patients undergoing cardiac surgery and FM tape recordings of abnormal EKG patterns have all been used to develop and test the heart monitor prototype. After consultation with appropriate leaders in cardiology, thoracic surgery, medicine and anesthesiology, it was decided to add high and low rate limit indicators to the monitor for the final prototype. This prototype is being assembled for field trials in Vietnam. Tests to date indicate that this monitor will bridge the gap between periodic EKG tracings and the fragile oscilloscope as it is used in intensive care and surgical suite monitoring.

2. Army Pulsatile Blood Pump. (A) Effects on blood. Twenty six dogs have been used. The first 14 were used to familiarize the team with the technique of left heart bypass and use of the Army blood pump. Serious difficulties in the technique of left heart bypass were encountered due to the discrepancy in size between the cannula used and the size of the jugular vein in the available dogs. Twelve dogs were placed successfully on left heart bypass using the Dennis-Jenning cannula and the Army blood pump for periods of up to six hours. A colloid free prime was used in all cases and no blood was transfused during the bypass or afterwards. After bypass the dogs were placed on a standard kennel diet. The only medication they received was penicillin and streptomycin for 10 days after bypass.

Determinations of hemoglobin, hematocrit, platelet count, serum bilirubin, serum hemoglobin, fibrinogen, total protein, albumin and mechanical and osmotic fragility were done before, twice during bypass, one hour after bypass, one day, one week, and one month after bypass. All twelve dogs survived the one month period. Analyses of the data obtained show that the animals undergo a dilutional effect on all parameters at the start of bypass which is maintained during bypass. One day after bypass the hemoglobin has dropped about 10 per cent and the total protein and fibrinogen are still not up to pre-bypass levels. At one week post-bypass the hemoglobin is almost back to normal and all other studies are normal. At one month post-bypass all parameters are within normal limits.

(B) Myocardial augmentation. The Army pulsatile blood pump has been shown to be capable of production of synchronized pulsation in normal dogs relative to the animal's electrocardiogram. The pump is initiated through an electrocardiogram signal in series with a Cordis Electronic Programmer and a solenoid valve attached to the pump. A monitor system designed by the Harry Diamond Laboratories filters out extraneous signals and relays only the desired EKG signal to the Cordis Programmer.

The R wave of the electrocardiogram is used as the signal and sufficient delay in delivery of the pulse is regulated through the computer so that the pulse from the dog arrives in diastole and the negative phase of the pump arrives in systole.

In normal dogs, with catheters placed in each femoral or iliac artery connected in series with a common channel, which divides into inflow and outflow routes for the pump, consistent changes have been seen in post-systolic augmentation. Systolic blood pressure is reduced, diastolic blood pressure is increased, and ventricular pressure is reduced. Reduction in ventricular pressure averages 25 mm Hg. Cardiac output remains unchanged as measured by a Beckman Cardio-Densitometer.

Immediate other studies include measurement of coronary blood flow and cartoid artery blood flow.

3. Ventilator. The volume-cycled ventilator was tested here at Walter Reed Army Institute of Research as well as at the Downstate Medical Center, New York, and the Rhode Island Hospital, Providence, Rhode Island. A prototype is at present being evaluated in Vietnam by the Surgical Research Team (WRAIR). The initial reports have been good except for the following points: (a) an excessive gas requirement for powering the respirator; (b) a fairly large compression effect when the machine is working against a severe compliance-resistance load; (c) calibration and air-oxygen mix not being accurate which is due to (b). Because of these factors, work has been suspended on this unit. A re-orientation of the approach is in progress including limitation to one moving part.

4. Membrane Oxygenator. Testing with animal blood has continued using modules of the capillary type. A change in personnel both medical and engineering has slowed this project during this fiscal year. A new in vitro test circuit has been designed and constructed and current regular testing has continued using horse blood. The permeability to oxygen of the tubing does not at present seem to be the limiting factor. At present we are seeking a new way to break up the boundary layer of blood which seems to interfere with oxygenation. A mathematical approach is now being sought to help predict the ultimate design criteria.

5. Oxygen Toxicity. A group of 500 gm guinea pigs has been exposed for 44-48 hours in oxygen at one atmosphere absolute in order to establish an LD_{50}. Those animals that died showed the classic pulmonary morphologic changes of O_2 toxicity. The living guinea pigs are sacrificed at intervals of 1 day, 2 days, 1 week, 2 weeks, 1 month, and 3 months. Young 250 gm guinea pigs display a change in their coagulation profiles. They are two times more resistant to O_2 toxicity than the more mature 500 gm guinea pigs. With serial sacrifice of the remaining 500 gm guinea pigs, there is a return of the lung towards normal function coordinated by light microscopy and pulmonary surfactant. The end result after 3 months appears to be mild to moderate emphysema.

6. Pulmonary function in the severely ill patients. This project has been underway for two months. In this time we have examined 14 patients prior to surgery. We have noted an increase in plasma volume in relation to red cell mass, a low normal arterial pO_2 in room air, a slight to moderate increase in physiologic dead space, and a mild to moderate degree of left to right shunting.

7. Cardiac arrest following succinylcholine. An initial series of dogs showed occasional rise in serum potassium with succinylcholine administration after mallet trauma followed by varying periods of time.

It was determined that it would be necessary to add sepsis to the mallet trauma insult. The traumatized area was then infected with dirt from Vietnam. These dogs then showed a significant rise in serum potassium after succinylcholine administration about two to three weeks after the insult.

Summary and Conclusions.

1. A heart monitor is nearly ready for field trials which incorporates the following features: high and low rate limit indicators, audible signal for each QRS when desired, alarm for ventricular fibrillation, and alarm for cardiac arrest.

2. (A) Effects on blood. The effects of prolonged bypass on blood trauma are being studied using the Army blood pump. Data obtained show that up to six hours of bypass produces minimal damage to the blood. This damage is well within the recuperative powers of the animal on his own. The experiments will be continued adding determinations of RBC survivals with Cr^{51} and extending perfusion for periods ranging to several days.

(B) Myocardial augmentation. The Army pulsatile blood pump is capable of delivering a pulse synchronous with the animal's electrocardiogram when used in series with a Diamond Monitor, Cordis Programmer and solenoid valve.

Consistent changes include decrease in systolic blood pressure, decrease in ventricular pressure and increase in diastolic blood pressure and no change in cardiac output.

Early studies suggest increase in coronary blood flow and no change in carotid blood flow but these studies are not complete.

3. A volume-cycled ventilator is being revised after successful operation in Vietnam to decrease power requirements, dead space, and the number of moving parts.

4. A membrane oxygenator utilizing capillary tubing of silicone rubber is being tested in an in vitro circuit. Mathematical analysis is being carried out using test data to determine ideal design characteristics.

5. Oxygen toxicity studies in guinea pigs show that immature guinea pigs are more resistant to the toxic effects of O_2 than adult guinea pigs. Coagulation defects appear in the immature guinea pigs.

6. Pulmonary function in the severely ill patients. Cardiopulmonary studies in patients with moderate trauma have been done prior to repeat surgery. Several parameters have been found to be outside of normal limits: (a) plasma volume, (b) arterial pO_2, (c) increased physiologic dead space, and (d) left to right shunting.

7. Cardiac arrest following succinylcholine. Serum potassium rises, which lead to cardiac arrest in human casualties following succinylcholine administration, have been produced in a dog model. These are seen about two to three weeks after mallet trauma of a limb with sepsis induced by implantation of Vietnamese dirt.

Publications.

1. Meyer, J. A.: Electrical Hazards in Medical Instrumentation, Chapter Six. In: CLINICAL CARE. F. A. Davis Co., Philadelphia, 1967.

2. Ragsdale, C. W., Wright, E. A., and Meyer, J. A.: An automatic diagnostic cardiac monitor. Ann. Conf. on Eng. in Med. and Biol., 13-16 November 1967, Proceedings, p.344.

PROJECT 3A025601A822
MILITARY INTERNAL MEDICINE

Task C1
Military Internal Medicine

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 049469	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF SUMMARY
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10. DOCUMENT NUMBER CODE: 62156611 3A025601A822 00 120

10a. PRIOR NUMBER CODE:

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12. SCIENTIFIC OR TECH AREA	13. START DATE	14. CMPL. COMPL. DATE	15. FUNDING AGENCY
012900 PHYSIOLOGY 002300 BIOCHEMISTRY 003900 CLINICAL MEDICINE	08 63	NA	OTHER DA

16. RESOURCES EST	a. PROFESSIONAL MAN-YEARS	b. EXPENDITURES (K)
FUNDING FY 68	20	290
CURRENT FY 69	20	290

17. PERFORMING METHOD	17. CONTRACT/GRANT	17. DATE	17. NUMBER	17. AMOUNT
C. IN-HOUSE	NA	NA	NA	NA

18. GOVT. LAB INSTALLATION/ACTIVITY

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21. TECHNOLOGY UTILIZATION: MEDICAL SCIENCE

22. COORDINATION: NA

23. KEYWORDS: ENDOCRINE GLANDS, ADRENAL GLANDS, ISLANDS OF LANGERHANS.

(U) TECH OBJECTIVE - DEVELOPMENT AND APPLICATION OF METHODS OF INVESTIGATION INTO BASIC MECHANISMS OF ENDOCRINE DISEASE AND ENDOCRINE ROLE IN MAINTENANCE OR FAILURE OF HOMEOSTATIC MECHANISMS DURING STRESS OF DISEASE AND INJURY TO PROVIDE RATIONAL APPROACH TO THERAPY.

(U) APPROACH- DEVELOPMENT OF RADIOIMMUNOASSAY PROCEDURES FOR HORMONES FOR APPLICATION TO INVESTIGATIONS OF CARBOHYDRATE METABOLISM AND ANABOLIC PROCESSES, WATER AND MINERAL METABOLISM IN METABOLIC AND ENDOCRINE DYSFUNCTION. APPLICATION OF STANDARDIZED METHODS FOR HORMONE DETERMINATIONS TO SUBJECTS WITH INFECTIOUS DISEASE, AND INJURY. BODY COMPOSITION MEASUREMENTS IN ENDOCRINE DISEASE.

(U) PROGRESS - JUL 67 THRU MAY 68 THE EFFECT OF STEROIDAL COMPOUNDS ON INSULIN AND GROWTH HORMONE AND CARBOHYDRATE METABOLISM ARE CURRENTLY BEING EVALUATED. A RAPID, SIMPLE METHOD FOR DETERMINATION BY RADIOIMMUNOASSAY TECHNIQUE OF SERUM THYROXIN CONCENTRATION HAS BEEN DEVELOPED. A SIMILAR METHOD FOR PLASMA CORTISOL IS UNDER DEVELOPMENT. EVALUATION OF TOTAL BODY POTASSIUM MEASUREMENTS IN PATIENTS WITH ACROMEGALY ARE IN PROGRESS. LONGITUDINAL STUDIES OF PLASMA AND URINARY STEROIDS IN SUBJECTS UNDERGOING PHYSICAL CONDITIONING IN HOT ENVIRONMENT HAVE BEEN COMPLETED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

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AR 705-5	NA

36. CONTRACTOR'S NAME	37. SPECIAL EQUIPMENT
38. CONTRACT NUMBER	39.

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Project 3A025601A822, MILITARY INTERNAL MEDICINE

Task 01, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Investigators.
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Description.

A. Radioimmunoassay procedures for the peptide hormones (insulin and growth hormone) and radioassay procedures (thyroxine and cortisol) for non peptide hormones were developed or are under development to provide more sensitive, specific, and rapid methods of measuring hormones of interest in biologic fluids in order to support research studies and provide diagnostic information in patients with various endocrine diseases.

B. Previous studies have shown that a combination of estrogenic and progestational steroids usually produce abnormalities of carbohydrate metabolism manifested primarily by glucose intolerance. The incidence of these steroid induced abnormalities of carbohydrate metabolism, the mode of production of these abnormalities and the susceptibility of humans with and without family histories of diabetes mellitus have not been clearly defined.

C. The liver has long been known to play an important role in energy metabolism and it has been postulated that hepatic extraction of varying amounts of insulin from portal vein blood is one of the critical determinants of peripheral plasma insulin concentrations and peripheral glucose utilization.

D. Hypertonic Glucose (7%) utilized in peritoneal dialysis commonly elevates blood glucose 2 - 5 times basal concentrations. Dialysate and plasma insulin concentrations were measured to gain insights into the well known carbohydrate intolerance of uremia.

E. Insulin secretion is known to be stimulated by various apparently unrelated compounds including glucose and some amino acids. It is not known whether these compounds stimulate insulin secretion via a common mechanism or whether insulin secretion may be stimulated through several different mechanisms.

F. Pseudomonas polysaccharide (Piromen®) was evaluated as a test of pituitary reserve.

G. The five hour glucose tolerance test as a measure of growth hormone reserve is being evaluated.

H. Studies of growth hormone in abnormal states of thyroid function.

I. The effect of altered thyroid function on hormone stimulated lipolysis in vitro.

J. An investigation of the interrelationships between magnesium and calcium in the Mg. deficient monkey is under study.

K. Whole body counting of the naturally occurring isotope K^{40}, which comprises 0.019% of total body K, provides an excellent theoretical method for estimating total body K and lean body mass. The availability of a sensitive, relatively simple method, not requiring administration of radioactive isotopes would provide a tool of great value in studies of human subjects with spontaneous or induced abnormalities of potassium metabolism.

Progress.

A. Insulin and Growth Hormone assays have been developed by extensively modifying the charcoal method of Herbert. This has resulted in assays with a lower level of sensitivity of 0.25 micro units/ml for insulin and 0.5 mug/ml for growth hormone. Approximately 300 individual assays can be performed per week by one technician. A modification of the Patee-Murphy procedure for measurement of serum thyroxine has been developed. Determinations of plasma cortisol by similar principles are in the early developmental stages.

B. The response of blood glucose, serum insulin, and plasma growth to an oral glucose load and infusions of glucose and arginine has been measured in female human volunteers prior to and following 3 months of treatment with a combination estrogen-progestin preparation. It is anticipated that this prospective study on 20 subjects will be completed in Fall 1968.

C. Preliminary studies in postoperative human subjects with T tubes in the common bile duct have demonstrated that the radioimmunoassay procedure is capable of measuring biliary insulin concentration. This technique may provide a simple, albeit, indirect method of quantifying hepatic insulin extraction. Preliminary studies in dogs have demonstrated the feasibility of determining pancreatic insulin secretion rates and factors influencing hepatic insulin extraction.

D. During early dialysis periods employing hypertonic glucose, the peritoneal clearance of insulin was relatively constant. During later dialysis periods and after blood glucose concentration had become elevated, peritoneal insulin clearance declined abruptly and at a time when the peritoneal clearance of all other measured solutes had increased. This abrupt decline in peritoneal insulin clearance was totally unexpected. This phenomenon may be due to pancreatic secretion of "big insulin," as is known to occur late in the course of an oral glucose tolerance test. If the observed decreased peritoneal insulin clearance is indeed due to

a predominance of "big insulin" in plasma, peritoneal dialytic techniques may provide the first in vitro model for studies of hormone transport mechanisms.

E. Studies in a patient prior to and following removal of a pheochromacytoma demonstrated that glucose stimulated insulin secretion is inhibited by elevated concentrations of plasma catecholamines but arginine stimulated secretion was not inhibited. Studies before and during epinephrine infusion in a normal subject gave similar results. These observations indicate that there are at least 2 different mechanisms governing insulin secretion.

F. Pseudomonas polysaccharide has been administered to approximately 30 patients during the course of evaluation of anterior pituitary function. Serum growth hormone rise to Piromen® was compared to insulin-induced hypoglycemia. Plasma cortisol response was compared with the response to a standard metapyrone test. Preliminary results indicate that the Piromen test alone gives as valid a measure of corticotropin and growth hormone reserve as the insulin and metapyrone tests together.

G. Approximately 40 patients have been studied by means of a five hour glucose tolerance test and at least one other standard test of growth hormone reserve (insulin-induced hypoglycemia, arginine, pseudomonas polysaccharide). The results indicate that the late rise in serum growth hormone concentration following glucose administration correlates well with the growth hormone response to other stimulatory agents. Determination of growth hormone levels during a glucose tolerance test provides a test of growth hormone reserve which has the advantages of ease and safety compared with standard tests presently in use.

H. Serum growth hormone levels during standard insulin tolerance tests were measured in 10 patients with hyperthyroidism and 13 normals. The results indicate that the growth hormone response to insulin-induced hypoglycemia in thyrotoxicosis does not differ significantly from normal.

I. Accurate and reproducible assay systemsfor free fatty acids and glycerol have been established. It has been confirmed that adipose tissue of thyroidectomized rats releases less FFA and glycerol when incubated in vitro than does tissue taken from intact animals. Tissue taken from thyroxine-treated rats has increased lipolytic activity. When epinephrine is added to the incubation medium absolute lipolytic responses are greater in tissue from thyroxine-treated rats and less in tissues of hypothyroid rats. However, the fractional increases induced by epinephrine are the same.

J. A diet developed with the assistance of General Biochemicals, Inc., has been successful in producing Mg. depletion in monkeys without significant weight loss. Studies with approximately 12 animals have shown that hypomagnesemia can be consistently induced within 7 to 14 days. Hypocalcemia is a predictable development after 3 or 4 weeks of Mg. depletion and this

occurs despite a large amount of dietary calcium and low urine Ca. Although hypocalcemia and hypomagnesemia occurred in all animals, only one monkey had convulsions and none of the other animals showed gross evidence of neuromuscular irritability. Sequential infusions of parathyroid hormone during control, depletion, and repletion periods did not show reduced responsiveness of bone to the hormone. No changes have been observed in serum phosphorus, potassium and albumin. No changes have occurred in erythrocyte, Na, K, and Ca concentration but erythrocyte Mg. falls slowly. Removal of the thyroid (the source of thyrocalcitonin) does not affect the development of hypocalcemia.

K. Metabolic balance studies have been utilized in 2 patients to determine if changes in potassium balance detectable by external balance techniques are correlated with changes in total body K estimates derived from the whole body counting procedure. Whole body counting appears sufficiently promising to warrant studies in additional human subjects.

Summary and Conclusions.

Summary and conclusions are listed at the end of each paragraph in the Progress section.

Publications.

None

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
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19. GOVT. LAB. INSTALLATION/ACTIVITY

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24.

(U) TECH OBJECTIVE - TO FIND IMPROVED PROCEDURES TO CONTROL DIARRHEAL DISEASE. PRESENT WORK INVOLVES THE TESTING OF ORAL VACCINES AGAINST BACILLARY DYSENTERY. IF SUCCESSFUL VACCINES WILL BE OF VALUE TO THE MILITARY AND TO CERTAIN CIVILIAN POPULATIONS.

(U) APPROACH- ATTENUATED DYSENTERY STRAINS ARE BEING DEVELOPED. THEY ARE BEING EVALUATED FOR SAFETY IN SEVERAL SYSTEMS AND ARE BEING TESTED FOR POTENCY IN MONKEYS.

25. (U) PROGRESS - JUL 67 THRU JUN 68 PARENTERALLY ADMINISTERED VACCINES CONSISTING OF VIRULENT DYSENTERY BACILLI FAIL TO PROTECT MONKEYS AGAINST VIRULENT SHIGELLOSIS. PREVIOUS STUDIES INDICATE THAT ORALLY ADMINISTERED LIVING ATTENUATED BACTERIA DO PROTECT. STUDIES IN VOLUNTEERS INDICATED THAT LIVING AVIRULENT MUTANT STRAIN OF S. FLEXNERI 2A REVERTS TO A VIRULENT FORM AND CAUSES CLINICAL DISEASE IN VOLUNTEERS WHEN FED IN DOSES ABOVE 100 MILLION CELLS. A HYBRID STRAIN OF THIS AVIRULENT MUTANT HAS BEEN PREPARED BY RECOMBINATION WITH E. COLI AND ITS STABILITY IS NOW BEING ASSESSED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A025601A822 MILITARY INTERNAL MEDICINE

Task 01, Military Internal Medicine

Work Unit 121, Pathogenesis of enteric disease

Investigators.

Principal: Samuel B. Formal, PhD

Associate: Eugene H. LaBrec, PhD; CPT R.M. Maenza, MC; CPT Thomas G. Lawrence, MC; COL Helmuth Sprinz, MC

Description.

The pathogenesis of enteric disease is studied to elucidate the mechanisms by which enteric pathogens produce symptoms. By understanding the disease process, improved procedures for prevention and treatment of diarrheal diseases will become evident.

Progress.

1. In previous studies we have described two kinds of dysentery strains of reduced virulence. One is a natural mutant, incapable of invading the bowel wall, and acts in experimental animals no differently from E. coli. The other was hybridized with Hfr E. coli and has a reduced capacity to multiply in the intestinal mucosa. Studies carried out at the University of Maryland have demonstrated that an avirulent mutant strain of S. flexneri 2a reverts to the virulent form when fed in high doses to volunteers. In an effort to make this mutant strain safe for human beings, we have hybridized it with E. coli and selected a clone which has incorporated the E. coli xylose-rhamnose region into its genome. This new strain has been tested as an oral vaccine for its ability to protect monkeys against experimental challenge. Two experiments were conducted in which 5 doses of vaccine, given at intervals of 3 to 4 days, were administered. The pooled results of this study are summarized in Table 1, and they indicate that a significant degree of protection was achieved. Two further tests with the same hybrid strain were carried out to determine if protection could be conferred by three doses of vaccine fed at intervals of seven days. The pooled data from these experiments are presented in Table 1 and offer evidence of good protection.

2. Because of the finding that an avirulent strain of S. flexneri 2a reverted to the ability to penetrate epithelial cells and thus cause disease when fed to man in relatively high doses, an investigation was initiated to determine the locus on the shigella chromosome which is responsible for epithelial cell penetration. Previously we had investigated the ability of E. coli-Shigella hybrids to penetrate epithelial cells and cause disease. In this study we were only able to test recombinants which had incorporated segments of E. coli chromosome covering approximately 60 percent of the E. coli genome, and we found no chromosomal region which controlled penetration. We were not able to study the remaining 40 percent of the chromosome because of the lack of suitable markers between lactose and histidine (going clockwise on the circular E. coli chromosome) in our naturally occurring Shigella strains. A suitable marker would be galactose fermentation, but both the E. coli donor and the recipient shigella strains are positive for this. By treating a strain of S. flexneri 2a with n-methyl-n-nitro-nitroso guanidine we obtained a galactose negative mutant which still retained the capacity to invade epithelial cells as measured by the guinea pig keratoconjunctivitis test. This galactose-negative mutant Shigella strain was used in hybridization experiments with Hfr E. coli strains to determine what portion of the E. coli chromosome which when incorporated into the shigella genome renders the pathogen unable to penetrate epithelial cells. In confirmation of our earlier work all hybrids containing segments of E. coli chromosome from lactose to histidine (counter clockwise) remained virulent. Many lactose-positive recombinants lost their S. flexneri type 2 specific antigen but their ability to cause keratoconjunctivitis was not noticeably affected. On the other hand, all hybrids which had incorporated the lactose - galactose region were, without exception, avirulent, but also lacked the type-specific antigen. Because of this loss of antigen, it is not likely that these recombinants would be satisfactory vaccine strains. However, some hybrids which retain their own lactose region and incorporate the E. coli galactose region into their genome lose virulence, but still have the S. flexneri type-specific antigen. Such strains may be of value. Since we have localized the virulence factor in a relatively small area of the chromosome, it is likely that we shall be able to effect the loss of virulence by transferring the small piece of critical area using transduction techniques. Thus, we should be able to retain the type antigen and cause virulence loss in 100 percent of the clones. Such an approach is now under investigation.

3. Studies on encephalomyocarditis (EMC) virus infection of mice have continued. The present work involved an attempt to distinguish the immunity which follows oral infection with an avirulent variant of EMC in the mouse from the immunity which follows the parenteral administration of a formalin-killed vaccine prepared from virulent EMC. Attention was directed at the fecal excretion of virus in order to determine whether local gut-associated immunity was important in preventing shedding after oral challenge with virulent virus. An avirulent strain of EMC had been previously prepared in this laboratory and was continued by serial passage through mouse fibroblasts growing in tissue culture. A formalin-killed vaccine was prepared by incubating virulent EMC (10^7-10^8 PFU/ml) at pH 7.1 - 7.4, 37 degrees C., in BME-Hanks salts containing a 1:6000 dilution of formalin. The inactivation was first-order, and complete killing (less than one PFU/ml.) was obtained by six days. Intracranial injection in weanling Balb/C mice confirmed the inactivation. Three groups of mice were then orally challenged. The first group consisted of animals which had been immunized with formalinized vaccine in Freunds complete adjuvant. The second group had been immunized with orally administered avirulent virus. A third untreated group served as controls. Fecal specimens were collected daily from day 1 to day 12. The results are presented in Table 2. As previously shown in this laboratory, oral immunization was effective. Ninety percent of the challenged mice survived, and only two of thirty-two survivors excreted virus. A single injection of killed virus in adjuvant was used to see whether minimal protection would be accompanied by fecal excretion in survivors. This immunization was less effective than the oral vaccine in terms of survival. In spite of an apparently weaker immune response as measured by survival only four of twenty-four survivors excreted virus. Two of six surviving control animals excreted virus.

Mice were then passively immunized with rabbit hyperimmune serum to study the contribution of circulating antibody and to exclude the immune response which might have been evoked in the gut by parenterally administered killed vaccine. Groups of 5 mice per group were given 1.0 ml of serial ten-fold dilutions of rabbit anti-EMC (1:25,000 caused 50% reduction in plaques) which was shown by sucrose gradient ultracentrifugation to be of the 7S class. In the first set of experiments (Table 3) all animals receiving more than 1×10^3 ml of rabbit antiserum survived oral challenge and pooled fecal samples harbored no virus. Three of five receiving 1 X 10-4 ml died. In this group, pooled fecal samples contained virus as indicated. To see whether virus excretion was restricted to dying mice, the experiment was repeated and individual fecal samples were collected daily (Table 4).

At serum doses of 1 X 10^{-3} ml and 1 X 10^{-4} ml fecal virus excretion occurred only in mice which subsequently died. It thus appeared in this system that circulating antibody suffices to prevent significant virus replication in the gut, and that a gut-associated local active immune mechanism is not necessary to confer resistance. Studies are in progress to evaluate early (19S) antibody in a similar way. Preliminary studies have shown that it is feasible to determine antibody content in bowel washings and homogenates. We plan to compare the coproantibody response in orally immunized and parenterally immunized mice. From the present data, it appears that viral shedding via the intestine does not occur to a significant degree if an antibody response is actively raised or if antibody is passively transferred, providing overwhelming fatal infection does not ensue.

4. Experiments reported from this laboratory over the past few years have shown that monkeys given live oral vaccines prepared from avirulent or attenuated virulent hybrid strains of Shigella flexneri serotypes were protected against infection when challenged orally with the virulent parent strains. However, parenteral immunization procedures with live or killed vaccines prepared from the virulent parent strains failed to protect monkeys against subsequent oral challenge with the virulent strains. Serum antibody responses in orally or parenterally vaccinated groups as measured by the passive hemagglutination (HA) technique failed to reflect the immune state of the animal. In fact, the serum antibody titers of parenterally immunized monkeys were often higher than those of orally immunized monkeys. Antibody titers of fecal extracts (coporantibody) as measured by HA or bacterial agglutination were low or not detected.

Studies on the nature of the antibody response in monkeys to oral versus parenteral routes of immunization and its relationship to the immune state of the animal have been started. Groups of monkeys were immunized with live S. flexneri 2a vaccines by either the oral or parenteral route using several dose schedules. The monkeys were sacrificed 5-7 days after the last dose. Intestinal content and sera were collected at time of autopsy to determine the nature and character of the antibody response. Specimens of intestine and lymphoid organs were collected for routine histological procedures and other portions frozen for analysis by fluorescent antibody (FA) procedures. The "sandwich" technique was used to detect antibody forming cells containing antibody specific for the immunizing antigen.

Cryostat sections of frozen tissues fixed in alcohol were incubated with a purified S. flexneri 2a lipopolysaccharide antigen, washed, stained with fluorescein-labeled rabbit anti S. flexneri 2a antibody and examined. Specifically fluorescing cells with the appearance of plasma cells or lymphocytic cells were identified as antibody producers. The specific fluorescence in the cells was seen as bright homogeneous fluorescence in the cell cytoplasm or as discrete droplet-like particles of varying size. The data presented in Table 5 show the distribution of anti-Shigella antibody cells in the tissues of orally and parenterally immunized monkeys. Sections of stomach, jejunem, and terminal ileum were grouped as small intestine; sections of cecum and colon as large intestine. If one of the sections in the group showed significant numbers of antibody cells the group was considered positive. If the tissue section contained fewer than 3 scattered fluorescing cells it was considered negative. The data presented clearly show that the distribution of antibody forming cells is related to the route of immunization. In the orally immunized monkeys the number of antibody forming cells in the small intestine was usually small and often associated with the lymphoid tissues. Many more antibody forming cells were found in the large intestine. Here, most fluorescing antibody cells in the lamina propria were found near the base of the glands very close to the epithelial basement membrane. The spleens of monkeys given oral vaccines were usually negative. If the spleen was considered positive, the number of fluorescing cells was usually small.

Antibody forming cells in the organs of monkeys receiving parenteral vaccine were seen almost exclusively in the lymphoid tissue draining the site of injection and in the red pulp of the spleen. Significant numbers of antibody cells were not seen in the specimens of the intestinal tract, with the exception of two monkeys in which Shigella flexneri 2a was cultured at autopsy and visualized in the tissues by the FA technique.

Attempts to corroborate the FA findings using other methods were not wholly successful. The Jerne plaque technique which worked well in experiments with rabbits immunized with Shigella antigens proved erratic in these experiments with monkeys. In most of the animals tested the number of antibody forming cells appearing as plaque forming units in this test was usually only slightly above background. Therefore, little credence was placed on the results. The reason for our failure has not been found.

The sera and fecal extracts collected from each animal were tested to determine the nature of the antibody response. The serological activity was measured by hemagglutination of sheep red cells coated with S. flexneri 2a O-antigen. With few exceptions, all animals exhibited a rise in titer following immunization. The other evidence presented below indicates that the antibody activity in the sera of these monkeys belonged to the immunoglobulin M (IgM) class of globulins.

a. All activity was found in the first peak when serum samples were chromatographed on Sephadex G-200. This peak contains most of the heavy globulins of IgM class. HA tests of individual fractions eluted from the column showed all the activity in this fraction. The presence of IgM globulins was confirmed by immunodiffusion analysis using specific antisera to IgM.

b. The hemagglutinating activity was abolished if the serum sample was treated with a mild reducing agent such as 2-mercaptoethanol or Dithiothreitol. Immunoglobulin G class of antibody is resistant to reduction by this method.

c. In sucrose gradient ultracentrifugation experiments, the antibody activity was found in the most rapidly sedimenting fractions which correspond to 19S or IgM globulins. The presence of IgM globulin was confirmed by immunodiffusion analysis.

In a few serum samples fractionated by gel filtration on Sephadex G-200, some individual fractions corresponding to the elution profile for immunoglobulin A gave positive HA titers. However the presence of other globulins was not ruled out. In these experiments attempts were also made to improve the methods of isolation of globulins from fecal extracts. The intestinal contents obtained at autopsy were precipitated with 2.1 M ammonium sulfate, washed, and dissolved in 1 ml of water and dialyzed vs. saline. Hemagglutination tests were performed. Most were negative. A few extracts representing samples with both positive and negative hemagglutinating activity were selected for analysis by the radioimmuno-electrophoretic technique. The purpose was two-fold, to determine the major classes of globulin in the extracts and to determine whether they possessed antibody. In preliminary studies fecal extracts from animals which received either oral or parenteral vaccines and also extracts from feces of normal monkeys

were electrophoresed in agar. Controls of normal monkey serum and monkey serum known to contain antibodies against S. flexneri 2a were included. This was done to 1) show the positions of the various precipitated globulins and other proteins, 2) to check for nonspecific combination of antigen and antibody. The precipitin arcs formed by the immunoglobulin classes were identified by using monospecific antiglobulin sera. Rabbit anti-monkey serum antibody was used to detect the maximum number of proteins present in the fecal extract. Immunoglobulins G and A were detected in most of the fecal extracts studied irrespective of whether or not the animals had been immunized. Immunoglobulin M was detected in some samples. The presence or absence of a particular globulin class in a fecal sample was not related to the HA or bacterial agglutinin titers. To determine whether the fecal globulins precipitated were also antibodies against Shigella flexneri 2a, the plates were washed of excess serum and then flooded with C^{14} labeled S. flexneri 2a O-antigen. After suitable incubation the excess antigen was removed by washing, the plates dried and x-ray film used to detect the presence of radioactivity. In most cases there was some degree of nonspecific adsorption of C^{14} antigen to most of the precipitin arcs developed. Therefore, no definitive conclusions as to the presence of antibody activity could be made. Attempts to reduce nonspecific adsorption are in progress.

Summary and Conclusions:

1. A vaccine strain has been prepared by hybridizing avirulent S. flexneri 2a with Hfr E. coli and selecting clones which incorporated the E. coli xylose-rhamnose region into the shigella genome. Three oral doses of 5×10^{10} hybrid cells protect monkeys against experimental oral infection.

2. Virulent S. flexneri 2a which has incorporated the lactose-galactose region of E. coli chromosome following recombination lacks the ability to penetrate epithelial cells and cause keratoconjunctivitis.

3. Encephalomyocarditis virus shedding from the intestinal tract of orally challenged mice following immunization by either the oral or the parenteral route.

4. Groups of monkeys were immunized by either the oral or parenteral route using live S. flexneri 2a vaccines. Cells containing specific antibody against S. flexneri 2a antigen were detected in relatively large numbers in the lamina propria of the colon of animals receiving the oral vaccine. This was not observed in animals vaccinated by the parenteral route.

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2. Formal, S. B., Maenza, R. M., Austin, S. and LaBrec, E. H. Failure of parenteral vaccines to protect monkeys against experimental shigellosis. Proc. Soc. Exp. Biol. Med. 125: 347, 1967.

3. Kent, T. H., Samuel B. Formal, E. H. LaBrec, H. Sprinz and R. M. Maenza. Gastric shigellosis in rhesus monkeys. Am. J. Path. 51: 259, 1967.

4. Takeuchi, A., Samuel B. Formal, and Helmuth Sprinz. Experimental acute colitis in the rhesus monkey following peroral infection with Shigella flexneri. Am. J. Path. 52: 529, 1968.

5. Maenza, R. M., D. W. Powell, G. R. Plotkin, T. H. Kent and S. B. Formal. Experimental diarrhea: Salmonella enterocolitis in the rat. Clin. Res. 16: 288, 1968.

6. Powell, D. W., G. R. Plotkin, R. M. Maenza and S. B. Formal. Experimental diarrhea: Intestinal electrolyte and water transport in rat Salmonella enterocolitis. Clin. Res. 16: 290, 1968.

Table 1

Signs of illness in control monkeys and in monkeys fed either three or five doses of a living hybrid strain of *S. flexneri* 2a*

Exp. No.	No. Vaccine Doses	Vaccine Group			Control Group			P
		No. with diarrhea	No. with dysentery	Total ill / Total challenged	No. with diarrhea	No. with dysentery	Total ill / Total challenged	
M102, 104	3	2	2	4/46	10	9	19/46	$< .001$
M100, M101	5	2	3	5/40	25	6	31/41	$< .001$

*Hybrid was prepared by mating Hfr *E. coli* with an avirulent strain of *S. flexneri* and selecting recombinants which had incorporated the xylose-rhamose region of the *E. coli* genome.

**A vaccine dose consisted of 5×10^{10} cells, and was fed at intervals of 3 to 4 days. The challenge administered 10 days after the last vaccine dose.

TABLE 2

Survival and virus excretion in mice immunized with oral attenuated vaccine or with parenteral killed vaccine.

	Oral[2] attenuated strain		Killed[3] virulent virus		Control	
	Survived	Died	Survived	Died	Survived	Died
Total	32	3	24	12	7	12
Specimens obtained	32	2	24	11	6	8
Excretors[1]	2	2	4	10	2	7

1 Single plaques were not scored.

2 Fed 1.0 cc containing 10^7 PFU attenuated EMC 2 weeks before challenge

3 0.2 cc of virulent EMC ($\sim 10^7$/cc) mixed 1:1 with complete Freunds adjuvant, 2 weeks before challenge

TABLE 3

Virus content of pooled fecal specimens from mice given 1.0 cc diluted rabbit antiserum i. p.

Dilution Serum[2]	Deaths	Virus Excreted[1] Day 1	2	3	4	5	6	7	8
10^{-1}	0/5	0	0	0	0	0	0	0	0
10^{-2}	0/5	0	0	0	0	0	0	0	0
$10^{-2.5}$	0/5	1[1]	0	0	0	0	0	0	0
10^{-3}	0/5	1	0	0	0	0	0	0	0
10^{-4}	3/5	10	0	0	5	0	14	0	0
Control	3/5	1	1	4	8	0	1	0	0

[1] PFU/0.2 cc sample. Sample is supernatant of suspension containing five fecal pellets in 2 cc BME-Hanks 5% fetal bovine serum.

[2] 1 ml contains 25×10^3 50 percent plaque reduction units.

TABLE 4

Survival and fecal virus excretion in mice receiving 1.0 cc diluted rabbit antiserum s.c.

Dil. Serum[1]	10^{-2}		10^{-3}		10^{-4}		Control	
Survival	Survived	Died	Survived	Died	Survived	Died	Survived	Died
Total	5	0	3	2	4	1	0	5
Excretor	0	-	0	1	0	1	-	5

(1) 4×10^{-5} ml. of antiserum caused 50 per cent plaque reduction using 100 PFU.

TABLE 5

Distribution of specific antibody forming cells in monkey tissues following oral or parenteral immunization

Route, No. of Doses and Type of Vaccine	No. of Monkeys	Small Intestine	Large Intestine	Spleen	Inguinal Node	Mesenteric Node
Oral						
2 Doses, Hybrid[1]	6	4/6[4]	5/8	1/6	0/6	4/6
3 Doses, Hybrid	2	1/2	2/2	0/2	0/2	1/2
4 Doses, Hybrid	5	2/5	4/4	0/3	ND	ND
5 Doses, Hybrid[2]	6	3/6	5/6	2/6	ND	2/6
Total	19	10/19	16/18	1/17	0/8	7/14
Parenteral						
2 Doses Virulent[3]	8	2/8[7]	3/8[7]	6/8[7]	4/8[7]	0/8[7]
4 Doses Virulent	6	0/4 Inc[6]	1/4	2/4	ND	ND
Total	14	2/12	4/12	8/12	4/8	0/8

1. Hybrid = S. flexneri 2a E. coli hybrid attenuated virulent strain M22-18X16
2. Avirulent strain a colonial mutant derived from the virulent parent of S. flexneri 2a
3. Virulent = S. flexneri 2a strain M42-43
4. No. Positive / Total = Sections showing significant numbers of specifically fluorescent antibody forming cells
5. ND = Not Done
6. Inc = Incomplete
7. In two of these animals Shigella flexneri 2a was cultured from intestinal content. Organisms were identified in the lamina propria by the FA technique

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KEYWORDS, TAXONOMY, MICROORGANISMS, DNA, ANTIGENS, VIRULENCE, SALMONELLA, DRUG RESISTANCE, MICROBIAL, ESCHERICHIA, BACTERIAL HYBRIDS, PROTEUS.

23. (U) TECH OBJECTIVE - THE TECHNICAL OBJECTIVE IS TO DEFINE IN GENETIC AND MOLECULAR TERMS THE METABOLIC, ANTIGENIC AND PATHOGENIC CHARACTERISTICS OF ENTERIC BACTERIA. THE WORK UNIT IS PRESENTLY INVESTIGATING THE MOLECULAR BASIS OF INFECTIOUS DRUG RESISTANCE, THE GENETIC REGULATION OF SPECIFIC ANTIGENIC DETERMINANTS, AND THE GENETIC AND BIOCHEMICAL BASIS OF VIRULENCE. 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.

(U) APPROACH- THE GENERAL APPROACH IS TO USE GENETIC RECOMBINATION BETWEEN STRAINS OF ENTERIC BACTERIA. WHERE POSSIBLE, THE GENETIC RESULTS ARE EXTENDED TO INCLUDE STUDY OF THE INFORMATIONAL MACROMOLECULES - SUCH AS DNA - INVOLVED.

(U) PROGRESS - JUL 67 THRU JUN 68 CHROMOSOMAL TRANSFER HAS BEEN OBSERVED BETWEEN ESCHERICHIA COLI DONOR CELLS AND PROTEUS MIRABILIS RECIPIENT CELLS. PROTEUS HYBRIDS HAVE BEEN CONSTRUCTED WHICH CONTAIN ESCHERICHIA COLI CHARACTERS. ANALYSIS OF THESE HYBRIDS BY GENETIC AND PHYSICAL CHEMICAL METHODS HAS CORRELATED THE PRESENCE OF A NUMBER OF ESCHERICHIA COLI GENES WITH THE TOTAL AMOUNT OF ESCHERICHIA COLI DNA ADDED TO THE PROTEUS CELLS DURING CONJUGATION. ONE OF THESE PROTEUS HYBRIDS CONTAINS AS MUCH AS 30 PERCENT ESCHERICHIA COLI DNA AND HAS SEVERAL ESCHERICHIA COLI FERMENTATION PROPERTIES AND IS SENSITIVE TO CERTAIN ESCHERICHIA BACTERIOPHAGES, AND HAS SOME ESCHERICHIA COLI SURFACE ANTIGENS. SALMONELLA HYBRID STRAINS HAVE BEEN FOUND TO BE ABLE TO REPRESS THE MULTIPLICATION OF COLIPHAGE LAMBDA. IT WAS POSSIBLE TO COTRANSDUCE THE HIS AND O-ANTIGEN 2 GENES TO BOTH SALMONELLA TYPHIMURIUM AND SALMONELLA TYPHOSA. SALMONELLA TYPHIMURIUM TRANSDUCTANTS WHICH RECEIVED SOMATIC ANTIGEN 2 CONCURRENTLY LOST O-ANTIGEN 4, AND SALMONELLA TYPHOSA TRANSDUCTANTS RECEIVING O-ANTIGEN 2, LOST THEIR NATIVE O-ANTIGEN 9. THESE RESULTS INDICATE THAT THE GENETIC DETERMINANTS OF O-ANTIGENS 2, 4, AND 9 OCCUPY THE SAME O LOCUS IN SALMONELLA PARATYPHI A, SALMONELLA TYPHIMURIUM, AND SALMONELLA TYPHOSA, RESPECTIVELY, AND ARE PROBABLY ALLELIC. A STUDY OF SEVERAL NATURALLY OCCURING SALMONELLA LACTOSE POSITIVE EPISOMES ISOLATED FROM CLINICAL SOURCES HAS REVEALED THAT THEY HAVE SEX FACTOR PROPERTIES. IT IS CONCLUDED FROM GENETIC STUDIES, THAT SOME OF THESE SEX FACTORS INTERACT WITH THE BACTERIAL CHROMOSOME, AND BEHAVE AS THE PROTOTYPE F FACTOR OF ESCHERICHIA COLI. FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

24. SECURITY CLASSIFICATION	25.	26. DOD CODE	27. BUDGET CODE
a. CLASSIFIED [X] UNCLASSIFIED b. RESTRICTED		02	1
28. MISSION RELEVANCE		29. PARTICIPATION	
NA		NA	

30. REQUEST NO ABSTR	31. SPECIAL EQUIPMENT
32. ESTIMATED COMPLETION	33.

DD FORM 1498 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 are identical to DD Form 1222)

Project 3A025601A822, MILITARY INTERNAL MEDICINE

Task 01, Military Internal Medicine

Work Unit 122, Microbial genetics and taxonomy

Investigators.

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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. Chromosomal transfer has been observed between Escherichia coli donor cells and Proteus mirabilis recipient cells. Proteus hybrids have been constructed which contain Escherichia coli characters. Analysis of these hybrids by genetic and physical chemical methods has correlated the presence of a number of Escherichia coli genes with the total amount of Escherichia coli DNA added to the Proteus cells during conjugation. One of these Proteus hybrids contains as much as 30% Escherichia coli DNA and has several Escherichia coli fermentation properties and is sensitive to certain Escherichia bacteriophages, and has some Escherichia coli surface antigens.

2. Salmonella hybrid strains have been found to be able to repress the multiplication of coliphage Lambda (λ).

3. It was possible to cotransduce the his and O-antigen 2 genes to both Salmonella typhimurium and Salmonella typhosa. Salmonella typhimurium transductants which received somatic antigen 2 concurrently lost O-antigen 4, and Salmonella typhosa transductants receiving O-antigen 2, lost their native O-antigen 9. These results indicate that the genetic determinants of O-antigens 2, 4, and 9 occupy the same O locus in Salmonella paratyphi A, Salmonella typhimurium, and Salmonella typhosa, respectively, and are probably allelic.

4. A study of several naturally occurring Salmonella lac^+ episomes isolated from clinical sources has revealed that they have sex factor properties. It is concluded from genetic studies, that some of these sex factors interact with the bacterial chromosome, and behave as does the prototype F factor of Escherichia coli.

Progress.

1. Chromosomal Transfer from Escherichia coli to Proteus. Previous studies in this laboratory have employed conjugation to promote genetic exchange between Escherichia coli K-12 donor strains and recipient bacteria classified in various genera composing the Enterobacteraceae.

Intergeneric hybridizations have been achieved by the conjugal transfer of small episomic elements, such as the F-lac merogenote, and multiple drug resistance factors. Such elements have been transmitted among Escherichia, Salmonella and Shigella, all of which have the same average guanine plus cytosine DNA base composition of 50%. Furthermore, these extrachromosomal elements have been transferred to genera whose DNA base composition differs significantly from that of E. coli, namely Serratia (58% GC) and Proteus species (39% GC). The promiscuity of episomes is further illustrated by the transfer of the F-lac episome of E. coli K-12 across family lines to Vibrio comma. In contrast to episomes, the transfer of chromosome from E. coli K-12 Hfr donors to different recipient genera has not been studied as broadly. Although recipient strains of Salmonella typhosa, Salmonella typhimurium and Shigella species have been hybridized with E. coli Hfr strains, such intergeneric hybridizations occur usually at lower frequencies than that found with E. coli recipients. Moreover, in the case of Salmonella, the hybrids very frequently behave as unstable partial diploids, presumably due to the differences in DNA homology between E. coli and Salmonella species. Recently it has been possible to transfer parts of the chromosome from E. coli to Proteus strains by conjugation. The Proteus hybrids that are formed are partial diploids which contain a complete Proteus chromosome and segments of the E. coli genome. The Proteus diploids may lose the E. coli chromosomal segment and revert to the original Proteus genotype.

We will describe the method and the strains used in the formation of these Proteus diploids. Three streptomycin sensitive, Hfr derivatives of E. coli K-12, which differ in their polarity of chromosome transfer, were used as donor strains. W1895 donates as an early marker the lactose (lac) genes, followed by the proline (pro) and arabinose (ara) chromosomal loci with galactose (gal) as the terminal marker. Hfr H has a different polarity of transfer, the gene order of transfer being ara-pro-lac-gal. Strain P4X6 has the same polarity as W1895, an early marker being pro, followed by ara and finally lac as terminal marker. The DNA base composition of these strains is 50% GC, typical of E. coli. As the recipient, we employed a streptomycin resistant, non-swarming Proteus mirabilis strain, WR11. It has a 39% GC base composition and is unable to utilize lactose, arabinose or galactose as a carbon source.

By means of plate matings using either W1895 or Hfr H donors and WR11 as a recipient, we have recovered lac^+ Proteus hybrids at very low frequencies, about one lac^+ hybrid per 5×10^7 Hfr males. The Proteus hybrids do not show any differences from the original Proteus in cell size, colonial morphology, or growth rate, but can be easily distinguished

from the original Proteus on selective media because of the acquired fermentation ability. Table I summarizes the characteristics of three such hybrids.

All the hybrids were unstable for the lac$^+$ character, continuously segregating lac$^-$ types on repeated platings. They were agglutinated by anti-Proteus serum, were urease positive, and showed strong sensitivity to a virulent Proteus mirabilis phage. No evidence of male properties, i.e. F-pili, male phage sensitivity and lac$^+$ donor ability, could be detected in these hybrids, as compared to a Proteus harboring a known F-lac merogenote which expressed these properties (Strain PM-1 F-lac). We therefore concluded that the lac$^+$ Proteus hybrids are unstable heterozygous partial diploids containing a lac K-12 chromosomal segment unassociated with a functional F-factor.

Physicochemical examination of hybrid DNA provided further evidence for the hybrid nature of these clones. DNA was extracted from hybrids by the Marmur method and centrifuged to equilibrium in a CsCl density gradient. The results of such an analysis on hybrid WR13 indicate that in addition to the main band, having a density equivalent to the Proteus parental DNA, there is a satellite DNA band at a density of 1.710, which corresponds to the density of E. coli DNA. We consider this satellite component — about 6% of the total DNA extracted — to be the lac$^+$ diploid segment.

Attempts to recover arabinose positive diploids from crosses of E. coli Hfr donors with P. mirabilis have been unsuccessful. In contrast, if a Proteus lac$^+$ diploid is used as a recipient in backcrosses with either of the Hfr donors, both arabinose and galactose positive hybrids have been isolated, the frequency being about 10-50-fold greater than that observed for the initial lac$^+$ hybridization. The following table summarizes the results of CsCl density gradient analysis of three hybrids and their segregants.

TABLE 2. AMOUNT OF DNA IN THE SATELLITE BAND OF PROTEUS HYBRIDS

Strain	Characteristics	Main Band GC Content	Satellite Band GC Content	Satellite Band Amount*
WR 11	Lac$^-$ Proteus parent	39%	—	nd**
WR 13	Lac$^+$ hybrid	"	50%	6%
WR 16	Lac$^+$ Ara$^+$ hybrid	"	50%	20%
	Lac$^+$ Ara$^-$ segregant	"	50%	6%
	Lac$^-$ Ara$^-$ segregant	"	—	nd
WR 17	Lac$^+$ Gal$^+$ hybrid	"	50%	16%
	Lac$^+$ Gal$^-$ segregant	"	50%	6%
	Lac$^-$ Gal$^-$ segregant	"	—	nd
WR 18	Lac$^+$ Ara$^+$ Gal$^+$ hybrid	"	50%	26%

* Percent of total DNA extracted
** None detected

TABLE I

CHARACTERISTICS OF LAC$^+$ PROTEUS HYBRIDS

STRAIN	MAC CONKEY LACTOSE	UREASE	SENSITIVITY[a] TO PROTEUS PHAGE	AGGL. PROTEUS ANTISERUM	F-PILI	SENSITIVITY[b] TO MALE PHAGE R-17	LAC$^+$ TRANSFER
WR11	STABLE LAC$^-$	+	+	+	-	-	-
WR13 (W1895 X WR11)	UNSTABLE LAC$^+$ LAC$^-$	+	+	+	-	-	-
WR14 (HFR H X WR11)	"	+	+	+	-	-	-
WR15 (HFR H X WR11)	"	+	+	+	-	-	-
PM-1 F-LAC	"	+	N.T.	+	+	+	+

(a) PLAQUE TEST

(b) TITER INCREASE TEST

WR16, a lac$^+$ ara$^+$ diploid has a satellite band of about 20% of the total extracted DNA. WR17, a lac$^+$ gal$^+$ diploid and WR18 a lac$^+$ ara$^+$ gal$^+$ diploid show satellite bands of 16% and 26% respectively. An analysis of segregants of these diploids enabled us to estimate the size of the ara$^+$ and gal$^+$ segments added during backcross matings. By subtracting the 6% satellite of lac$^+$ ara$^-$ and lac$^+$ gal$^-$ segregants from the percent satellite DNA in lac$^+$ ara$^+$ and lac$^+$ gal$^+$ hybrids, values of 14% for the ara segment and 10% for the gal segment have been estimated.

The amount of satellite DNA associated with these fermentation properties is more than that needed to code for the enzymes involved in carbohydrate utilization and suggests that other non-selected E. coli markers may have been inherited along with the selected genes. We are now trying to determine in detail how many of the E. coli genes are transferred and functioning in the Proteus diploids.

With the diploid strains that we have available at the present time, it has been feasible to examine as unselected markers the inheritance of E. coli type I pili genes, which map near the arabinose locus, and the inheritance of the receptor for coliphage T1 which maps between the arabinose and lactose loci. These results are presented in Table 3.

TABLE 3. INHERITANCE OF NON-SELECTED MARKERS IN LAC$^+$ - ARA$^+$ PROTEUS HYBRIDS

HYBRID TYPE	NO. TESTED	E. COLI TYPE I[(a)] PILI	COLIPHAGE T1[(b)] RECEPTOR
LAC$^+$ ARA$^+$ (P4X6 X WR13)	21	20	18
LAC$^+$ ARA$^+$ (HFR H X WR13)	24	14	18
LAC$^+$ ARA$^+$ (W1895 X WR13)	24	5	22

(a) AGGLUTINATION TEST; ANTISERUM VS PURIFIED E. COLI TYPE I PILI

(b) TESTED BY SPOT TEST, FOR EVIDENCE OF THINNING

A number of lac$^+$ ara$^+$ hybrids, derived from matings with P4X6, Hfr H and W1895 donors and the lac$^+$ WR13 recipient were examined. The presence of E. coli type I pili was scored by slide agglutination tests employing antiserum prepared against purified E. coli type I pili. As expected, a high proportion of the clones had inherited this marker. The presence of coliphage T1 receptor was scored by spotting a high multiplicity of T1

phage on faint lawns of the clones. After about four hours of incubation, clones possessing the T1 receptor were lysed, presumably a lysis from without type of phenomenon. Although we have been unable to plaque T1 on such hybrids, the validity of this test is supported by adsorption experiments and experiments showing inhibition of growth by high input ratios of phage T1. We are currently examining other coliphages for their behavior on hybrids in an effort to demonstrate other non-selected E. coli genes.

After studying the various Proteus diploids, the consistent pattern has emerged that the more E. coli genes that a Proteus diploid carries, the larger the satellite DNA band. This provides a method for the direct measurement of chromosomal segments of diploids that has not been possible before. The only difficulty has been in establishing methods to demonstrate the expression of some E. coli genes in Proteus diploids. Some progress has been made in methods of determination which E. coli genes have been transferred to the Proteus diploids. At the present time, carbohydrate genes can be easily detected. The E. coli surface antigen, type I pili, and also susceptibility to E. coli phages has been shown to be transferred to some Proteus diploids.

2. Repression of Coliphage λ by a Native Salmonella Repressor. Bacteriophage λ has been studied in great detail since its discovery in 1951 as a lysogenic phage in Escherichia coli K-12. These experiments have been limited to K-12 and a few other strains of E. coli. In the past few years, we have been interested in extending the λ system to Salmonella species. This approach was made possible by crossing appropriate recipient strains of Salmonella typhosa or Salmonella typhimurium with E. coli K-12 Hfr donors.

By selection for a distal marker of the donor strain, Hfr Cavalli, diploid hybrids can be obtained which possess approximately 30% of the E. coli chromosome. Such diploid Salmonella hybrids encompass the λ receptor site locus thus allowing for adsorption of the phage. In general, the Salmonella hybrids remain unstable and various types of segregants can be isolated from them. The diploid colonies are characteristically smaller in size and more dense in appearance than are the segregant colonies. The larger segregant colonies closely resemble the Salmonella parent in colonial appearance. While some segregant types appear to have stably integrated portions of the E. coli chromosome, others seem to have lost various segments of the original diploid region.

The original diploid Salmonella strain and a series of segregant types possessing different regions of the K-12 genome were examined. All of the strains listed are able to adsorb λ, but none of the strains were able to plaque the phage or produce infectious centers even at exceedingly high multiplicities of infection. In addition, the transfer of λ prophage from E. coli donors to such Salmonella hybrids does not

result in zygotic induction in the recipients. Furthermore, examination of a large number of gal$^+$ Salmonella recombinants from crosses with λ lysogenic K-12 donors showed that none of these recombinants had become lysogenic for λ.

The possibility of restriction of the λ phage was considered an explanation for these observations. Experiments were performed to test the restriction hypothesis using as experimental controls, E. coli K-12 and E. coli B mal$^+$ a strain known to restrict λ DNA. Two Salmonella hybrids, one with and the other without the E. coli rm locus, were tested at the same time. No appreciable degradation of C^{14} labelled λ DNA was observed with either of these Salmonella hybrids, whereas typical results are obtained with the E. coli K-12 and B strains. These experiments have led us to conclude that restriction as defined by DNA degradation was not involved in the inability of λ to produce plaques on these Salmonella hybrids.

Although plaques are not produced, the gal genes can be transduced to Salmonella hybrids containing the rm locus of E. coli. HFT lysates of the thermo-inducible mutant, λ_c, obtained by heat induction of E. coli K-12 heterogenotes produce gal transductants of λ_c dg in the Salmonella hybrid. These λ_c dg transductants fail to be induced under temperature conditions causing induction and lysis of λ_c in K-12. At present, we interpret the above data as indicating that Salmonella species have a repressor substance which acts to prevent the multiplication of λ either after infection, zygotic induction, or lysogenic induction.

By plating high-titered lysates of wild-type λ, λ vir, and λ^{i434} on the Salmonella hybrids, we have been able to isolate mutants which have apparently overcome the Salmonella repressor. These mutant phages plate with equal efficiency on both E. coli and the Salmonella hybrid regardless of the host on which they were previously grown. The appearance of the mutant plaques, however, is considerably different on the two hosts. The mutant phages produce clear plaques on E. coli which are typical in appearance to the usual c mutants previously described in λ. When plated on the Salmonella hybrid hosts, the mutant plaques formed are clear but considerably smaller than on K-12. The λ mutant phages plate equally well on Salmonella hybrids whether or not they possess the E. coli rm locus.

We have attempted to isolate mutants of the Salmonella hybrid defective in repressor which would permit the production of plaques by wild-type λ and its derivatives. The Salmonella hybrid strain was subjected to the mutagen methyl-nitro-nitroso guanidine and the treated colonies were screened by plating 500 p.f.u. of λ vir on them. Out of approximately 300 colonies examined to date, three mutants were detected on which plaques were produced. Wild-type λ and λ^{i434} lysates were tested on these mutant strains. None of the three mutants plating λ vir were susceptible to wild-type λ, while only one of the mutants was susceptible to λ^{i434} in addition to λ vir. We interpret these results as indicating that the mutants are now unable to repress λ vir, and in the case of mutant #40, λ^{i434} as well, but are still able to repress wild-type λ.

3. Somatic Antigen 2 Inheritance in Salmonella Groups B and D. We have shown previously that the genes which determine Salmonella somatic (O) antigens 4 (Group B) and 9 (Group D) are mutually replaceable in genetic transfer experiments between Group B and Group D Salmonella. In other words, these genes behave as alleles of the same chromosomal locus. This O-locus is closely linked to the chromosomal determinant of histidine biosynthesis (his), and is cotransducible with his (by phage P22) in both S. typhimurium and S. typhosa. In the present study, transduction and conjugation experiments were employed to examine the genetic basis of O-antigen 2 of S. paratyphi A, a representative of Salmonella Group A. The results of these experiments indicated that the genes determining O-antigens 2 of S. paratyphi A, 4 of S. typhimurium, and 9 of S. typhosa occupy the same O-locus in their respective species and are probably allelic.

An S. paratyphi A donor strain was obtained by mating an S. paratyphi A var. durazzo recipient strain (WR7000) with the S. typhosa Hfr strain WR4000, and selecting those S. paratyphi A hybrids which inherited the terminal lac^+ marker of WR4000. One such hybrid, which had inherited also the closely linked sex factor, F, of WR4000 and now behaved as an Hfr donor, was selected for further use. It was designated WR4002.

The S. paratyphi A Hfr strain WR4002 was mated with the S. typhimurium recipient strain WR5000 and selection was made for those WR5000 hybrids which inherited the his^+ marker of WR4002. Serological examination of 100 such hybrids revealed that 70 of these agglutinated in single factor 2 antiserum and displayed no reaction in Group B (factors 4, 5, 12) antiserum, having lost their native O-antigen 4. The remaining 30 gave positive reactions in Group B O-antiserum and did not react with factor 2 antiserum. As was the case in our previous Group B X Group D crosses, no intermediate types expressing both or neither of the antigens were detected. These findings are consistent with the view that the genetic determinants of O-antigens 2 and 4 (as well as 9) occupy the same chromosomal locus in their respective Salmonella species and are probably allelic.

Phage P22 was grown on an S. typhimurium WR5000 his^+ hybrid which had received the O-2 determinant from WR4002, and the resulting lysate was employed to transduce the his^+ marker to S. typhimurium WR5000. Of 100 his^+ transductants tested, 14 had acquired O-antigen 2 and concurrently lost O-antigen 4. It was observed that all hybrids which lost antigen 4 lost O-antigen 5 as well. Since we have shown previously that the genetic determinant of O-antigen 5, although located near his, is not cotransducible with this marker, it is inferred that the loss of antigen 5 is caused by the loss of antigen 4, and does not involve, in this instance, the replacement of the S. typhimurium O-5 gene. This dependency of O-antigen 5 expression on the presence of O-antigen 4 has been observed previously in this laboratory, as well as by Mäkelä, in crosses involving Group B and Group D Salmonella.

The same P22 lysate of the his$^+$, O-antigen 2 containing hybrid of WR5000 was used also to transduce the his$^+$ marker to the S. typhosa strain WR4205. Of the 72 his$^+$ transductants examined, eight had acquired O-antigen 2 and, as expected, lost their native O-antigen 9. As was the case with the S. typhimurium conjugation and transduction hybrids, no recombinants were detected which displayed both or neither of the antigens in question. The findings again indicate that the genetic determinants of O-antigens 2, 4, and 9 are probably alleles of the same O-locus in S. paratyphi A, S. typhimurium, and S. typhosa, respectively.

4. Lac$^+$ Elements of Salmonella. Preliminary characterization of several lactose positive (lac$^+$) strains of Salmonella has been previously reported. These strains were isolated from hospital and other natural sources by Dr. W. H. Ewing of the Communicable Disease Center at Atlanta, Georgia. These lac$^+$ Salmonella strains have been shown to contain transmissible genetic elements and studies were undertaken to compare these elements isolated from a wide variety of sources to the classic F-lac episome of E. coli. If these elements are similar to the F-lac of E. coli, it means that these infectious genetic elements occur in at least one other species of bacteria and have a wide geographic distribution.

These Salmonella lac$^+$ genetic elements are transferred to E. coli at high frequencies and have also been transferred to Proteus strains. In Proteus it has been possible to characterize the physical-chemical properties of the DNA associated with the episomes. In a general way these properties of the Salmonella genetic elements are similar to the F-lac episome of E. coli.

These strains have been examined for other episomic properties. One of the Salmonella genetic elements, lac$^+$ 32, has been shown to promote the transfer of chromosomal markers from one strain to another at low frequency. Even though the frequency is low, it is significant that chromosomal transfer occurs at all and indicates additional similarity to F-lac.

Another property of F-lac is associated with the surface antigens of the F pili and this has not been demonstrated to be present in these Salmonella genetic elements. Cells with F-lac episomes have F pili on the surface which can be detected by the adsorption of F-specific phages. Tests of the Salmonella lac$^+$ genetic elements in both E. coli and Salmonella strains with the F-specific phages indicate that the phages did not adsorb to these cells. This shows some difference between the surface antigen of these genetic elements and the F-lac episome.

Genetic elements are sometimes associated with colicin production and the Salmonella strains with the lac$^+$ genetic elements were examined for their ability to produce colicins. The lac$^+$ Salmonella strains were spotted on colicin sensitive cells. If these cells produced a colicin, then zones of lysis would be expected; but none were observed. Thus, these lac$^+$ genetic elements of Salmonella do not carry genes for colicin production.

Acridine orange is a proflavine dye, which will selectively eliminate or "cure" non-chromosomal DNA material in sensitive strains. Lac$^+$ elements of E. coli are known to have a higher sensitivity to acridine orange than elements of Salmonella or Proteus. The lac$^+$ 32 element was therefore transferred by conjugation to E. coli 200-U lac$^-$. The resulting 200-U lac$^+$ 32 was then compared with 200-U F-lac for elimination of the lac$^+$ element or "curing." The results showed that from increasing concentrations of 5, 10, 15, and 20 μg/ml of acridine orange, 200-U F-lac ranged from .4% to 90% curing. However, 200-U lac$^+$ 32 was not cured at similar concentrations and higher. Thus, while there seems to be some basic similarities between the lac$^+$ Salmonella genetic elements and the F-lac episomes of E. coli; the evidence also indicates that there are many differences and cast doubt on the hypothesis of the identical origin of these genetic elements and the F-lac episome.

Summary and Conclusions.

1. After mating Proteus mirabilis with Escherichia coli K-12 Hfr donors, P. mirabilis lac$^+$ hybrids were recovered at low frequency. These hybrids behave as unstable partial diploids. When a P. mirabilis lac$^+$ hybrid is used as a recipient in backcrosses with E. coli Hfr donors, transfer of the ara$^+$ chromosomal marker was detected, whereas ara$^+$ hybrids have not been observed in crosses with the original P. mirabilis recipient. Genetic analysis of unselected markers in such P. mirabilis lac$^+$ ara$^+$ diploids revealed the presence of E. coli genes for type I pili and the receptor site for phage T1, suggesting that the diploid segment can consist of the lac$^+$ through pil$^+$ chromosomal region. Physical examination of the deoxyribonucleic acid (DNA) from such hybrids by CsCl density-gradient centrifugation showed a large satellite band of E. coli DNA (50% guanine plus cytosine) in addition to the Proteus parental DNA component (39% guanine plus cytosine). The size of the satellite DNA band was correlated with the size of the chromosomal segment transferred to the Proteus diploids.

2. Coliphage λ is adsorbed by Salmonella hybrids possessing the λ receptor site locus obtained by recombination with Escherichia coli K-12. The formation of plaques or infectious centers, however, does not occur when λ is plated on these hybrids. In addition, the transfer of prophage λ from E. coli donors to Salmonella recipients does not result in zygotic induction of the recipient. These observations cannot be explained by restriction of λ because the DNA of the phage is not degraded by the Salmonella host. It appears therefore that Salmonella species have a repressor substance which prevents multiplication or zygotic induction of λ. Induction of λdg in Salmonella hybrid transductants also is repressed regardless of the inducing agents used or the thermoinducibility of the phage. Mutants of λ which have overcome this native Salmonella repressor were isolated by plating high titered lysates on the Salmonella hybrid. These mutant phages plate with equal efficiency

on both E. coli and the Salmonella hybrid without regard to the host on which they were previously grown. Repressor mutants of the Salmonella hybrid also have been isolated on which derivatives of wild-type λ are now able to produce plaques.

3. Somatic (O) antigen 2 of Salmonella paratyphi A replaced somatic antigen 4 of an S. typhimurium recipient as the consequence of mating with an S. paratyphi A var. durazzo Hfr strain. The genetic determinants of these O-antigens behaved in this cross as alleles of a common O-locus, which is linked to the determinant of histidine biosynthesis, his. By employing phage lysates obtained by growing P22 on an S. typhimurium hybrid which had received his and O-antigen 2 genes from the S. paratyphi A Hfr, it was possible to cotransduce the his and O-antigen 2 determinants to both S. typhimurium and S. typhosa. S. typhimurium transductants which received O-antigen 2 concurrently lost O-antigen 4, and S. typhosa transductants receiving O-antigen 2 lost their native O-antigen 9. These results indicated that the genes determining O-antigens 2, 4, and 9 occupy the same O-locus in S. paratyphi A, S. typhimurium and S. typhosa, respectively, and are probably allelic.

4. Lactose (lac$^+$) fermenting Salmonella strains were isolated from hospital patients and other sources. These strains have lac$^+$ genetic elements that have been transmitted at high frequencies to E. coli and to Proteus strains. In Proteus these genetic elements show satellite DNA bands and it is possible to measure the amount of DNA associated with each of the genetic elements. While there are similarities to the F-lac episome of E. coli, these Salmonella genetic elements have differences in acridine orange sensitivity and susceptibility to F-specific phages. While some chromosomal transfer is promoted by these genetic elements, the frequency of transfer is much lower than that initiated by F-lac integration into the chromosome. Thus, these genetic elements of Salmonella are similar, but not identical to the F-lac episome of E. coli.

Publications.

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3. Johnson, E.M. Somatic Antigen 2 Inheritance in Salmonella Groups B and D. J. Bacteriol. 94:2018-2021 (1967)

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RESEARCH AND TECHNOLOGY RESUME			DA CA6443		
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(U) BIOMEDICAL INVESTIGATIONS OF TYPHOID FEVER

3. AGENCY ACCESSION DA CA6443			
4. DOCUMENT CLASS C			
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9. KEY WORDS (U) BIOLOGY			
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a. NUMBER C. IN-HOUSE	b. PHASE NA b. TYPE NA	c. DATE NA d. AMOUNT NA	
11. GOVT LAB INSTALLATION/ACTIVITY			
NAME	WALTER REED ARMY INST OF RES		
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12. TECHNOLOGY UTILIZATION	NA		

13. START DATE 09 63	14. COST CODE DATA NA	15. FUNDING AGENCY OTHER DA	
16. PROFESSIONAL MAN YEARS	CURRENT 68	PROJECTED 69	BUDGET 69
	C	C	90
	C	C	90
17. PERFORMING ORGANIZATION			
NAME	WALTER REED ARMY INST OF RES		
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ASSOCIATE INVESTIGATOR TEL	202-576-2077	TYPE	DA
22. COORDINATION	NA		

SYNOPSIS, CHOLERA, DYSENTERY, INTESTINAL DISEASE, PARASITIC, SALMONELLA INFECTIONS, TYPHOID, TYPHOID, INTESTINAL SECRETIONS, INTESTINAL MICROORGANISMS, RADIATION BIOLOGY.

(U) TECH OBJECTIVE - THE PATHOLOGY AND PATHOGENESIS OF VARIOUS DISEASES OF THE GASTRO-INTESTINAL TRACT OF MAN AND EXPERIMENTAL ANIMALS IS STUDIED BY MULTIDISCIPLINARY APPROACHES WITH EMPHASIS ON MORPHOLOGY. THESE INVESTIGATIONS ARE CONSIDERED ESSENTIAL PARAMETERS FOR A COMPREHENSIVE AND SCIENTIFICALLY BASED THERAPY OF DIARRHEAL DISEASES AND RADIATION INJURY TO THE INJURY TO THE INTESTINE.

(U) APPROACH- PRINCIPALLY MORPHOLOGIC, INCLUDING LIGHT, FLUORESCENT AND ELECTRON MICROSCOPIC EXAMINATIONS. KINETIC STUDIES USING TRITIUM-LABELLED THYMIDINE AND HISTOCHEMICAL INVESTIGATIONS ARE ALSO EMPLOYED.

(U) PROGRESS - JUL 67 THRU JUN 68 19 MANUSCRIPTS AND REPORTS WERE COMPLETED. THE AREAS OF INVESTIGATION INCLUDED, 1) PATHOLOGY OF EXPERIMENTAL SHIGELLOSIS, 2) IMPACT OF BACTERIAL INVASION ON CELLULAR ENZYMES, 3) STUDIES ON EXPERIMENTAL ENTERITIS DUE TO SALMONELLA TYPHIMURIUM, 4) PATHOLOGY AND PATHOGENESIS OF CHOLERA, 5) ENTERIC VIRUS DISEASE, 6) EVALUATION OF AUTOPSY FINDINGS IN ACUTE, FATAL DIARRHEA IN MEXICAN CHILDREN, 7) RADIATION INJURY AND REPAIR OF SMALL INTESTINES OF RATS FOLLOWING SUBLETHAL IRRADIATION AND 8) MISCELLANEOUS STUDIES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

23. PROJECT/TASK/WORK UNIT	24.	25. DOD CODE 6R	26. BUDGET CODE 1
27. KEY WORDS		28. FURNISH FOR PUBLICATION NA	
29. SPECIAL ASSIGNMENT			

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM MAY BE USED UNTIL EXHAUSTED. SUPERSEDES AR 705-5, 20 MAY 1963, WHICH IS OBSOLETE.

Project 3A025601A822, MILITARY INTERNAL MEDICINE

Task 01 - Military Internal Medicine

Work Unit 123, Histopathologic Manifestations of Diarrheal Disease

Investigators.

Principal: COL Helmuth Sprinz, MC
Associate: CPT Ronald M. Maenza, MC
CPT Earl J. Kasdon, MC
Helen R. Jervis, D. Sc.
Akio Takeuchi, M. D.
Thomas G. Merrill, Ph. D.
Paul A. Shurin, LT, PHS, part-time

Description.

In accordance with the basic orientation of the department, collaborative and independent studies of the pathogenesis and the evolution of morphologic lesions of various forms of enteritis were continued or started. Joint projects with the Departments of Applied Immunology, Gastroenterology, and Radiation Biology were successfully completed and new ones are being pursued.

Progress.

1. Dr. Merrill completed our work on the acute effects of a non-lethal dose of staphylococcal enterotoxin on the mucosa of the gastro-intestinal tract. The results were published in a series of 3 papers, the last in Laboratory Investigation (see Bibliography); the preceding 2 papers appeared in the American Journal of Pathology, Volume 48, 1966. The work was also presented at a staff conference at Fort Detrick. No further studies in this area are being performed by our group.

2. The work on experimental shigellosis has received international recognition and is now being cited even by Russian and Japanese Investigators who in the past have been sceptical towards us as newcomers to the field. Dr. Takeuchi was invited to address the 46th Congress of the Japanese Microbiological Society in Tokyo and to present the data of the WRAIR team, in particular his own optical and electronmicroscopic studies on the pathology of shigellosis. He was also invited to lecture at the Keio University School of Medicine in Tokyo (his alma mater), at the Jikei University School of Medicine, Tokyo, at the Institute of Tropical Medicine, Nagasaki University, Nagasaki, at the Aichi Cancer Center, Nagoya, and at the National Institutes of Health in Tokyo. His scientific exhibit,"Patterns of Invasion of the Intestinal Mucosa by Various Microorganisms", was first shown at the 1968 Spring Meetings of the American Association of Pathologists and Bacteriologists and International Academy of Pathology and was very well received.

3. There exists a great need for an *in vitro* model to study the penetration of the intestinal mucosal barrier by enteric microorganisms. So far all investigations performed in this area, the study of virulence of enteric bacteria, must utilize intact, live animals. The use of whole animals introduces many complex factors which influence and modify the host-parasite relationships. Since the demonstration of the dramatic role of the intestinal brush border in bacterial invasion by our group, especially by Dr. Takeuchi, we attempted over the past 3 years to reproduce this phenomenon *in vitro*, utilizing a brush border preparation. CPT Kasdon thoroughly investigated this subject, unfortunately with a negative result. Brush border preparations which fulfill presently known criteria for 1) morphologic integrity of their plasma membranes, as determined by the electron microscope, and 2) for functional integrity as determined by measurements of their disaccharidase activity, do not interact with bacteria, contrary to our hopes and some evidence previously obtained. It is still unknown which factors present *in vivo* are responsible for our failure. This experience has focused our attention on a key problem of enteric infections: the nature of virulence and its morphologic expression in a host parasite system.

4. A related project deals with the impact of bacterial invasion on intestinal epithelial enzymes. In last year's report the hope was expressed that Gomori acid phosphatase methods could be adapted for our purposes. Unfortunately, so far we have been unsuccessful due to the vagaries of tracer methodology. However, we have pinpointed the sources of our failure. We now foresee the possibility that lysosomal enzymes can be demonstrated by high resolution autoradiography utilizing unfixed, unembedded, freeze-dried tissues, sectioned in a special cryostat. We are presently engaged in adopting this method for our laboratory.

5. *Salmonella typhimurium* enteritis. Work on this important experimental model was continued with the help of 2 associates. CPT Maenza completed studies on the anatomical aspects and left a manuscript entitled, "Experimental Diarrhea: *Salmonella* Enterocolitis in the Rat." He described the clinical course and morphologic characteristics of the enteritis and attempted to correlate anatomic with physiologic alterations. LT Shurin completed his studies on the pathogenesis of mouse typhoid in normal and immune mice. Lethal infections with *S. typhimurium* have been induced intraperitoneally in normal, *Salmonella* free mice and in mice immunized with living, avirulent organisms. The pattern of mortality and the histogenesis of the disease were indistinguishable in the two groups. However, a hundredfold larger inoculum of bacteria was required to induce the disease in the immunized animals. Studies by other investigators using the same dose of bacteria in challenging control and immune animals have led to the conclusion that immunity in mouse typhoid confers the ability to completely clear a challenge dose of virulent bacteria from the host's tissues. Our findings do not corroborate these observations. The identical progression and spread of the lesions of the disease in both groups of animals studied by us was an unexpected finding. It suggests that, in a potentially lethal infection, host defense factors are effective only in the period shortly following infection. This is

the period at which a defensive role for serum antibody has been clearly demonstrated. The enhanced bacterial killing power conferred by acquired cellular immunity, on the other hand, is potentially a factor throughout the course of the disease, but has not been shown to be effective at the sites at which bacterial multiplication takes place. It is quite possible that the environment of the granulomas in the liver and spleen protects the organisms from host defensive factors. These findings are in agreement with those obtained by Hornick and Woodward in human volunteers infected with S. typhi. In both experimental models it was demonstrated that immunity to typhoid is partial, affecting only the dose of bacteria required to induce the disease. The clinical and pathologic course of the disease, once the infection becomes established, is unaffected by the previous state of immunity.

6. Studies on cholera are being continued. Our results and interpretation have been challenged by Yardley and Elliot using the canine and adult rabbit model and by Norris employing the infant rabbit model. Work during the past year has narrowed the area of disagreement. However, only we have described a cytotoxic effect on intestinal vascular endothelium. Further work is in progress to resolve this problem.

7. Studies on radiation injuries of the intestine revealed several striking parallels with inflammatory bowel disease as seen in bacterial infection. New to us was the absence of intestinal epithelial denudation and the striking regenerative capacity of crypt cells in animals dying from the intestinal radiation syndrome. The results of our work have encouraged us to attempt to broaden our studies on intestinal injury due to ionizing radiation.

Summary and Conclusions.

As may be seen from the bibliography the individual contributions were directed to the over-all continuity of our research. This department has become a leader in experimental gastro-intestinal research which has been recognized also by the election of the Principal Investigator to membership in the American Gastroenterologic Association.

During the past year the direction of activity was slightly shifted and greater emphasis is being placed on correlation with clinical, physiologic and radiobiologic data. During the year there was a nearly one hundred per cent turn-over of the enlisted technicians participating in these studies. Three of the Associate Investigators, Maenza, Kasdon, and Shurin, completed their obligatory military service. Likewise, Captains Donati and Powell, our principle collaborators in Radiation Biology and Gastro-intestinal Physiology, returned to civilian life. Their replacements are due to arrive in the Fall.

Publications.

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2. Kent, T. H., Formal, S. B., and LaBrec, E. H. Salmonellae Virulence Testing. A. M. A. Arch. Path., 84:300-303, 1967.

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6.1 Scientific or Technical Area: 14100 RADIOBIOLOGY; 012900 PHYSIOLOGY

5. Project No.: 3A062110A819 00 Task No.: 00 Work Unit No.: 122

4. Title: (U) ABSORPTION AND LOSS OF RADIOISOTOPES BY THE GUT

12. Start Date: 07 63	13. Completion Date: N A	14. Reporting Period: OTHER DA		
15. Resources Estimate: FY 68	Man-Years: 2	Funds: 85		
Cumulative: 73	2	85		
7. Procurement Method: C. IN-HOUSE	8. Contract/Grant: a. Number: NA b. Type: NA	c. Date: NA d. Amount: NA		

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Investigators Principal: CONRAD, LTC R. E.
Associate:
Tel: 202-576-2358 Type: DA

21. Technology Utilization: NA

22. Coordination: NA

23. Keywords: ABSORPTION, INTESTINE, IRON, HEME, HEMOGLOBIN, POLYMER.

(U) TECH OBJECTIVE - STUDIES OF MECHANISMS REGULATING THE ABSORPTION, EXCRETION AND BODY CONTENT OF TRACE ELEMENTS.

(U) APPROACH- STUDIES OF INTRALUMINAL, MUCOSAL AND CORPOREAL FACTORS INFLUENCING THE ABSORPTION OF ORGANIC AND PORPHYRIN IRON.

(U) PROGRESS - JUL 67 THRU JUN 68 STARVATION AND PROTEIN DEPRIVATION DECREASE THE QUANTITY OF DIETARY IRON WHICH IS AVAILABLE FOR ABSORPTION. IN ADDITION, THEY DECREASE THE CAPABILITY OF THE GUT TO ABSORB AVAILABLE IRON. ANIMAL STUDIES INDICATED THAT THIS ABSORPTIVE DEFECT WAS RELATED TO THE RELATIVE UNAVAILABILITY OF AMINO ACIDS FOR GLOBIN SYNTHESIS. THE IRON DEFICIENT STATE BECOMES MORE APPARENT IN ANIMALS MAINTAINED ON LOW PROTEIN HIGH CALORIE STARCH OR RICE DIETS BECAUSE TISSUE CONCENTRATIONS OF IRON ARE REDUCED MORE RAPIDLY THAN IN STARVATION. THE ADMINISTRATION OF ERYTOXIN CAUSES MARKED REDUCTION IN THE CAPABILITY OF EXPERIMENTAL ANIMALS TO ABSORB IRON. THUS MALNUTRITION AND INFECTION ARE ADDITIVE CAUSES OF IRON DEFICIENCY. THE BASIC MECHANISM FOR THESE ABNORMALITIES WERE INVESTIGATED. THE MECHANISM FOR ABSORPTION OF HEMOGLOBIN-IRON WAS INVESTIGATED AND SHOWN TO INVOLVE SPLITTING OF HEME FROM GLOBIN WITH ABSORPTION OF HEME INTO THE INTESTINAL CELL. IRON IS SPLIT FROM HEME WITHIN INTESTINAL CELLS FOR SUBSEQUENT ABSORPTION INTO THE BODY BASED UPON NEED. ABSORPTION OF HEME FROM THE LUMEN OF THE GUT COULD BE MARKEDLY ENHANCED BY DEPOLYMERIZATION OF MACROMOLECULAR AGGREGATES OF HEME. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

27. Security Classification: [X] Secret [ ] Restricted	28.	29. DoD Code: PA	30. Project Code: 1
31. Mission Objective: NA		32. Participation: NA	
33. Man-Years Cumulative	34. Special Purpose Code		
35. Keywords	36.		

DD Form 1498m PREVIOUS EDITION OF 1 JAN 65 IS OBSOLETE. Supersedes AR 705-5 which may be used. (Incl. 1 thru 26 - Complete AR 705-5 Form 1122).

Project 3A025601A822 MILITARY INTERNAL MEDICINE

Task 01, Military Internal Medicine

Work Unit 125, Absorption and loss of radioisotopes by the gut

Investigators.
Principal: LTC Marcel E. Conrad, MC
Associate: LTC F. D. Garretson, MC, CPT Stanley G. Schade, MC and Harold L. Williams

Description.

Studies of the mechanisms regulating the body content of trace metals by investigation of both absorption and excretion of radioactive labeled compounds.

Progress.

Iron deficiency is a major nutritional problem throughout most of the world, which reduces the capability of affected populations to maintain a sustained effort and remain self sufficient. Iron deficiency is most frequent in those geographical areas where (1) the diet contains little protein; (2) dietary iron is mostly inorganic iron and (3) infection is commonplace in the native populations. The role of these factors upon iron absorption and excretion has not been considered previously. To ascertain whether they had an effect upon the state of iron repletion, studies were performed in both experimental animals and humans.

Starvation and protein-deficient diets caused marked ferrokinetic abnormalities and decreased absorption in rats fed diets containing a normal content of iron. Observations at intervals after the onset of fast and in rats fed various amounts of protein suggested that the changes in iron metabolism were caused by depressed erythropoiesis and retarded growth. The diminished weight gain and decreased hemoglobin synthesis increased the concentration of iron in the plasma and various body organs. Starved rats with iron deficiency or hemolysis increased erythropoiesis to normal levels but not to values observed in unstarved or phenylhydrazine treated animals. Although sufficient quantities of substrate can be mobilized for hemoglobin synthesis, a relative unavailability of precursors for hemoglobin synthesis may be important in the etiology of diminished red blood cell production. Animals attempted to re-establish and maintain a normal body concentration of iron by decreased absorption and increased excretion of iron. The decreased absorption of iron was not accompanied by an increased iron content or iron concentration in the intestinal mucosa. However, in starved rats increased amounts of dialyzable iron were incorporated into the duodenum from the body stores. This indicated that iron absorption was not affected by

the total quantity of iron within intestinal cells but that unbound iron within mucosal cells might be an important regulator of iron absorption.

Experiments were performed in rats maintained on either rice or starch diets. These diets were chosen because iron deficiency is commonplace in populations who consume rice as their principal dietary staple and the habitual ingestion of laundry starch is a common practice among certain populations and is often associated with severe iron deficiency. Obviously, much of the iron deficiency which occurs among persons maintained on either rice or starch diets is caused by the relatively low iron content of these foodstuffs. However, in animals studies in which these foodstuffs were made iron-replete by the addition of iron to the diet, decreased absorption of iron was observed. This was not caused by a direct intraluminal effect of the starch or rice upon dietary iron but seemed related to protein deprivation and the decreased erythropoiesis described above.

The frequent association of chronic infection with iron deficiency and ferrokinetic abnormalities led us to study the effects of bacterial endotoxins upon iron absorption and repletion. The parenteral administration of endotoxin causes marked changes in iron absorption and metabolism within one hour after administration of small sublethal doses. The changes observed during the first day following injection were unique; there was decreased absorption of iron with a normal intestinal iron content, an accelerated rate of iron clearance from plasma and a decreased serum iron concentration. That a generalized cytotoxic effect upon the gut was not the cause of these changes was suggested by the normal intestinal histology and lifespan of mucosal cells, normal absorption of glucose and unchanged excessive absorption of iron by iron-depleted, endotoxin treated animals. Two days after the administration of a single injection of endotoxin most abnormalities became normal except that the intestinal iron content increased, and a significant decrease in iron absorption persisted. It was only during this later period that iron-depleted rats had decreased absorption of iron from the gut. We postulated that the acute absorptive defect was caused by a decreased capability to transfer iron from the mucosal cell into the body, whereas the late defect was associated with impaired entry of intraluminal iron into the intestinal absorptive cells.

The high incidence of iron deficiency in geographical portions of the world where meat is relatively unavailable, and the differences in the chemical form of iron between meat and most other dietary constituents, led us to study the absorption of hemoglobin-iron and make comparisons with the absorption of iron from inorganic salts. Fasting subjects absorbed greater quantities of iron from test doses

of ferrous salts than from hemoglobin. However, more physiologic experiments in which the labeled iron preparations are added to food, much greater quantities of iron were absorbed from hemoglobin than from ferrous iron salts. Thus, hemoglobin can be an important source of dietary iron. That hemoglobin-iron is absorbed selectively based upon body requirements was shown in iron absorption studies in humans before and after phlebotomy. The mechanisms for the enhanced absorption of iron from hemoglobin were studied both in vitro and in human experiments. Heme is split from globin within the duodenal lumen by proteolytic enzymes and the heme is absorbed into the duodenal mucosal cell as an intact and metalloporphyrin. Iron is split from the heme within the intestinal cells and transferred into the plasma as inorganic iron. The globin degradation products were found to be important for the absorption of heme from the intestinal lumen. Test doses of heme are poorly absorbed in comparison to similar quantities of hemoglobin-iron. This was shown to be caused by the polymerization of heme into macromolecular structures which cannot be absorbed into the intestinal epithelial cell. The amino acids from globin act as depolymerizing agents to prevent the formation of heme macromolecules. Similarly, the addition of other compounds which form ligands with heme will markedly enhance the absorption of iron from the metalloporphyrin, i.e, niacin, carbon monoxide, cyanide. Comparison of human studies with animal experiments performed during the previous fiscal year suggested that the selective absorption of heme-iron was regulated either by controlled release of iron from heme within intestinal cells or by regulation of the quantity of iron transported from intestinal cells into the plasma or both.

Demonstration of the importance of polymerization in the regulation of absorption of hemoglobin-iron led us to investigate the possible role of macromolecular formation with inorganic iron. "In vitro" studies of ferrous iron salts showed that this iron formed significant quantities of large sized molecular compounds at the slightly alkaline pH of the duodenum. Polymerization of inorganic iron was markedly enhanced by the addition of small quantities of sodium bicarbonate to the solutions. Studies in animals showed that the quantity of iron absorbed from test doses was markedly decreased by polymerization. That this phenomenon might have physiologic significance was investigated in animals given injections of secretin. Secretin stimulates the production of bicarbonate by the pancreas and reduces iron absorption. These phenomena may explain the siderosis observed in patients with chronic pancreatis, cystic fibrosis and perhaps cirrhosis and add to a basic understanding of the importance of the chemical form of iron in both the diet and pharmacologic preparations used for the treatment of iron deficiency.

Summary and Conclusions.

Starvation and protein depletion markedly decrease the capability of the intestine to absorb test doses of iron. This phenomenon can be demonstrated in animals maintained on either rice or starch diets and may partially explain the high incidence of iron deficiency in many geographical areas of the world. The capability of intestinal cells to absorb increased amounts of iron from meat was studied and the basic mechanisms delineated. Bacterial endotoxins were shown to markedly decrease iron absorption. Experimental studies suggested that this was an impaired capability to transport iron from intestinal cells into the plasma. The role of polymerization of both inorganic iron and hemoglobin-iron was studied and provided an etiology for siderosis in certain disease states, a role for the pancreas in iron absorption and information of value in understanding differences in the absorption of iron from various foodstuffs and pharmacologic preparations.

Publications.

Benjamin, B. I., Cortell, S. and Conrad, M. E.: Bicarbonate-induced iron complexes and iron absorption: One effect of pancreatic secretions. Gastroenterology 53:389, 1967.

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Rieber, E. E., Conrad, M. E., and Crosby, W. H.: Gastrectomy and iron absorption: Effects of bleeding, iron loading and ascorbic acid in rats. Proc. Soc. Exper. Biol. Med. 124:577, 1967.

PROJECT 3A025601A823
MILITARY PSYCHIATRY

Task 01
Military Psychiatry

[illegible]

62[illegible]

(U) SOCIAL AND PRIVATE PSYCHIATRY

[illegible] CLINICAL MEDICINE [illegible] PSYCHOLOGY

[illegible]	[illegible]	NA	[illegible]
C. IN-HOUSE	NA	NA	[illegible]

[illegible]	69	4	[illegible]
[illegible]	69	4	[illegible]

15. GOV'T LAB/INSTALLATION/ACTIVITY

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17. TECHNOLOGY/MISSION

SOCIAL + COMMUNITY PSY

22. COORDINATION

NA

COMMUNITY PSYCHIATRY, CROSS-CULTURAL COMMUNICATION, SEMANTIC CLASSIFICATION, THERAPEUTIC COMMUNITY, PSYCHIATRY, GROUP PROCESS.

(U) TECH OBJECTIVE - TO INVESTIGATE SOCIOCULTURAL FACTORS IN PRIMATES AND MAN INFLUENCING PRODUCTION OF MALADAPTIVE PSYCHOPHYSIOLOGICAL AND GROUP BEHAVIOR, USING RESULTS TO MAXIMIZE INDIVIDUAL ADAPTIVE BEHAVIOR IN MILITARY ORGANIZATIONS, E.G., REDUCTION OF MAN DAYS LOST DUE TO PSYCHOPHYSIOLOGICAL COMPLAINTS, AWOL, BASIC TRAINING STRESS, AND IMPROVEMENT OF THE ABILITY OF THE US SOLDIER TO COMMUNICATE WITH NEMESIS OF OTHER CULTURES.

(U) APPROACH- TO STUDY INFLUENCE OF INDIVIDUAL AND SOCIOCULTURAL FACTORS IN CLINICAL AND FIELD CIRCUMSTANCES IN THE US ARMY, SE ASIA AND CERTAIN PORTIONS OF AMERICAN CIVILIAN SOCIETY ON HUMAN BEHAVIOR. THE TECHNIQUES EMPLOYED ARE INDIVIDUAL PSYCHIATRIC INTERVIEWS, EXPERIMENTAL GROUP MANIPULATIONS, GROUP PROCESS ANALYSIS, SOCIOMETRIC DEVICES, QUESTIONNAIRES, SOCIAL ANTHROPOLOGIC INVESTIGATIONS, SEMANTIC ANALYSIS, OPERANT CONDITIONING, ANALYSIS OF FORMAL AND INFORMAL STRUCTURE OF ORGANIZATIONS, AND MEASUREMENTS OF PSYCHOENDOCRINE REACTIVITY. SINCE THESE STUDIES ARE CARRIED OUT USING HUMANS OPERATING IN VARYING ENVIRONMENTS, THE MAJOR TECHNICAL PROBLEMS ARISE FROM UNEXPECTED ENVIRONMENTAL CHANGES, E.G., INTENSIFICATION OF THE STRUGGLE IN VIETNAM.

(U) PROGRESS - JUL 67 THRU JUN 68 NEW STUDIES ARE UNDERWAY INVESTIGATING INFLUENCES OF INDIVIDUALS GROUP PAST HISTORY ON PSYCHOENDOCRINE RESPONSES AND INVESTIGATIONS INTO ORIGINS AND MAINTENANCE OF ALCOHOLISM AT FT. MEADE, MD, WITH EXPERIMENTAL APPROACHES TO TREATMENT OF PATIENTS AND SPOUSES. WORK CONTINUES ON RESTORATION TO DUTY BY OPERANT CONDITIONING IN A THERAPEUTIC COMMUNITY AND GROUP PSYCHOLOGICAL PROCESSES INFLUENCING TRAINING AND DUTY PERFORMANCE. PAPERS HAVE BEEN PRESENTED ON ENDOCRINE RESPONSES IN GROUPS IN COMBAT, INFLUENCE OF GROUP PROCESSES ON DUTY PERFORMANCE, AND COMMUNITY PSYCHIATRY TECHNIQUES ON MILITARY POSTS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

[illegible]		[illegible]	1
[illegible]		NA	

[illegible]

Project 3A025601A823 MILITARY PSYCHIATRY

Task 01, Military Psychiatry

Work Unit 030, Social and preventive psychiatry

Investigators.
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Description.

1. Conditioning eye movements. Operant conditioning techniques have been applied to the task of conditioning eye movements as free operants. A stand-type Hackworth television Eye-marker Camera has been adapted for recording of the movements of both eyes simultaneously. A picture of the target of gaze, together with spots of light representing the positions of the eyes, is fed to a visual monitor and a video tape recorder. Reinforcements for desired eye movement patterns are delivered by hand by the experimenter viewing the monitor screen and pressing a switch. The effectiveness of reinforcements in controlling eye movements is evaluated quantitatively by independent observers viewing the video tape eye movement record without the reinforcement record.

2. Traumatic parietal lobe disease. Visual hemi-inattention syndromes have been studied in cases of traumatic unilateral parietal lobe disease. An eight-choice automated visual discrimination task was presented, in which one of the choices is a horizontal line bisected in the exact center by a short vertical line, and the other choices are similar horizontal lines bisected at varying distances from the center. After appropriate teaching procedures, a twenty-five trial set to such discriminations was given to five patients with right cerebral hemisphere disease, five patients with left cerebral hemisphere disease, and ten patients with no brain disease.

3. Visual brightness thresholds. Visual brightness thresholds are being assessed in the albino rat by a behavioral technique employing the conditioned suppression paradigm first described by Estes and Skinner. A flashing light is used as the conditioned stimulus for suppression of bar-pressing behavior, and contingencies are arranged such that evidence of suppression during a given presentation of the stimulus automatically programs a decrease in the intensity of the stimulus for the next presentation, and vice versa.

4. Operant conditioning in psychotherapy. This project was begun four years ago by Dr. Goldiamond and Dr. Dyrud, who attempted to apply operant conditioning principles to describe the standard intensive psychotherapy situation. These investigators studied the process of the therapy through one-way mirrors and written transcripts of psychotherapy sessions. Dr. Colman joined the project in September 1966, at first as a trainee in this new method of analysis and later investigating a patient of his own with these methods.

5. Experimental psychiatric ward. Many non-effective soldiers diagnosed as character and behavior disorders are admitted to the psychiatric services of Army General Hospitals. They represent a small fraction of a similar group of men seen at mental hygiene clinics and stockades. Recent policy has lowered mental induction standards for the Armed Services and has determined to teach poorly prepared men to adjust to military life. Since few guidelines exist for instituting effective programs, the Army psychiatric service has the urgent mission of finding new approaches for treatment of the character and behavior disorders. During the past year an experimental ward has been established for the study of this problem.

The Army's use of positive and negative reinforcements to shape its troops, plus the military mission's requirement for highly predictable performance and controls, closely matches the philosophy and technical constructs of operant conditioning which provides a useful model for clearly formulating operational principles for reconditioning men to active duty.

The model of an operant conditioning ward linked to an extra-ward or "field" program, in combination, affords the following therapeutic milieu:

a. A social system in which explicit and immediate information concerning adequacy and acceptability of social and work performance is provided, a system in which the soldier can regularly observe that desirable behaviors "pay off" and other behavior does not.

b. The control intrinsic to such a system is directed to teach the soldier specific behaviors, including occupational, educational, and recreational skills, and the interpersonal skills required for success in the groups in which men live, work, and fight in the Army.

c. The program moves from communication of control through extrinsic reinforcements toward more internalized control systems for each man during the treatment period.

d. The work program is coordinated with a nearby military post so that behavior learned on the ward can be "generalized" into an application model for measure of role competence before return to duty.

Patients comprising the reported group are selected from soldiers admitted to Walter Reed General Hospital, and diagnosed as character and behavior disorders. (Homosexuals, alcoholics, and men under sentence are excluded.) Half of the group is randomly picked for the research ward; the other half, provided the more traditional hospital treatment and disposition, serve as Controls. Longitudinal follow-up is carried out on both. The ward utilizes a point economy in which reinforcements (TV, card playing, authorized absences, educational courses, etc.) are paid for by points earned in military activities, problem-solving groups, formal instruction, and work projects. Across time, the program shifts toward more delayed point reinforcement schedules, and, in a few cases, the men may be taken off the point system entirely when nearing discharge.

Follow-up of each soldier at his next duty assignment is coordinated through the military post's Mental Hygiene Consultation division. This promotes strengthened communication between an Army General Hospital, Walter Reed, and the post mental hygiene clinic, and between the clinic and the local military units.

6. Alcohol study. Last year at the research field station at Ft. George Meade, Md., the department established a study of soldiers with problems in excess intake of alcohol. The goal of the study was to define the problems in establishing an alcohol treatment unit in the army, to characterize the population served, and to identify staff time and capability requirements. In addition, the study attempted to characterize the social and behavioral patterns of the patients in their homes and units with emphasis on relationship to their spouses and the interaction of the units in which the problem of alcoholism was manifested.

Progress.

1. Conditioning eye movements. It has been possible to condition fixation of gaze alternately to and away from a given area of the target in eight out of eight normal subjects, and to demonstrate specific control of the tone reinforcer over gaze fixation by an "A-B-A" paradigm. It has also been possible to establish color as a discriminative stimulus for direction of gaze in five out of five normal subjects, and to generate a simple pattern of eye movement (side-to-side and up-down) in five out of five normal subjects.

2. Traumatic parietal lobe disease. An examination of errors per opportunity as a function of discrimination difficulty shows a significant increase in error rates with increasing task difficulty in the five patients with right brain lesions, as compared with the five patients with left brain lesions and the ten patients with no brain lesions, who did not differ significantly from each other. Stimulus generalization curves generated by graphic choice frequency as a function of distance of the error choice from the center show a significant "peak shift" in all five patients with right brain lesions, not

in the others. The peak shift disappears when the task is treated as a sample-matching task, i.e., when no judgement of "which is in the center" is required.

3. Visual brightness thresholds. Relative stability and reproducibility of brightness thresholds under conditions of dark-adaptation have been achieved in four normal rats over multiple determinations. In addition, a significant change in the brightness threshold has been elicited in two rats as a result of damage to the retina by fluorescent light for a prolonged time. Retinal damage has been confirmed by histologic examination in one of the experimental rats.

4. Operant conditioning in psychotherapy. For the past year the psychotherapy project has continued with the emphasis shifting from observation of conventional therapy to utilization of the operant procedural description to modify and rationalize the therapeutic process. The project terminated in June 1968.

5. Experimental psychiatric ward. Fifty men from the comparison group and 50 men from the ward experimental group have been sent back to active duty over the past year. Through June 1968 two-thirds of the experimental group have either been honorably discharged or are successfully completing their military duty as compared with less than 10 percent of the comparison group.

The ward program has been stabilized so that we are now able to begin to describe the ward as a feasible and exportable model for treating character disorders in the military. The educational program, work program, point system, phase two plan, work and educational contracts, and general staff organization are described in forthcoming papers, as are theoretical issues of this treatment mode.

The focus of the study has been upon examination of certain procedures in a rigorous experimental fashion to help guide practical operations. Specific studies have included: (1) manipulation of points as reinforcement for specified behavior; (2) shaping a higher performance level with differential amounts of reinforcement; (3) demonstration of "model" behavior by a ward officer (psychiatric resident); (4) punishment by a point fine to control undesired behavior; (5) use of a chaining-type reinforcement contingency to increase desired behavior; and (6) differential reinforcement of the individual versus the group to increase a verbal performance.

6. Alcohol study. An alcohol treatment center has been established at Ft. Meade. Referred patients have been evaluated through extensive interviewing and the administration of two psychological tests. In the units of affected patients a formalized interview has been carried out with the Commanding Officer and First Sergeant. In addition, where spouse is available, she has been seen and evaluated by interview and psychological test. Approximately 40 patients have

been evaluated and approximately 30 have been treated or are being followed. Current treatment has consisted of individual, couple and group therapy with and without concomitant administration of medication.

Summary and conclusions.

1. Conditioning eye movements. Eye movements and gaze fixation may be conditioned as free operants by manipulating their external consequences. Current study is attempting to generate conjugate gaze in subjects with congenital strabismus, and steady gaze in a subject with congenital nystagmus. These efforts should be greatly facilitated by an automatic photocell recording matrix now under construction.

2. Traumatic parietal lobe disease. Patients with right parietal lobe disease show a relatively specific deficit in spatial "criterion" or "decision making," although actual visual discrimination is unimpaired. This finding contributes to the resolution of a long-standing controversy in the neurologic literature over whether the non-dominant parietal lobe serves any specific "spatial orientation" functions, or whether apparent deficits are merely the artifact of absence of aphasia in the patient with non-dominant parietal lobe disease. Operant techniques which are known to affect "peak shifts" in stimulus generalization will be employed in an attempt to work out a rational therapeutic approach to visual hemi-inattention syndromes.

3. Visual brightness thresholds. The combination of conditioned suppression and a response-adjusting method of varying stimulus intensity provides a feasible tool for evaluation of brightness thresholds in the albino rat. This technique will next be applied to the study of the effects of chloroquine and other retinotoxic agents on visual receptor function.

4. Operant conditioning in psychotherapy. This project has investigated the possibility of utilizing the operant psychological model to describe psychotherapy and use these descriptions to modify the therapeutic process. The language of operant psychology is an elegant one for describing the therapeutic transaction, yet lends itself to thinking in terms of more practical procedures in psychotherapy. Considerable headway has been made in more accurately programming therapeutic operations particularly with obsessive and hysterical characters. A more complete report of the project's findings and conclusions is in progress.

5. Experimental psychiatric ward. (1) Using the behavior of running a half-mile course, it was found that soldier participation could be increased by awarding larger amounts of reinforcement (points) for running. (2) The speed of running was greatly increased when the running speed required for a larger point award was gradually increased from week to week. (3) When the ward's psychiatric resident made the run with the men (thereby serving as a behavioral "model"), the

soldiers' participation in the run was unchanged. (4) An effort was made to increase attendance at the unit meeting by punishing with a 10 point fine the conflicting behavior, namely, sleeping in bed. The effect was to sharply reduce attendance at programmed activities, including the unit meeting. (5) In contrast, a chaining-type positive reinforcement contingency was quite successful in increasing attendance at the unit meeting. The contingency was simply that a soldier must attend the meeting in order to receive points for other activities. (6) To increase the frequency of verbal reports at a group meeting, a large number of points were given to the group (the audience) when a soldier presented a verbal report. At a later time the same number of points was allocated to the individual speakers. The more effective procedure with several low frequency speakers was reinforcement of individual speakers.

The project gives hope of developing an application model for the treatment and rehabilitation of the character and behavior disorder in the army. It is already planned to expand the program and integrate it into the routine psychiatric service of Walter Reed General Hospital. In addition, the ward has been used to contribute basic research findings relating to control of group behavior by an operant milieu.

RESEARCH AND TECHNOLOGY RESUME

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STRESS, MENTAL HYGIENE, HUMAN PERFORMANCE, LEARNING, MEMORY, PSYCHOPHYSIOLOGY.

(U) TECH OBJECTIVE - THIS RESEARCH FOCUSES UPON ANALYSIS OF PRINCIPLES UNDERLYING BEHAVIOR INVESTIGENT RELATIONSHIPS LEADING TO PERFORMANCE DECREMENT AND ASSOCIATED PHYSIOLOGICAL RESPONSES. EMPHASIS IS PLACED UPON DEVELOPMENT OF MODELS WHICH ARE PREDICTIVE OF PERFORMANCE AT DIFFERENT LEVELS OF TASK COMPLEXITY. RESEARCH CONTENT INCLUDES VIGILANCE DEGRADATION, HUMAN DATA PROCESSING, BEHAVIORAL STRESS, CONCEPT FORMATION, MEMORY, PSYCHOLOGICAL FATIGUE, PROGRAMMED INSTRUCTION, AND PHYSIOLOGICAL CONDITIONING PROCESSES.

(U) APPROACH- LABORATORY EXPERIMENTATION WITH HUMAN VOLUNTEER SUBJECTS IS PERFORMED TO DEFINE UNDERLYING PROCESSES WHICH CONSISTENTLY LEAD TO STRESS AND FATIGUE PHENOMENA. PSYCHOPHYSIOLOGICAL MEASUREMENTS ARE USED TO ASSESS PHYSIOLOGICAL COST ASSOCIATED WITH EXPOSURE TO EXPERIMENTALLY DEFINED ENVIRONMENTS. PERFORMANCE LIMITS ARE EXPERIMENTALLY DEFINED UNDER A VARIETY OF NORMAL AND UNUSUAL ENVIRONMENTAL CONDITIONS.

(U) PROGRESS - JUL 67 THRU JUN 68 STUDIES ARE IN PROGRESS CONCERNING STRESS EFFECTS ON JUDGMENT PROCESSES, AVOIDANCE CONDITIONING OF CARDIODECELERATION AND CARDIOACCELERATION, STRESS EFFECTS UPON PAIRED ASSOCIATE LEARNING, AND PSYCHONEUROSAINE RELATIONSHIPS UNDERLYING CONDITIONED EMOTIONAL DISTURBANCES. EXPERIMENTS HAVE BEEN COMPLETED ON STRESS EFFECTS UPON VERBAL RETENTION, PERFORMANCE ON MULTIPLE CONCURRENT SCHEDULES OF SIGNAL DETECTION, AND ON CIRCADIAN RHYTHMS IN VIGILANCE PERFORMANCE. ONE PSYCHOENDOCRINE SERIES HAS BEEN COMPLETED TO STUDY PLASMA LEVELS OF PITUITARY-ADRENOCORTICAL AND PITUITARY GONADES DURING AVOIDANCE CONDITIONING. RESULTS TEND TO SUGGEST THAT THE CONSTRUCTION OF AVOIDANCE CONTINGENCY RELATIONSHIPS IS A MORE IMPORTANT FACTOR IN INDUCING TYPE OF BEHAVIORAL AND PHYSIOLOGICAL CHANGE THAN TASK STRUCTURE. RESULTS SUGGEST THAT PHYSIOLOGICAL CHANGES IN STRESS SITUATIONS TEND TO VARY FROM EARLY CONCEPTS OF A TYPICAL STRESS REACTION. VIGILANCE STUDIES HAVE SUGGESTED THAT CIRCADIAN RHYTHMICITY IN VIGILANCE PERFORMANCE CAN BE MODULATED BY MEAN SIGNAL FREQUENCIES WHICH MAY BE RELATED TO MOTIVATIONAL AND FATIGUE VARIABLES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A025601A823, MILITARY PSYCHIATRY

Task 01, Military Psychiatry

Work Unit 031, Analysis of behavior and of mediating mechanisms: Measurement of performance and decrement of performance

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Description.

This work unit studies influences of social and environmental variables upon human performance. It includes psychophysiological measurements to determine physiological costs associated with maintaining demanding task performance in aversive laboratory and natural settings. Research content includes vigilance decrement, behavioral stress, verbal learning and retention, concept formation, and socialization processes.

Progress.

1. Vigilance decrement.

The research on human vigilance performance has been extended to study control over human monitoring performance by multiple concurrent schedules of signal programming and to provide further data on the importance of circadian variations as a source of variability in monitoring performance.

The primary results which have been supported by the studies of multiple concurrent schedules are as follows:

a. The periods of training necessary to establish stable patterns of monitoring performance are often much longer than the periods over which past studies have obtained measurements. Consequently, past work has based conclusions about human vigilance performance upon transitory stages, which do not tend to reflect the patterns of responding characterizing the individual after steady-state responding is reached.

b. The manner in which signals are presented on a display does induce clearly observed control over the human observer's responding to the display. The probability of detecting a randomly inserted signal decreases as the observer develops stereotyped response patterns to the display's idiosyncracies.

c. It has been found that observing or scanning patterns can be controlled simultaneously by three schedules of signal programming, each inducing a very different pattern of visual scanning of a given display area. When other schedule combinations are employed for signal programming, a single schedule can assume control over scanning of the entire display, while the other component schedules exert no observable effect. Since the patterns in rate of observing panel meters were readily predictable, in terms of schedules of reinforcement data, it is evident that assessment of a man-machine system is not complete until it is known how the programming of display information affects the long-term work pattern of the human operator.

The results from studies of circadian rhythmicity in human vigilance performance are as follows:

(1) Further studies have confirmed that circadian rhythms are present in rate of observing panel meters and in efficiency of signal detection.

(2) Independent spectral analyses of rate of responding to individual areas of display operated by multiple concurrent schedules show very similar spectral intensity distributions, even though responding on the different schedules can be very different on gross inspection of records. This indicates that different patterns of responding to different signal presentation schedules can be affected similarily by circadian influences.

(3) Spectral analysis of rate of response information from monitoring performance frequently reveals multimodal intensities. These multimodal spectral intensities have been accounted for by a spectral convolution model. In this model these multimodal rhythms are viewed as side bands generated by the slow modulation of an essentially basic proximate 24-hour rhythm. The model implies that the spectral dispersion noted in the results we have obtained may be attributable to very slowly varying processes such as learning or habituation to the testing environment. The model does provide a quantitative estimation of the effects of the unknown (modulating) variable.

At the present time, plans are in progress to induce experimentally clear disruptions of the normal activity routine in order to explore the issue of predicted modulation of the circadian rhythm. This issue will be explored through exposing test subjects to 72-hour sleep deprivation and phase-shifting of the normal day. The resulting effects will be compared with predictions made on the basis of the spectral convolution model.

2. Behavioral stress.

The behavioral stress research program has been continued, with further investigations of factors which lead to performance decrement in aversive environments. One portion of our current research in this area consists of studying effects of an acute two-hour stress experience on the secretion of plasma 17-OHCS, plasma insulin, plasma growth hormones, plasma luteinizing hormone, and excretion of urinary catecholamines. This investigation represents a collaboration between the Department of Experimental Psychophysiology and the Department of Neuroendocrinology. A second portion of this program concerns the study of threat of punishment upon tracking performance. The third portion consists of studying effects of unavoidable punishment upon verbal retention. The fourth portion of this program is concerned with the study of avoidance conditioning of cardioaccelerative and cardiodecelerative responses. Finally, a project is underway to study predictability of individual stress tolerance.

a. Effects of psychological stress on endocrine activity. A pilot investigation of psychoendocrine responses to behavioral stress in seven male volunteers has been completed. This investigation is preparatory to a larger statistical study of controlled laboratory stress effects upon a number of pituitary hormones. Measurements have been made of plasma 17-hydroxycorticosteroids plasma insulin, plasma growth hormone, plasma luteinizing hormone, and excretion of urinary catecholamines. Information on effects of stress in humans has been accumulated for measures of pituitary-adrenocortical and medullary hormones. Little systematic data are available on possible effects of behavioral stress upon growth hormone, insulin secretion, or upon luteinizing hormone. In the present work, a nondiscriminative avoidance conditioning procedure is used to induce acute stress levels in young male volunteers. Through venous catheterization, blood samples are withdrawn on repeated occasions during control and stress session days. Urine is collected before, during, and following each session. Fractionated samples are used to study the course of hormonal changes, relative to time of day and specific responses to the avoidance conditioning experience. Results from the pilot study have shown the predicted elevation of plasma 17-hydroxycorticosteroid levels in blood, increased excretion of epinephrine, and some evidence of rises in growth hormone. The growth hormone changes are much less clear, due to large uncontrolled elevations which appear to occur on a spontaneous and intermittent basis at different times of day.

b. Effects of behavioral stress on tracking performance. A study in current progress has been designed to study effects of a punishment avoidance contingency upon zero input tracking performance. The goal of this study is to delineate factors which lead to increased tracking accuracy from those which induce performance degradation. One test group has been given the basic tracking task alone. The stress condition superimposed upon task performance is avoidance of electric shock punishment when error is kept within prescribed limits. This avoidance contingency has resulted in consistent increases in number of control stick commands, decreases in integrated error scores, and decreases in the number of time error reaches the prescribed limits. These results will be compared with those of several other groups, who are given a secondary task superimposed upon the tracking task. In one of these groups, the avoidance contingency will be attached to the tracking task and performance on the auxiliary task will lead to no punishment. In a second one of these groups, the contingency will be attached to the auxiliary task and tracking errors will not lead to punishment. Another group will be given the auxiliary task alone, with the avoidance contingency attached. We anticipate that group comparisons will reveal striking differences in performance levels among the different groups.

c. Stress-associated forgetting of verbal learning. A replication and extension of recent findings by Glucksberg and King (Science, 1967, 158, 517-519) has been performed to study stress-associated forgetting of paired-associates learning. These investigators had reported that forgetting in A-list - B-list paired-associates could be obtained by shocking D-list words which were implicitly associated (from word-association norms) with the B words. Forgetting was significantly greater for B words implicitly associated with shocked D words than for B words associated with non-shocked D words. This finding was described as "motivated forgetting." To replicate and extend these results, half of the test subjects in our study were given D words and half C words, which could be shocked. It was argued: if forgetting occurs in B words which are associated with shocked D words, more forgetting should occur in B words when associated C words are shocked. It was found that when original learning of the A-B list was controlled, there were no differences in forgetting attributable either to C or D word lists, or to shock or no-shock treatments. These findings contradicted the original Glucksberg-King findings, and a replication which they recently completed. We plan a further replication which will obtain a physiological measure (GSR) of the assumed mediated stressful effects of the shock-associated word chains.

d. Avoidance conditioning of heart rate in humans. Previous investigations on avoidance conditioning of heart rate in humans have suggested several issues pertinent to conceptualization of behavioral stress and associated physiological costs. A study is in current progress and near completion to investigate the following:

(1) The feasibility of conditioning both cardioacceleration and cardiodeceleration concurrently within individual subjects.

(2) The effects of contingent and non-contingent punishment upon conditioning.

(3) The effects of correct vs. incorrect instructions regarding contingency relationships upon success in conditioning cardioacceleration and cardiodeceleration.

(4) The interaction of the above variables.

The basic technique employed consists of discriminative avoidance conditioning procedure, in which punishment can be avoided in some cases by increases in heart rate, and in other cases by decreases in heart rate. The specific contingency between heart rate and electric shock avoidance depends upon the presence of two discriminative stimuli (lights). When a blue light is presented, heart rate must increase above the previous minute for shock to be avoided. When a white light is presented, heart rate must decrease below the previous minute's total to avoid shock. Pairs of test subjects participate in the experiment simultaneously, using two different test chambers. Half of the subjects are given correct instructions. The other half are given incorrect instructions. Half of the subjects are given contingent (earned) punishment and the other half noncontingent punishment. This results in four different groups. Results from the correct instruction - contingent punishment group showed significant evidence both of accelerative and decelerative responses in the presence of the blue and white lights respectively. The correctly informed noncontingent punishment group failed to show significant differences in heart rate from one condition to another. The subjects told incorrectly that punishment was contingent upon their heart rates have shown evidence of cardioaccelerative and decelerative responses. The group given contingent punishment and noncontingent instructions has shown no real differences between responses during the white and blue lights. These results lead to a modification of previous conclusions: The basic hypothesis that the cardiovascular effects of shock are dependent on the conditions under which punishment is presented continues to be supported. However, it is now suggested that instructions, as a pretraining or preconditioning procedure must be considered a very important aspect of conditioning humans. Pretraining through verbal instructions can

have the effect of drastically modifying subsequent conditioning of cardioacceleration and cardiodeceleration.

e. Prediction of individual stress tolerance. Data are now being collected to test the possibility of predicting individual tolerance of acute behavioral stress on the basis of conventional psychometric instruments. Test subjects are divided into several groups based upon tolerance of avoidance conditioning in the laboratory. Approximately one-half of all test subjects tested with our current techniques for avoidance conditioning of heart rate find themselves unable or unwilling to complete the requirements of the experiment. Those who fall into this category typically display numerous indications of psychological stress. In our current work, every test subject utilized in the avoidance conditioning work is given a lengthy battery of specific and general-purpose tests. It is hoped that one of these tests, or a combination of test items can be developed into a reliable measure for predicting tolerance of the individual to avoidance experiences. The current test battery includes the following tests: Minnesota Multiphasic Personality Inventory; Sixteen Personality Factor Test; Rosenzweig Picture Frustration Test; and the Cold Pressor Test. The dimensions of special interest for this investigation include the neuroticism and extroversion factors measured by the 16-PF test; the ego strength and anxiety scales from the MMPI; and the specific cardiovascular response to the Cold Pressor Test.

3. Concept formation.

Our current studies in the concept formation area include studies of analogy-solving performance and multiple cue probability learning. The former area is especially pertinent to problems in the measurement of basic intellectual abilities. The latter is particularly pertinent to the development of measurements of judgment processes and degradation of judgment.

Previous work by Gentile has indicated that a large portion of total variance associated with solution of analogy items could be attributed to an association or association-like process. Two projects attempting to demonstrate the influence of the associational relatedness of words in analogy items on the solution of those items are near completion. The first represents a transfer design, in which test subjects receive a pretraining session to prime selected associates of the analogy item stem word pairs and then receive the analogy items to test the influence of priming on the analogy performance. Data are now being analyzed. The second project concerned the prediction of subjects' rank orderings of the same sets of words on a word relatedness rating scale. The average rank order correlation over

ten items is of the order of .64. Further work is planned to cross validate this substantial correlation. One important implication of these findings is that analogy tests are not nearly as pure a measure of reasoning ability as had been thought. Associational factors are largely determined by individual cultural and educational backgrounds. Consequently, culturally disadvantaged individuals may be handicapped when they are given analogy tests for screening or selection purposes. This hypothesis will receive further examination.

4. Groups, organizations, and institutions.

a. Military discharge procedures governing the management of those found "unfit" or "unsuitable" for further military service. Work has continued on this project, with emphasis on the comparison between the use of the former AR 635-208 and AR 635-209 with the present AR 635-212. One item of particular importance is the counselling and rehabilitation provision (Section II) of AR 635-212 and the extent and under what conditions these requirements are waived by command authority. Also of interest are the number of discharges under the present regulation, and the question of whether actual enforcement of AR 635-212 is fundamentally different from its predecessors. An important procedural question that emerged from an examination of discharge figures for the earlier regulations was the rather high number of military personnel who waived their right to an administrative board hearing. The extent to which these waivers were actually based on the subject's knowledge of the consequences of a general or an undesirable discharge is not known, nor is it clear from the figures why so many persons preferred a quick solution rather than administrative procedures that could possibly have resulted in a higher discharge. It would also be useful to compare these waiver figures with those under the present AR 635-212. These and other questions are being studied, and it is hoped that the research data on the first group of persons to enter the Armed Services under "Project 100,000" (selection of quotas from Mental Group IVs for military service) will answer the question of whether an inordinate number of servicemen in this project find their way into the channels of AR 635-212 or its equivalent in the other services.

b. Socio-legal studies of the experimental ward and field program for treatment of character and behavior disorder. Work has continued on this project, with emphasis on two aspects of the program: the enforcement of ward rules and penalties, and the systematization of legal precedents in the enforcement of these ward rules, regulations, and policies; and the extent to which "revolt" against ward authority has taken place. The latter question is especially significant because the two major organized revolts against ward authority took place within the rules of the experimental program, namely, through a kind of sit-down strike or refusal to work in the system, rather than an attempt to destroy the system by physical or illegal means. Why the revolts took place, the role of ward personnel in precipitating these

events, and their responses to them, are all part of a complex set of events that can throw some light on the soldiers' attitudes toward authority, constitutional (lawful) means for making changes, and especially their ability to use gradual means rather than more extreme attempts to bring the ward program to a complete stop. As the results of the individual followup studies come in, an effort will be made to compare the results of ward experience with authority and the democratic process with the aftermath of being sent back to a military unit, or even eventual discharge from the Army.

c. Legal problems of experiments on human subjects. As the result of individual and group discussions of some of the problems of conducting experiments on human subjects, an effort was made to bring together some of the recent cases and more dramatic events that have occurred in this area since the thalidomide case of 1962 and the more recent cancer research cases at the Brooklyn Jewish Chronic Disease Hospital in 1964-66. Discussions with three departments were conducted, and as a result of these discussions, an effort will be made to systematize this data into some kind of general guideline for research on human subjects and the possible problems that can emerge from this type of research.

d. Effect of federal court decisions regarding alcoholism on the Uniform Code of Military Justice. Several recent lower federal court decisions making public drunkenness a disease, rather than a crime, and a recent decision by the United State Supreme Court in the case of Powell v. Texas, raise several questions pertinent to the Uniform Code of Military Justice and the attitude of the military services toward chronic alcoholism. If chronic alcoholism is regarded as a disease by some portions of the civilian community, the question arises as to its relation to Line of Duty disabilities, its treatment in military medicine and psychiatry, and its relation to military criminal law. Although the recent Supreme Court decision did not overturn existing practices and policies, the entire area of the treatment of alcoholism is going through medical as well as legal ferment. Military medicine and psychiatry should perhaps take a more careful and conscientious look at the whole question in the light of these recent developments, especially before test cases are made in the courts to apply civilian legal precedents to the treatment of chronic alcoholism.

Summary and Conclusions:

This year's research efforts represent a continuation of research inquiry in areas particularly relevant to analysis of behavior in aversive environments. There has been an increase in scope of inquiry pertaining to behavioral stress. Active research now is in progress on many different kinds of task performance and comparative influences of different contingent and noncontingent punishment conditions. At the psychophysiological level, measurement has been expanded to assess physiological cost of exposure to aversive environments on a more

comprehensive scale. Our findings tend to provide growing support for the hypothesis that the conditions under which punishment is used may be more significant in prediction of behavioral change than the differences in mental functions or task complexity involved in a group of performance tasks. Additionally, there is growing support for the conclusion that the characteristics of change in physiological responsivity to aversive environments do not reflect some single stereotyped pattern of change across many situations. Instead, the very direction of change can be determined by factors such as what the subject learns about experimental contingencies and variations in the exact contingency relationship employed. These indications contrast with prevailing thought, although there is evidence from other laboratories of growing support for these hypotheses. The study of the responses, upon which signal detection depends, as a class of operant behavior continues to lead to fruitful results, and continues to support predictions made on the basis of reinforcement principles. However, it also points to some gaps in the status of knowledge in this area, in demonstrating the sensitivity of rate of response and signal detection measurements to circadian influences. It also shows that inspection of cumulative record data alone can lead to failures to observe longer-term changes in behavior. Spectral analysis and other varieties of automatic analysis seem to be much more informative in identifying these slow changes in behavior and in describing them quantitatively. The work on processes involved in analogy solving tends to indicate that one of the "purest" tests of reasoning ability actually is highly susceptible to the educational and cultural histories of the individual. This is especially significant in view of the dependence upon tests of analogy performance in selection of college students. We have not yet conducted a formal analysis of "Project 100,000" men in terms of military suitability regulation actions, but anticipate performing this analysis within the next year.

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PROJECT 3A025601A824
IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 01
Ionizing Radiation Injury, Prevention and Treatment

RESEARCH AND TECHNOLOGY WORK UNIT

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(U) CHEMICAL PROTECTION AGAINST IONIZATION

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MERSEY, CDL W. P. 202-576-3391	JACOBUS, DR D. P. SLOMAY, DR T. R. 202-576-2276 TYPE DA
DRUG DEVELOPMENT CHEMICAL INDUSTRY	NA

KEYWORDS:
ACTIVITY, CHEMICAL, COMPOUND, DOSE, DRUGS, PROTECTION, RADIATION INJURY, STRUCTURE.

(U) TECH OBJECTIVE - THE OBJECTIVE OF THIS RESEARCH IS TO DEVELOP A MILITARILY USEFUL PILL TO PROTECT PERSONNEL AGAINST THE LETHAL EFFECTS OF IONIZING RADIATION. IN ADDITION TO A STRICTLY TACTICAL MILITARY USE AN EFFICIENT ANTIRADIATION COMPOUND WOULD BE USEFUL TO THE [illegible] FROM THE CLINICAL STANDPOINT.

(U) APPROACH- 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 ANTAGONIST STUDIES ARE BEING CARRIED ON. THE DEVELOPMENT OF EFFICIENT METHODS OF SUPPLYING CHEMICAL AND BIOLOGICAL INFORMATION WHICH CAN BE APPLIED TO THE PROGRAM ARE BEING DEVELOPED.

(U) PROGRESS - JUL 67 THRU JUN 68 TWO NEW CHEMICALS ARE NOW IN PRECLINICAL WORKUP PREPARATORY TO PRESENTATION TO THE PHARMACOLOGY ADVISORY COMMITTEE FOR CLEARANCE TO ADMINISTER TO MAN. SOME OF THE WATER-SOLUBLE COMPOUNDS IN THE AMINOALKYLATED MERCAPTOBENZYL CLASS HAVE BEEN FOUND TO BE SPECIFIC ADRENERGIC BLOCKING AGENTS WHICH MAY PROVE USEFUL IN THE TREATMENT OF SHOCK. PROGRESS CONTINUES TO BE MADE IN THE NUMBER OF AGENTS CLEARLY SUPERIOR TO PEROXYPOTASSIUMATE AND THE DEVELOPMENT OF COMBINATIONS SUPERIOR TO MOST SINGLE AGENTS. ADDITIVE PROTECTION IN COMBINATIONS OF AGENTS WITHOUT ADDITIVE TOXICITY HAS BEEN DEMONSTRATED. SIXTY NEW COMPOUNDS HAVE BEEN EVALUATED IN MICE. TWO OF THESE IN THE WATER-SOLUBLE AMINOALKYLATED MERCAPTOBENZOATE SERIES SHOWED GOOD PROTECTION. FOUR COMBINATION STUDIES HAVE BEEN DONE IN DOGS, THE MOST PROMISING BEING A COMBINATION OF PROPYL AND ETHYL ESTERS OF THE AMINOALKYLATED MERCAPTOBENZOATES. THE NUMBER OF CHEMICAL SYNTHESES PRODUCTS HAS DROPPED DURING THE LAST YEAR. DESPITE THIS, SEVERAL INTERESTING NEW LEADS HAVE BEEN RECOGNIZED. ONE IS A NEW SULFUR-BEARING GROUP WHICH HAS TURNED UP IN THE USUALLY UNCOMPROMISING UNSYMMETRICAL DISULFIDE CLASS. ANOTHER IS THE INTRODUCTION OF A [illegible]-ALKYLALKYL GROUP ON THE NITROGEN OF THE PARENT AMINOALKYLMERCAPTOBENZOIC ACID SERIES. THE ACTIVITY HERE IS HIGHLY POSITION-SPECIFIC AND IT MAY PROVIDE THAT A ROLE FOR ACTIVE-CYCLICS IN ANTIRADIATION AGENTS HAS FINALLY BEEN UNCOVERED. FINALLY, ACTIVITY HAS BEEN UNCOVERED IN A NEW CLASS OF COMPOUNDS, THE PHENOTHIAZOLES. FOR TECHNICAL DETAILS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

1 JUL 67		NA	

Project 3A025601A824 — Title: Ionizing Radiation Injury Prevention and Treatment

Task 01 — Ionizing Radiation Injury Prevention and Treatment

Work Unit 055 — Chemical Protection Against Irradiation

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Project No. 3A025601AS24

Title: Ionizing Radiation Injury, Prevention and Treatment

Task No. 01

Ionizing Radiation Injury, Prevention and Treatment

Work Unit 055

Chemical Protection Against Irradiation

Progress

I. General

The Antiradiation Drug Development Program during the past year has made remarkable progress in the areas of chemical synthesis, pharmacologic evaluation and clinical trials. In the area of chemical synthesis new analogs have been developed which are appreciably different from those which were previously known to be defective. These agents not only have a still greater range of safety but appreciably broaden the opportunities for synthesis in this area.

New pharmacologic actions were discovered in the antiradiation compounds. The most important activity is probably the highly specific alpha blockading action, WR-2723 and related compounds. The development of additional medical uses for antiradiation compounds is to be expected in view of their fundamental relationship to essential natural cellular components.

A radiation drug has been partially worked up for clinical trial which, if the preclinical toxicity experienced in monkeys is translatable to man, will offer a DRF of at least 1.1 for people without observable side effects. The clinical trials for tolerance on WR-638 have been completed. The primate data gave a correct prediction of our clinical experience to date.

The overall level of the program remains approximately the same as last year with the exception that there has been some increased spending in the area of clinical trials.

II. Chemical Synthesis Program

A. Contract Program: The chemical synthesis program for FY-68 was operated on a $450,000 budget. The fiscal year started with nine active research type contracts, one preparations laboratory contract and one special services contract for the synthesis of tagged compounds. During the year, 3 contracts expired and 2 are in a terminal period, scheduled to expire early in FY-69. Thus, as of the end of FY-68, the program is left with 6 active synthesis contracts, 1 preparations laboratory and 1 special services laboratory. A breakdown of the budget with respect to synthesis contracts, excluding the preparations laboratories and the special services laboratory, yields 18% with academic institutions, 22% with research house and 60% with with industry. No new contracts were let during the year. During this fiscal year there were submitted 299 compounds at an average cost of about $1450 per compound. This figure is on the low side because two of the contracts that submitted a fair number of compounds during FY-68 were carried into FY-68 by virtue of an extension of the contracts without additional funds, and hence the money put on their contracts is not reflected in the FY-69 budget.

Modification of the 2-aminoethanethiol molecule (MEA) on the two positions found to be most fruitful, namely on the sulfur or nitrogen atoms, in a search for better antiradiation agents, has continued. Thus, the N-alkyl group of the aminoethylthiols, Bunte salts and phosphorothioates has been modified by the introduction of an array of functionality in the terminal position, e.g., amines, alkyl ethers, aryl, substituted aryl, aryloxy, aliphatic, halogen, guanyl, heterocyclic, heteroaryl, hydrazide, hydrazine, hydrazone, hydroxylamine, nitrile, oxime, and thiol. During this reporting period increased emphasis has been put on the search for sulfur-blocking groups which may facilitate transportation or distribution and be physiologically labile to release an active aminoethanethiol moiety. Examples of new sulfur blocked compounds include dialkyldithiocarbazates, dialkylthiophosphates, diphenyldithiophosphinates, thiohemiacetels, sulfinamides, perthiocarbamates, perthioacetates, trisulfides, tetrasulfides, acyldisulfides, thioarsonates, and sulfenylisothioureas. A number of other novel sulfur-blocking groups are being studied but actual compounds containing them have not yet been synthesized. The blocking of the sulfur of the MEA moiety as an unsymmetrical alkyl disulfide, an unpromising device studied some time ago, has taken on new significance by virtue of the functionalization of the alkyl group. This will be discussed below.

Work has continued on the synthesis of alpha mercapto- and thiosulfate amidinium salts. The corresponding phosphorothioates have proved to be difficult to obtain because of instability but the parent compound has now been synthesized. Nitrogen substitution in the amidino series has centered on amidinoalkyl moieties, the terminal amidino being cyclized, e.g., as an imidazole and benzimidazole. Increase in lipid solubility has been achieved by the incorporation of a terpenoid moiety on the amidino nitrogen. Interesting activity was obtained in this area which again emphasizes the apparent importance of a hydrocarbon moiety of about 10 carbon atoms in conferring lipid solubility with activity, regardless of the actual structural nature of the hydrocarbon moiety.

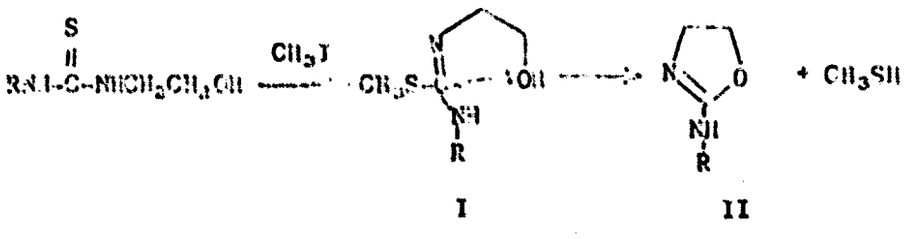
Work on the introduction of silicon into potential antirads as a means of changing solubility, distribution, electronegativity, etc., has proved to be disappointing insofar as the production of active compounds is concerned and this work is, therefore, being phased out.

Another potentially important area, viz., the cyclopropyl and cyclobutyl mercaptoamines, also was a disappointment. The chemistry proved to be intractable and beyond the capability of the investigator and has been discontinued.

The outstanding developments insofar as structure--activity relationship is concerned are the outstanding activity created by functionalizing the butyl moiety used to sulfur-block N-acetylaminoethanethiol as an unsymmetrical disulfide. The compound is WR-76843, $CH_3CONHCH_2CH_2SS(CH_2)_4SO_2Na$. The aminoalkylaminoalkylphosphorothioates continue to be of outstanding interest in the water-soluble antirad class. Perhaps the most significant finding in this area is the discovery of the high activity of the aminoalkyl-substituted aminopropyl phosphorothioates. With the possible exception of AET and APT, this is the first class of compounds in the entire program in which an aminopropylthiol derivative has equalled or surpassed the activity of the corresponding aminoethylthiol. Another breakthrough is in the incorporation of heterocycles in active compounds. It has been found that aminoethanethiosulfuric acid with an N-(halogen-substituted pyridinyloxyalkyl) substituent is highly active in the mouse screen. This is significant because it is the first promising lead in which heterocycles are involved and may indicate that the role of heterocycles in designing antirads is being elucidated. Isomerism in this class appears to play an important role in determining activity.

B. Organic Laboratory: Our interest in S-methyl derivatives of thioureas has not only led to the development of a scheme whereby thioureas can be classified according to the extent of N-substitution, but has also resulted in the discovery of several new reactions which may be useful in the synthesis of antiradiation agents.

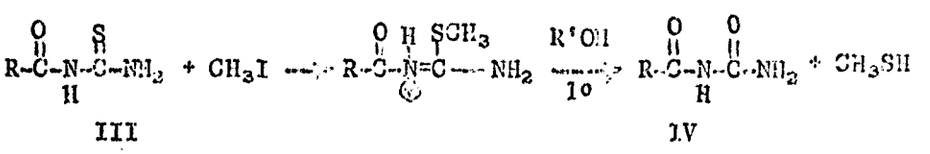
1-(2-Hydroxyethyl)-2-methyl-2-thiopseudoureas (I) have been noted to eliminate methyl mercaptan on heating. The evolution of methyl mercaptan is especially rapid when the thiourea is substituted with a phenyl or a benzoyl group in the 3-position. The intermediates were found to have cyclized giving 2-amino-2-oxazolines (II). Six-membered homologs,



I II

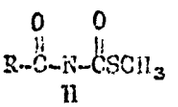
2-amino-2-dihydro-1,3-oxazines, have also been prepared from 1-(3-hydroxypropyl)-2-thioureas.

The S-methyl derivatives of 1-acetyl- and 1-benzoylthiourea (III) have been found to undergo reactions with hydroxylic compounds. With primary alcohols, methyl mercaptan is eliminated giving the corresponding acylurea (IV). Secondary



III IV

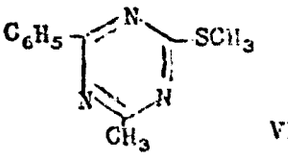
alcohols give rise to mixtures of the acylurea and the acyl thiocarbamate (V), the latter being formed by the elimination of ammonium ion. Tertiary alcohols



V

and water give V almost exclusively. The other products of this complex reaction are being investigated to determine its mechanism.

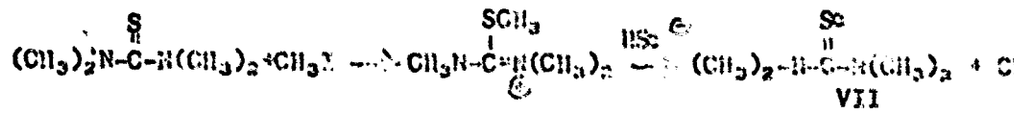
When the S-methyl derivative of benzoylthiourea was heated for several days in acetonitrile to determine its stability in that solvent, a cyclized product (VI) was formed in good yield. This represents the synthesis of a symmetrical



VI

triazine with a substitution pattern not reported previously. This reaction has also been successfully tried with trichloroacetonitrile as well as with the free base form of the S-methylthiopseudourea.

Further use of S-methylthioureas has been in the development of a new general synthesis of selenoureas. By performing the nucleophilic displacement of the $-SCH_3$ group by selenide ion at pH 8 it is possible to form selenoureas in moderately good yields. 1,1,3,3-Tetramethyl-2-selenourea (VII), reported previously to be obtainable in only 5% yield, was made easily by

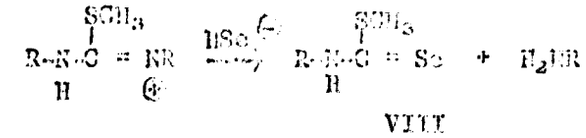


VII

new method in 70% yield. This reaction also gives unsubstituted, mono-, di- and trisubstituted selenoureas. By performing the selenide displacement

at ca. pH 5, instead of the $-SCH_3$ moiety being displaced, an amine function is eliminated to give the previously unreported class of compounds, selenothiocarbamic esters (VIII). Nuclear magnetic resonance studies are being performed on one of these compounds.

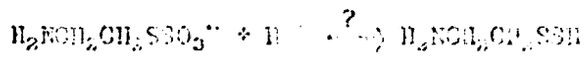
$$\underset{H}{R-N}-\overset{SCH_3}{\overset{|}{C}} = \underset{\oplus}{NR} \xrightarrow{HSe^{\ominus}} \underset{H}{R-N}-\overset{SCH_3}{\overset{|}{C}} = Se + H_2NR$$



VIII

The reaction of organic thiosulfates (Bunte salts) with sulfide ion has been investigated as a possible route to the as yet unknown, 2-aminoethylhydrodisulfide (IX). This reaction was found to follow a variable course depending

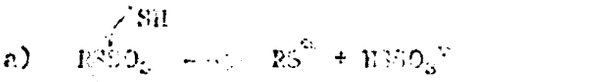
$$H_2NCH_2CH_2SSO_3^{\ominus} + H^{\ominus} \xrightarrow{?} H_2NCH_2CH_2SSH + SO_3^{\ominus}$$



IX

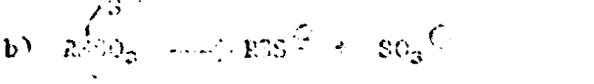
on the pH of the medium. At low pH (ca. 5) the sulfide ion attacks the hexavalent sulfur atom (equation a.). At high pH (ca. 11), the sulfide ion attacks

a) $RS\overset{SH}{\overset{|}{S}}O_3 \longrightarrow RS^{\ominus} + HSSO_3^{\ominus}$



the divalent sulfur atom (equation b.) These conclusions are based on the

b) $RS\overset{S^{\ominus}}{\overset{|}{S}}O_3 \longrightarrow RSS^{\ominus} + SO_3^{\ominus}$



quantitation of the sulfite and thiosulfate (as barium salts) formed as by-products. Additional studies along these lines may have some bearing on the current synthesis of insulin.

Organic variants of hydroxylamine are showing promise as antimalarial and anti-neoplastic agents (e.g., hydroxyurea). We have made, therefore, three hydroxythiourea, $RNH-\overset{S}{\overset{|}{C}}-NHOH$, for screening purposes. These compounds are difficult to prepare because of their property of suddenly decomposing violently at room temperature and above. Regrettably, these hydroxythiourea showed no activity in either screen.

Evaluation of Chemicals for Antiradiation Activity

A. The Rodent Testing Program

The primary screening test in which new chemicals are evaluated for antiradiation activity in the mouse system, in which animals are lethally irradiated following administration of the test compound, and 30 day survival is recorded to evaluate the protective activity. Primary screening in rodents is performed in three laboratories: The Woodard Research Corporation under the direction of Dr. Marvin Bleiberg and Dr. Henry Horn, the Toxicology Laboratory of the University of Chicago under the direction of Dr. Kenneth DuBois, and the Walter Reed Army Institute of Research. The Walter Reed test facility screens new compounds specifically synthesized for antiradiation testing and performs secondary tests on those compounds identified as active by the two other laboratories. The following table summarizes the testing performed in each of the three laboratories during the past fiscal year.

	Number of Compounds Tested	Number of Compounds with Radioprotective Activity
Woodard Laboratories	858	38
University of Chicago	3006	113
Walter Reed	467	150
TOTAL	4331	301

A compound exhibiting radioprotective activity on primary test is immediately retested in the same laboratory and a sample transmitted for confirmation to one other laboratory. Following confirmation of radioprotective activity, active compounds are further evaluated for effectiveness by oral administration, effectiveness at reduced drug levels, duration of action, dose reduction factor, effectiveness in combination with other antiradiation agents, etc. As much data as possible regarding the acute toxicity and pharmacology of the agent are collected and recorded at the time of the primary test. Prior to the testing in lethally irradiated mice, an acute toxicity study is performed in which gross clinical symptoms are observed and recorded, and estimates of the $LD_{50}/10$ days and "maximum tolerated dose" are made. Toxicity, as well as antiradiation activity, is considered in selecting agents for further evaluation in rodents and in other species.

Many of the drugs for the antiradiation screening program are tested at the Woodard Research Corporation. All active agents (those agents affording greater than 33% protection to mice) are reevaluated by the Rodent Testing Section of the Division of Medicinal Chemistry. In some cases, where questionable results were obtained by Woodard, complete evaluation of compounds was accomplished including preliminary toxicity testing.

Of the 38 active compounds retested by WRAIR, 17 (43%) offered little or no protection. Comparable results were obtained on the balance of the compounds by the two laboratories.

A possible explanation for discrepancy in results might be associated with differences in mouse strains and technical competence.

The search for new strains of mice suitable for doing screening programs was continued. Outside mouse sources are still being assayed in the hope of finding a strain suitable for use in the drug screening program. ICR strains (females, 8 - 9 weeks old, from several sources (Forest Glen, Charles River and Manor Farms) were all assayed for their response to lethal radiation.

Results showed that the ICR mice from Forest Glen were still quite hardy with a consistent death pattern (8 - 15 days). Very few early deaths were noticed. The new strain of "Astro" mice from Charles River - (COBs - Caesarean originated, barrier - sustained) appeared to be more satisfactory than any of the new strains so far screened.

Manor Farms mice which had previously been used with success by the Woodard laboratory in their screening program, were no longer suitable. Early deaths, 3 - 5 days, have grown progressively worse.

Table 1. Radiation sensitivity (LD_{50} 30) of some strains of female mice exposed to Cobalt 60 irradiation.

Strain	Age	LD_{50} ± 2 S.E. (Rads)
ICR/FG	8 - 9 wks	813 ± 23
Manor Farms	8 - 9 wks	760 ± 14.4
Manor Farms	9 - 10 wks	760 ± 18.2
ICR/CR (Astro)	8 - 9 wks	900
ICR/CR (not pathogen free)	6 - 7 wks	740 ± 14

In addition to the screening program, the following ancillary services are provided:

1. Maintenance of a mouse malaria strain (P. berghei) for the Division of Nuclear Medicine for their special studies.

2. Assistance to other investigators with their studies on radiation protection. Collaborative studies with the Division of Nuclear Medicine include:

(a) Radiation and stress experiments (Cinkober, et al).

(b) Radiation and drug antagonism studies, (Mahin, et al).

(c) Radiation and drug protection in germ free mice.

All drugs, as well as preparation of some, and pertinent data regarding drugs are supplied by the Rodent Testing Section of Medicinal Chemistry.

B. Bacterial Testing Program

A screening test in which E. coli B/r are irradiated in the presence of test agents for determination of radioprotective activity was conducted at the Woodard Research Corporation under the direction of Dr. Daryl Bates. The screen was initiated with the hope of providing information useful in interpreting test results in rodents. Results in bacteria which either contradict or confirm rodent test results might suggest possible mechanisms of action, particularly with respect to agents which might be acting in the mouse solely through a pharmacologic mechanism. Budget limitations and a less than hoped for correlation with the mouse screening system resulted in termination of the bacterial test program in November 1967. Since the beginning of the bacterial screening system, 9327 compounds have been tested, including 1097 tested during fiscal year 1968.

C. Radiation Exposures in Large Animals

Chemical agents exhibiting significant radioprotective activity in the rodent screening test have been selected for evaluation in dogs, monkeys or swine. Those compounds protecting mice at dose levels well below the maximum tolerated dose are of particular interest. Compounds protecting mice only at doses near toxic levels have infrequently exhibited protective activity in the larger animal species.

A preliminary toxicity study was performed in each species prior to the use of a new agent for radiation studies. The chemical was first administered in graduated doses to a few animals to determine the maximum tolerated dose. The initial radiation study was generally performed using the highest possible dose level, administered intravenously. Gross clinical manifestations were observed and recorded. In addition, pharmacologic studies in anesthetized dogs were performed to characterize particularly the cardiovascular effects of drug administration at or near the radioprotective level.

Radiation exposures were performed using a Triga Mark F Nuclear Reactor. Exposures were made possible through the cooperation of the Diamond Ordnance Fuze Laboratory reactor staff under the direction of Mr. Walter Giesler. Animals were exposed in the wood-lined exposure room adjacent to the reactor tank. The room is 20 ft. by 20 ft. in diameter with a 10 ft. ceiling. Animals were confined during irradiation in rectangular lucite exposure cages arranged in a four cage array parallel to the gamma isodose curve produced by the reactor flux. The individual cages are

12 inches by 12 inches by 20 inches. The mid-line of the cages was 130 centimeters distant from the tank wall. By maintaining a thickness of 70 centimeters of tank water between the core of the reactor and the exposure room neutrons were selectively reduced. In this configuration the gamma radiation contributed over 98% of the dose in Rads delivered to the animals. Of the less than 2% neutron contribution to total dose, energies were predominantly thermal. The total distance between the reactor core and the cage mid-line was 2 meters. The measured radiation dose varied by less than 5% within the exposure cages. The gamma dose rate with the reactor operated at 250 kilowatt power steady state was 100 to 108 R per minute measured at the midline in air using a tissue equivalent ionization chamber.

Under the above conditions the radiation $LD_{50}/30$ day was found to be 432 R (Figure I). Six hundred and fifty R is the test dose of radiation for drug evaluation studies. Of 63 control dogs radiated at this exposure level there were no survivors. The death peak (Figure II) occurred at 9 days and the mean at 10.9 days.

Radiation Protection Studies in Dogs

The radioprotective action of 2-aminoethylphosphorothioate (WR 638) in rodents and in dogs was described in the Annual Report of 30 June 1964. Testing during Fiscal Year 1968 centered primarily around analogs of WR 638. WR 2721, WR 8186, WR 36,978 and WR 5144 are phosphorothioates with amino alkyl substitutions on the nitrogen of mercaptoethylphosphorothioate. The structures and results of antiradiation tests in dogs are presented in Table I. These agents exhibit excellent radioprotective activity and a good therapeutic index in rodents. Most are protective by oral administration in the mouse. Data on WR 2721 were obtained in FY 67 but are included for comparative purposes. WR 44,923 differs from WR 2721 by one methylene group on the phosphorothioate and exhibits protective activity quite similar to WR 2721.

The thiosulfates tested, WR 4245, WR 7467 and WR 3342, showed good activity in protecting mice but failed to significantly protect dogs at sub-toxic levels. The disulfide WR 19,635 and the mercaptan WR 2961 also failed to protect dogs, although protective in mice. The thiazolidine WR 85,562 can be considered as a closed ring of 2-mercaptoethylamine (MEA). The compound was excellent in protecting mice when administered by the oral route but failed to protect dogs following oral administration.

Several combinations of drugs were tested for potentiation of their protective activity. Results are presented in Table II. Dose levels were one-half to two-thirds of the minimal dose of the drug which offered significant protection when administered alone. The combination of paramethoxypropiophenone (PMPP) and WR 2721 offers the greatest protection of those combinations studied in FY 68.

Radiation Protection Studies in Swine

Two drugs were evaluated in swine. Results are shown in Table III. Fifty-three mixed Minnesota pigs were utilized, weighing 10 to 12 kg at the time of testing. Compounds were administered intraperitoneally rather

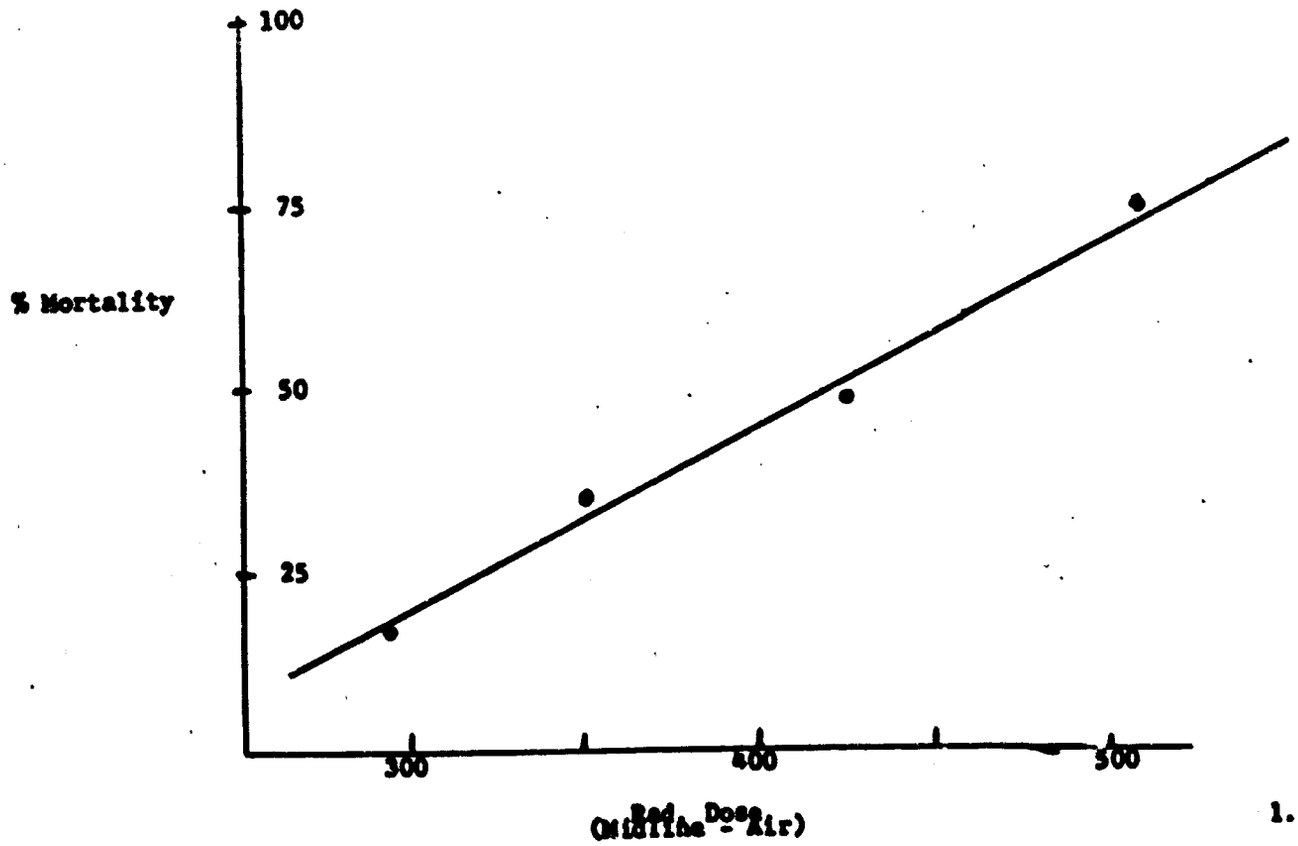
FIGURE I

Radiation Lethality Study

DORF REACTOR - Gamma

100 R/Min. Dogs

	Dead	% Mortality
292 R	2/12	17%
356 R	4/12	33%
432 R	6/12	50%
518 R	6/8	75%
650 R	63/64	98%



1.

FIGURE II

DOG MORTALITY

650 R Whole Body Gamma (Reactor)

63 Dogs

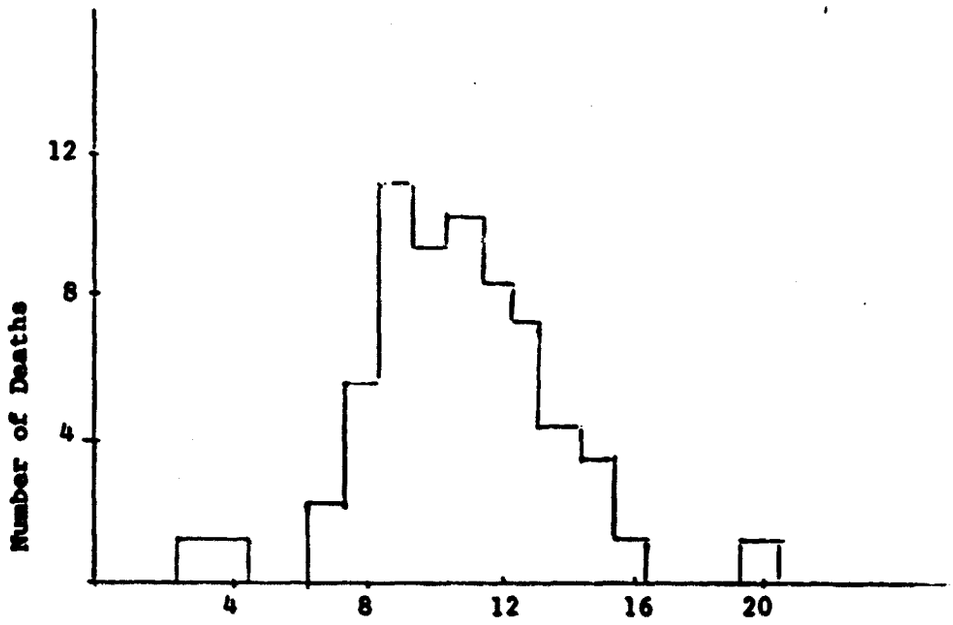


TABLE I

Dog Studies

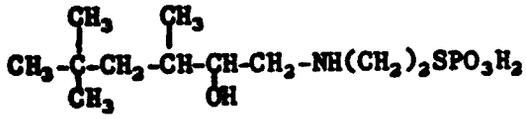
	Toxic Deaths	% Survival
WR 2721		
$H_2N-(CH_2)_3NH-(CH_2)_2SPO_3H_2$	1/6	83 (5/6)
200 mg/kg IV 30 Min pre-rad	2/16	50 (8/16)
150 mg/kg IV 30 Min pre-rad		
WR 8186		
$HO-(CH_2)_2NH(CH_2)_2SPO_3H_2$	3/9	11 (1/9)
500 mg/kg IV 30 Min pre-rad		
WR 36978		
	0/18	0 (0/18)
5 mg/kg IV 30 Min pre-rad		
WR 5144		
$CH_3CH_2NH(CH_2)_3-NH(CH_2)_2SPO_3H_2$		
200 mg/kg IV 30 Min pre-rad	0/9	0 (0/9)
250 mg/kg IV 30 Min pre-rad	0/9	67 (6/9)
WR 44923		
$H_2N-(CH_2)_3NH(CH_2)_3SPO_3H_2$		
200 mg/kg IV 30 Min pre-rad	1/9	78 (7/9
100 mg/kg IV 30 Min pre-rad	0/6	0 (0/6

Table I - Con't

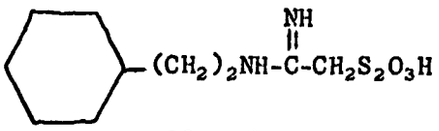
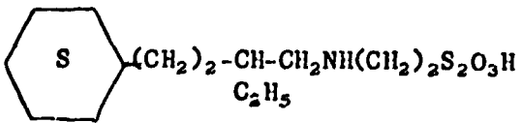
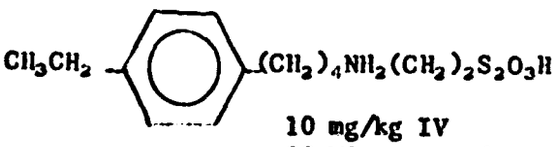
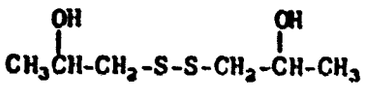
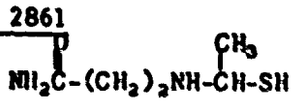
	Toxic Deaths	% Survival
WR 4245  20 mg/kg IV 30 Min pre-rad	1/9	11 (1/9)
WR 7467  20 mg/Kg IV 30 Min pre-rad	1/18	6 (1/18)
WR 3342  10 mg/kg IV 30 Min pre-rad	0/8	0 (0/8)
WR 19635  150 mg/kg IV 30 Min pre-rad	0/9	11 (1/9
WR 2861  200 mg/kg IV 30 Min pre-rad	0/9	0 (0/9)

Table I - Con't

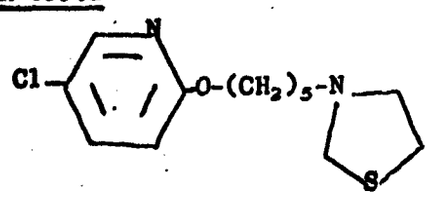
	Toxic Deaths	% Survival
WR 85562 		
200 mg/kg PO 45 Min pre-rad	0/6	0 (0/6)

TABLE II

Dog Studies

Drug Combination		Toxic Deaths	% Survival
WR 2727 WR 44923	100 mg/kg IV 100 mg/kg IV	0/15	53 (8/15)
WR 5144 WR 44923	200 mg/kg IV 100 mg/kg IV	3/9	33 (3/9)
WR 2721 p-amino-propiophenone	100 mg/kg IV 5 mg/kg IV	0/9	78 (7/9)

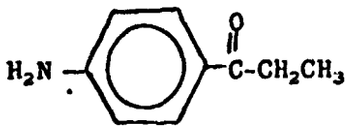
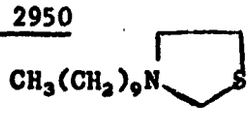


TABLE III

Swine Studies

	Toxic Deaths	Survival
WR 2950		
		
200 mg/kg IP 30 Min pre-rad	3/6	17% (1/6)
1000 mg/kg PO 65 Min pre-rad	0/3	33% (1/3)
1000 mg/kg PO 90 Min pre-rad	0/3	0% (0/3)
WR 2822		
$H_2N(CH_2)_4NHCH_2CH_2SPO_3H_2$		
100 mg/kg IP 30 Min pre-rad	0/6	17% (1/6)

than intravenously as in the dog. Other conditions of radiation remained unchanged. WR 2950 was toxic in the dog at 20 mg/kg. Swine were found to tolerate approximately 10 times this dosage. The compound, however, failed to significantly protect pigs following either the IP or oral routes of administration.

WR 2822 closely related to the very effective WR 2721 could be tolerated by the pig at twice the level found toxic in dogs. Again the compound failed to protect swine at 100 mg/kg even as it has previously failed to protect dogs at 50 mg/kg.

D. Related Studies with Antiradiation Drugs

Liver Perfusions

Nitrogen mustard mimics the effect of radiation on cells and has been used as a substitute for radiation therapy in certain neoplastic conditions. Several of the drugs effective in protecting against radiation effects are also protective against the action of nitrogen mustard.

Hepatic neoplasia is generally considered an inoperable condition. Classic treatment has been perfusion of the organ with nitrogen mustard, a technique which produces gross insult to the normal parenchyma as well as the neoplastic cell. Breedis and Young (Am. J. Path 30: 969-977, 1954) demonstrated that malignant neoplasms growing in the liver tend to acquire an exclusively arterial blood supply, regardless of the route by which tumor emboli reached the liver. In contrast, normal parenchyma receives blood primarily via the portal circulation. The two distinctive circulatory patterns of the neoplastic liver enable one to be selective in perfusion of the organ. Nitrogen mustard introduced via the arterial route should go primarily to the neoplasm and hepatic tissues adjacent to them. Introduction via the venous route of a nitrogen mustard antagonist should protect the normal parenchyma. It was the purpose of this study to see if the normal parenchyma could, in fact, be protected against known lethal doses of nitrogen mustard.

The experimental subjects were mature, healthy, purebred Beagle dogs in the 9 to 12 kilogram weight range. Following anesthesia, entry was made into the abdominal cavity and the portal vein, hepatic artery, gastro-duodenal artery and vein, and bile duct were isolated. The gastro-duodenal artery and vein were cannulated, the latter cannula extending into the portal vein. Umbilical tape was placed around the portal vein, caudal to the point where the venous cannula entered the portal, to facilitate future blockade. The femoral artery was isolated and a balloon catheter inserted to a point just anterior to the coeliac artery. Surgical preparation for the perfusion was complete at this point, with two patent catheters, one each in the gastro-duodenal artery and the gastro-duodenal vein; ligatures or clamps ready to provide temporary blockade of the hepatic artery, bile duct, and portal vein; and a balloon catheter ready to provide blockade anterior to the coeliac artery.

The level of nitrogen mustard to be utilized was determined on a series of 18 dogs. Results are shown in Table A . Drug levels of 0.5 and 1.0 mg/kg resulted in 100% survival; drug levels of 2.0 and 3.0 mg/kg resulted in 100% deaths in five days or less. The level of nitrogen mustard selected for use in combination with the protective drugs was 2.0 mg/kg.

Table A Response of dogs to nitrogen mustard liver perfusion

mg/kg	Survivors	Day of death
0.5	2/2	---
1.0	2/2	---
2.0	0/7	3,3,4,4,4,5,5
3.0	0/7	1,1,1,4,4,4,4

For perfusion of the liver through the venous system a drug combination of D-L-threo-3-2(2-mercaptoethyl) amino-1,2,4-butanetriol hydrochloride (WR 2347) at 300 mg/kg and cysteine (WR 348) at 400 mg/kg was utilized. Structures are indicated in Figures 1 and 2 .

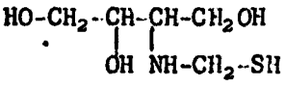


Figure 1

DL-threo-3-(2-mercaptoethyl) amino-1,2,4-butanetriol hydrochloride

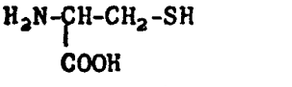


Figure 2

L-Cysteine

Seven dogs were given the drug-mustard treatment, each dog having its own control which received nitrogen mustard and isotonic saline.

Following inflation of the femoral balloon catheter, blockade of the portal vein and the bile duct, perfusion of the liver was started with the WR drug combination via the gastro-duodenal-portal vein route. Between one and two minutes later the nitrogen mustard was started via the gastro-duodenal hepatic artery route. Mustard administration was completed in 10 to 15 seconds. The WR drugs required about 5 minutes for complete administration. The liver was kept isolated for an additional 5 minutes to assure complete neutralization of the mustard before release to the general circulation. Following the perfusion, removal of catheters, restoration of the afferent hepatic circulation, and removal of the aortic block, routine closure was performed.

A drug combination of WR 2347 at 300 mg/kg and WR 348 at 400 mg/kg was successful in protecting the liver against the 2.0 mg/kg level of nitrogen mustard in 4 of 7 dogs. The four survivors were sacrificed on days 41, 41, 48 and 56. One animal sacrificed on day 41 exhibited a slightly

yellow friable liver with an atrophied right lobe. The remaining 3 animals showed livers grossly normal in appearance. The three animals which failed to survive the drug-mustard combination exhibited post-mortem lesions similar to those of the control dogs, i.e., severe liver necrosis and accumulation of sero-hemorrhagic fluid in the abdominal cavity.

The number of survivors is not felt to be the significant point, but rather that normal cells can be protected sufficiently in order that they may recover. In the neoplastic liver the effect of the mustard should be primarily on neoplastic cells while the protective drug action should be primarily on the normal cells. Such a situation offers distinct possibilities for the drug therapy of hepatic neoplasis. Further experiments are in progress.

Primate Antiallergy Drug Study

DESCRIPTION

The transfer of allergic antibody (reagin) from man to other primates has shown to sensitize the skin of other primates. Administration of antigen will produce a reaction in the sensitized animal. By injecting the reagin containing sera intradermally, the reaction can be localized as a wheal or erythemia. Intravenous injection of Evans blue dye results in a circumscribed blue area due to extravasation of dye at the sensitized site. Since reagin has been shown to be thiol sensitive, it should be sensitive to certain thiol containing antiradiation compounds. In this procedure, 1 ml of Reaginic sera from clinical patients was injected intradermally into mature, healthy Macaca mulatta monkeys. After 24 hours, the candidate drug was given to the selected subjects. Thirty minutes later, both treated animals and the untreated controls were given 2 ml of 1 percent Evans blue dye and .1 ml commercially prepared antigen by intravenous administration. Injection sites were checked after 15 minutes for color change resulting from extravasation of the dye. Interpretation of the effectiveness of the drug was based on a comparison of the reaction in treated animals with that of the controls. Intravenous injection of WR 2529 at a level of 750 mg/kg produced no reduction of severity of the reaction. Administration of 250 mg/kg/day for 3 days prior to administration of the antigen also proved unsatisfactory, however, when serum was incubated with the drug prior to injection, a less severe reaction was noted indicating that reagin may be sensitive to the drug WR 15504 (mercaptoethanol) is presently under investigation. At present, it appears results will closely parallel those of WR 2529.

The Effect of D-penicillamine on the Immune Response

D-Penicillamine is a potent chelating agent used in Wilson's disease, (1) rheumatoid and degenerating arthritis (2) and cystenuria (3,4).

Previous experiments conducted on mice in this laboratory showed that penicillamine inhibited growth, produced severe neurological disorders, and death. It also resulted in changes in the connective tissue proteins, collagen and elastin. Decreased collagen synthesis occurred. Half of

this collagen is extractable in contrast with the very small amounts extractable from normal tissues. As a result skin tensile strength is altered, along with an increase in skin fragility.

In view of potential importance of D-penicillamine on the treatment of human diseases (rheumatoid arthritis, Wilson's disease, cystenuria), experiments were designed to study the effect of D-penicillamine on the immune response of mice.

When rabbits were treated with D-penicillamine, the immune response was suppressed (5). Herd and Orbison also showed that in rats fed BAPN, and made lathyritic, there was no difference in the immune response (6).

Female weanling mice 3 - 4 weeks of age, ICR strain,were fed diets of ground Purina chow containing .5% D-penicillamine. Appropriate controls were fed the same standard ground mouse biscuit without the drug. Supplements of Vitamin B6 or copper were not added although reports in the literature have shown the antagonistic effect of penicillamine on Vitamin B6 (7,8) as well as its potent chelating effect on copper. Mice were fed D-penicillamine daily for 5 weeks. Daily weights were taken and food consumption was calculated from the total food intake per cage.

The weight changes of mice are shown in Table B after 5 weeks on diets of either D-penicillamine or ground Purina chow.

Table B

	Group	Wt at start (g ± S.E.)	Wt at 35 days (g ± S.E.)	g fd cons. per day/mouse
I	Penicillamine	11.8 ± .3	21.75 ± .43	3.8
II	Penicillamine	12.2 ± .6	22.90 ± .50	4.0
III	Penicillamine	10.85 ± .25	22.00 ± .33	4.2
IV	Control	11.40 ± .30	23.10 ± .60	4.5
V	Control	11.80 ± .30	26.0 ± .70	4.0
VI	Control	10.70 ± .60	23.13 ± 1.5	4.2

At 35 days following start of experiment, appropriate groups of mice were exposed to a lethal dose of Co-60 gamma radiation. The mice were distributed into the following treatment groups:

I .5% Penicillamine (No radiation) (10)
II .5% Penicillamine + 950 Rad Co-60 (18)
III .5% Penicillamine + 950 Rad Co-60 + rat bone marrow (20)
IV Control diet + Rads Co060 (15)
V Control diet = 950 Rads Co-60 + rat bone marrow (19)
VI Control diet (No R)

The mice,after exposure and treatment, were maintained on the respective diets until the termination of the experiment. Rat B.M. cells were suspended in Hank's solution and .5 ml of the suspension (equivalent of 1 rat femur, mouse) was injected intravenously 6 hours post exposure into the appropriate groups.

Daily mortality checks of the mice were made as well as careful scrutiny for evidence of graft rejection in those groups given the heterologous bone marrow.

The mean survival time of all groups was tabulated and results are shown in Table C . There wereno significant differences between the two treatment groups.

Table C

Treatment	MST S.E.	
Penicillamine + 950 Rad	11.65 ± 4.7 days	
Penicillamine + Rad and BM	23.0 ± 4.7 days	(0 surv at 60 days)
Control + 950 Rad	11.46 ± 4.5 days	
Control + 950 Rad + BM	24.7 ± 6.0 days	(2 surv at 60 days)

Weight changes following irradiation showed the typical pattern.

Table D

Treat	R day	R + 7 days	R + 14 days	R + 21 days	R + 28 days
Pen (No R)	21.75 ± .43	23.1 ± 2.2	24.1 ± .6	25.25	25.65
Pen + 950 R	22.0 ± .33	18.2 ± .27	—	---	---
Pen + 950 R + Rat BM	22.9 ± .50	19.1 ± .44	17.8 ± .8	$18.3^{(10)}$	$17.5^{(5)}$
Cont (No R)	26 ± .7	25.5 ± .6	26.7 ± .5	27.0	28.10
Cont + 950 R	23.1 ± .6	19.9 ± .71	---	---	---
Cont + 950 + Rat BM	23.1 ± 1.5	20.3 ± .4	20.8 ± .8	$19.0^{(14)}$	$20.4^{(6)}$

All groups showed a decrease in weight. The penicillamine groups appeared to show the greater loss. In the bone marrow treated groups, the weights tended to be stabilized for 14 days to 21 days. At this time, the surviving mice began to show signs of graft rejection. Typical symptoms were apparent (diarrhea, hunch backed appearance, respiratory difficulties, etc.). Slides of the blood taken at this time showed the presence of very few rat cells and almost all mouse cells. Those mice which appeared normal showed granulocytes which were predominantly rat cells.

At 30 days after irradiation the number of survivors in the penicillamine group was 3, vs 5 for the control groups. By 60 days all of the Penicillamine treated mice were dead while only 2 of the mice on the control diet survived.

From the results of this preliminary experiment, it would seem that there was no apparent difference in the immune response of the two groups.

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III. Pharmacology Radiation Program

A. The Pharmacology Department has prepared IND's on WR-638 and WR-2529. Work on the IND for WR-2721 has also been started. WR-638 has completed its Phase I trial. Twelve volunteers tolerated 11.18 grams by mouth per day without any observable side effects.

The Pharmacology Department has continued exploring the interesting pharmacological properties of a series of amino-alkyl-aminoethane phosphorothioic acids. A series of these agents had previously been found to possess excellent radioprotectant properties when given as single agents. When one combines these chemicals, their radioprotectant value is additive, but there is no addition of toxicity. Since this was clear evidence that toxicity could be separated from radioprotection, this department decided to concentrate on studying the pharmacology of these agents. This series of agents blocks the alpha adrenergic receptor sites in dogs, cats, rabbits, guinea pigs, rats and mice. Until the present time, this effect upon the sympathetic nervous system had not been evident nor disclosed as a characteristic of an aliphatic series delineated by the general structure $H_2N(CH_2)mNH(CH_2)nSY$, where m and n represent small integers (less than 10) and SY is a sulfur containing function which can be metabolically convertible into an -SH group.

B. Blood pressure effects were studied in a number of species. There are a number of drugs which have been reported to depress the blood pressure response to catecholamines, but only a few families of drugs cause "epinephrine reversal." The Indolethylamine Alkaloids (of which serotonin, lysergic acid, ergot alkaloids, yohimbine and reserpine are examples), the Benzodioxanes (of which piperoxan is an example), the Beta-Haloalkylamines (of which dibenamine and phenoxybenzamine are examples), the Azapetines, and the Benzazolines (of which tolazoline and phentolamine are examples) are the generally accepted families of drugs that are considered to be alpha adrenergic blocking drugs.

Experiments with WR-2822 $/\bar{H}_2N\text{-}(CH_2)_4\text{-}\underset{H}{N}\text{-}CH_2CH_2S\text{-}PO_3\bar{H}/$, WR-2833 $/\bar{H}_2N\text{-}(CH_2)_5\text{-}\underset{H}{N}\text{-}CH_2CH_2\text{-}S\text{-}PO_3\bar{H}/$ and WR-2824 $/\bar{H}_2N\text{-}(CH_2)_6\text{-}\underset{H}{N}\text{-}CH_2CH_2\text{-}S\text{-}PO_3\bar{H}/$ caused epinephrine reversal in dogs, cats, rabbits, guinea pigs, rats and mice. They depressed (by 50% or greater) the pressor response to norepinephrine. These agents cause only a slight hypotension (25 mmHg) which lasts approximately 15 minutes. The blood pressure returned to normal values before testing for blockade. This made evaluation of the blockade much easier. The response of the blood pressure to isoproterenol, acetylcholine and histamine was not modified by these agents. All animals were anesthetized with pentabarbital sodium and the blood pressure was measured directly from either the femoral or carotid arteries. The onset of action was approximately 45 minutes and by 60 minutes there was classical epinephrine reversal. This effect lasted for over 24 hours in dogs and over 36 hours in cats. All drugs were administered intravenously in doses of 50 mg/kg

(except in mice which were given 75 mg/kg) over a 1-3 minute period. The pressor response to epinephrine could be surmounted by administration of doses greater than 10 μg/kg. However, 15 to 20 minutes later there was still an alpha blockade to test doses of catecholamines. This indicates that these agents are reversible alpha blocking drugs.

These experiments led us to postulate the following working hypothesis to explain how alpha adrenergic blocking drugs combine with the alpha adrenergic receptor site.

1. Known Facts: Phenoxybenzamine (Dibenzyline®) is a powerful alpha adrenergic receptor blocking drug which requires approximately 45 minutes to an hour for onset of its action. This action is not only irreversible, but lasts for approximately 7 to 10 days.

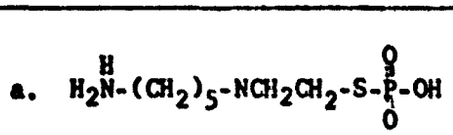
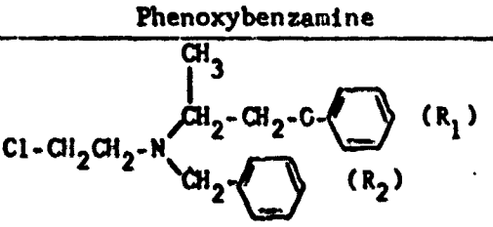
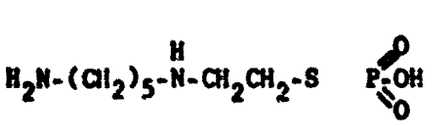
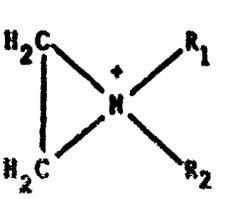
WR-2822, WR-2823, and WR-2824 are phosphorothioic acids with a potential free -SH group. Indeed, cysteamine -S-phosphate (WR-638), a phosphorothioic acid derivative, was found by Herrigton et al. to be hydrolyzed to cysteamine (MEA), a compound containing a free -SH group, and orthophosphate. The reaction was rapidly catalyzed by highly purified preparations of calf intestinal (duodenum) alkaline phosphatase. The kidney, duodenum and liver are the most active tissues. These compounds (WR-2822, WR-2823 and WR-2824) are reversible alpha adrenergic blocking drugs.

2. Assumptions:

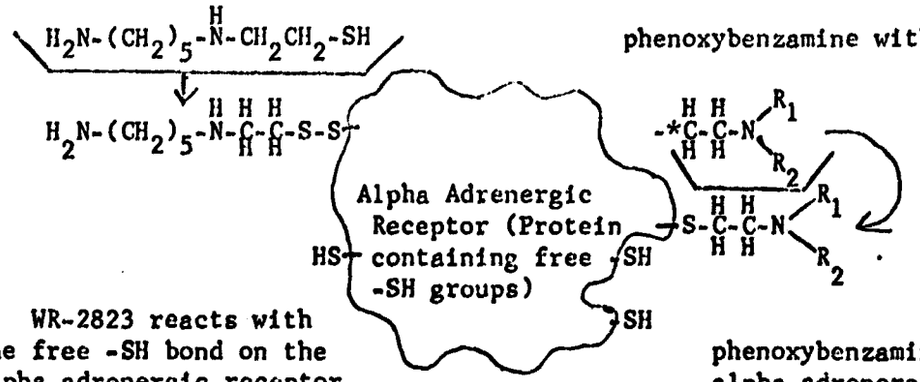
a. The alpha adrenergic receptor is a protein.

b. Since many proteins in the body contain free -SH groups, the alpha receptor also contains these groups.

3. Hypothesis:

WR-2823	Phenoxybenzamine
a. 	
b. During first hour after administration, WR-2823 is subject to enzymatic action (primarily in kidney, duodenum and liver):  (cleaved)	During first hour after administration phenoxybenzamine is biotransformed in the liver to an ethylenemmonium ion: 

c. WR-2823 is biotransformed to:



Aziridine ring breaks leaving phenoxybenzamine with a free radical:

d. WR-2823 reacts with the free -SH bond on the alpha adrenergic receptor forming a disulfide bond (which is a reversible bond)

phenoxybenzamines reacts with alpha adrenergic receptor forming a thiol-ether linkage (which is an irreversible bond)

To test this hypothesis we synthesized $H_2N-(CH_2)_5-\overset{H}{N}-CH_2-CH_2-SH$ (which we shall now call WR-1729) from WR-2823 and injected this compound into the dog to see if we could obtain an alpha adrenergic blockade in a shorter time period. Indeed, within 15 minutes we observed the blockade and the dose could be reduced by 50% (25 mg/kgm).

C. Experiments last year were started in collaboration with the Department of Gastroenterology, Division of Medicine, whereby we studied the effects of WR-2823 on the isolated rabbit aorta, and the isolated ileum from guinea pigs, rats and rabbits. WR-2823 blocked the alpha response caused by epinephrine, norepinephrine and phenylephrine on the rabbit aorta, but not on the ileum from any species tested. However, when WR-1729 was added to the medium instead of WR-2823 we obtained blockade to both aorta and ileum and the blockade to the aorta was established in a shorter time period. These data strengthen our hypothesis.

D. Further experiments were performed to determine whether our preliminary studies were correct last year in regard to the effects of WR-2823 in the perfused dog hind limb. We confirmed those earlier findings, where WR-2823 blocked the decrease in blood flow in the isolate dog hind limb caused by small amounts of catecholamines. In fact, after pretreatment with WR-2823, we observed that epinephrine and norepinephrine caused an increase in blood flow through the dog's hind limb in doses that decreased the flow during the control period.

E. WR-2823 converted the epinephrine and norepinephrine pressor response on the coronary arteries in the dog Langendorff preparation to a depressor one. The onset of action was about 30 minutes. WR-1729 produced the same effect immediately.

F. This year a report from another institution demonstrated that the positive inotropic action of the electrically driven, left guinea pig atrium caused by doses of less than 10^{-6} molar of phenylephrine could be blocked by phentolamine (an established alpha blocking agent). We began some experiments using the right spontaneously beating guinea pig atrium, using either WR-2823 or WR-1729. Preliminary results show that WR-2823 does not block phenylephrine while WR-1729 does block the positive inotropic action to phenylephrine. We are now determining whether this response is a specific or a non-specific one before stating anything about alpha blockade.

G. Antagonism of norepinephrine by WR-2823 in the isolated mouse spleen:

Ignarro (1967) reported that the mouse spleen contracts in response to alpha adrenergic stimulation. This contraction could be blocked by phenoxybenzamine. Higher concentrations of the catecholamine caused a depression of response which could be antagonized by propranalol. This system was used to test for the possible alpha adrenergic antagonizing properties of WR-2823.

METHOD: Sixteen adult female C57BL/6 mice were used. The animals were killed by cervical dislocation; the spleen was removed and blotted. The spleen was placed in a 50 ml bath and attached to a Sanborn force/displacement transducer by means of hooks secured to the spleen capsule using n-butyl-2-cyanoacrylate tissue adhesive. The resting tension was maintained at 1 gm. The spleen was bathed with Krebs-Henseleit solution at 37 degrees C containing 100 ug/L of propranalol and aerated with 95% O_2/5% CO_2.

After the resting tension was stabilized (usually about 30 minutes), acetylcholine (Ach) was added at 63 and 700 ng/ml in three consecutive trials. Appropriate concentrations of l-norepinephrine (NE) diluted with sodium bisulfite (1:1000) and EDTA (1:1000) in distilled water was added in consecutive volumes of 0.1 ml to obtain a cumulative dose-response curve over the range of 3×10^{-10} to 3×10^{-5} molar. Each concentration of the amine remained in contact with the spleen for 180 seconds before the next higher concentration of agonist was added. The bath was drained and replaced with fresh solution for a minimum of three washes. After 20 minutes, the preparation was tested for loss of autodesensitization by challenges of 3×10^{-9} molar NE and Ach (63 and 700 ng/ml). When sensitivity returned, a second cumulative dose-response curve to NE was run. Following the return of sensitivity following this test, the bath was replaced with solution containing WR-2823 in appropriate concentrations. Dose-response curves for NE were run 45 and 105 minutes after initial contact with WR-2823 (these are designated as one hour and two hour contact times - mean contact times). Sensitivity to NE and Ach was also tested between these latter two dose-response curves.

RESULTS: Maximum responses were estimated from a modified Lineweaver-Burk plot (Empirical fit: $1/Y_i = 1/Y_{i_{max}} \; 10^{-(\overline{b \log_{10} X})2}$

where Y_i = response; $Y_{i_{max}}$ = maximum response; b = slope; X = dose NE).

The resultant dose-response curves are shown in Figures 1 and 2. Logit transformation yields excellent linearity at concentrations of NE above 10^{-9} molar (See Figure A).

Concentrations of WR-2823 above 20 mg/L seemed effective in antagonizing the concentrations to NE at one hour. The maximum contraction did not appear to be depressed by WR-2823 at one hour by any of the concentrations used suggesting that the antagonism may be competitive. That equilibrium had not been attained at one hour is indicated by the further depression of the NE response at two hours. At this time, the maximum response was depressed by 70 mg/L suggesting non-competitive or non-specific antagonism.

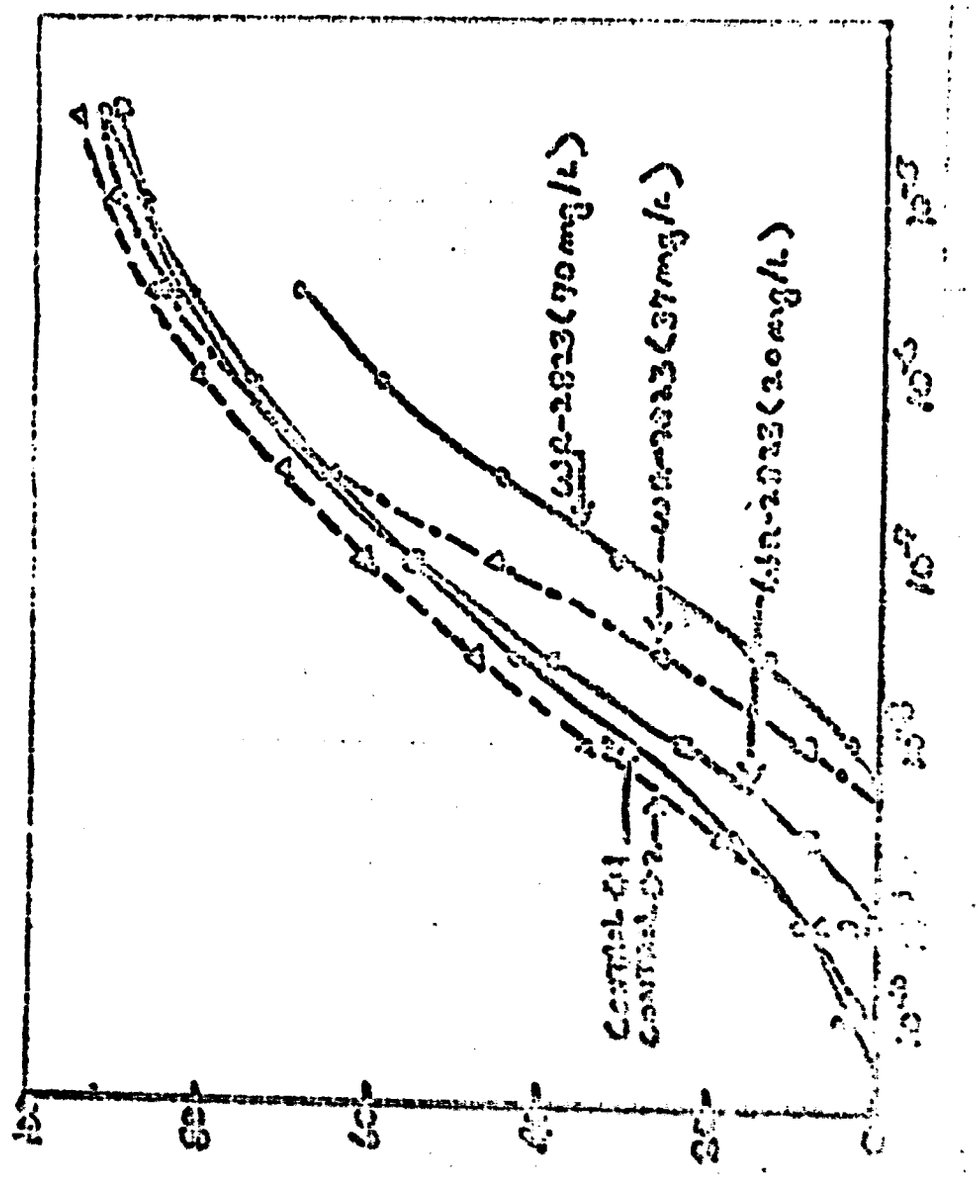
Responses to Ach are shown in Figure 3. The responses of the spleen to Ach are depressed by WR-2823 at the time and in a manner similar to the depression of the NE response. This would suggest that (1) 2823 possesses atropine-like activity in potency equal to its anti-adrenergic properties, (2) Ach acts indirectly in this preparation by release of NE or (3) 2823 depresses the responsiveness of the spleen non-specifically. No further experiments were done to determine the most probable of these explanations. Intuition would predict the latter to be the most likely. The slow equilibration is consistent with slow diffusion of a polar molecule through a lipid barrier.

Conclusions: (1) WR-2823 antagonizes the response of the mouse spleen to NE. (2) At 70 mg/L with 2 hour contact, WR-2823 causes non-competitive or non-specific antagonism. (3) Equilibrium is attained slowly in vitro; probably longer than 2 hours. (4) On the basis of these experiments one can conclude only that WR-2823 antagonizes the response of the mouse spleen to NE.

Experiments this year using WR-1729 will be conducted to determine if we obtain more prompt alpha adrenergic blockade. The spleen has very little activity in splitting of the phosphorothioate bond.

Reference: Ignarro, L. J. The presence of alpha and beta adrenergic receptors in the spleen of the mouse Fed Proc 26: 401 (1967)

GRAPHIC NOT REPRODUCIBLE



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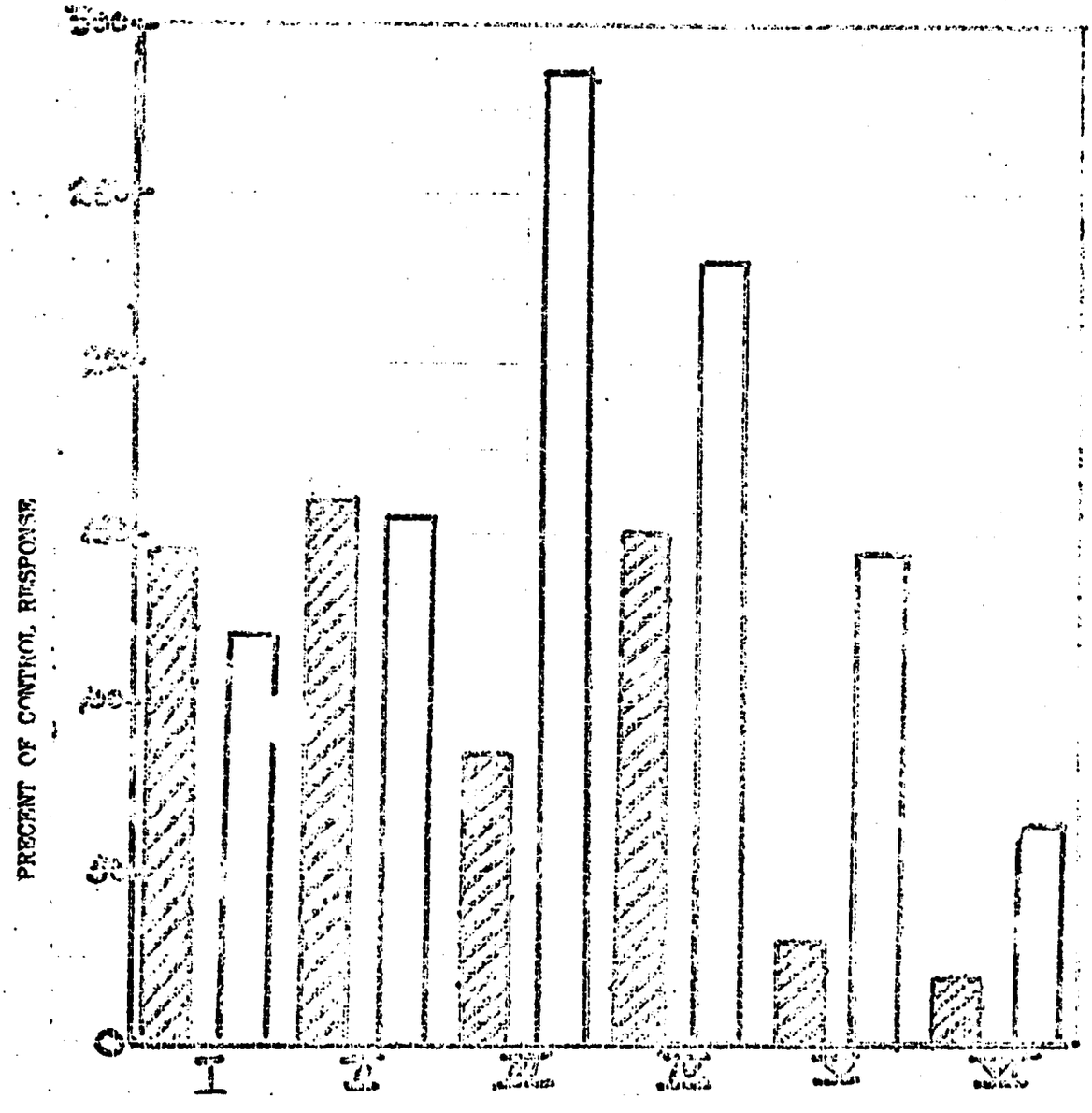
GRAPHIC NOT REPRODUCIBLE

FIGURE 3

EFFECT OF WR-2823 ON THE RESPONSE TO ACETYLCHOLINE(Ach) IN THE ISOLATED MOUSE SPLEEN.
63 ng/ml of Ach approximates ED_{10} For Norepinephrine (NE).
700 ng/ml of Ach approximates ED_{50} For Norepinephrine (NE).



I-RESPONSE AFTER CONTROL NE DOSE RESPONSE CURVE # 1.
II-RESPONSE AFTER CONTROL NE DOSE RESPONSE CURVE # 2.
III-2 mg/L of WR-2823.
IV-20 mg/L of WR-2823.
V-37 mg/L of WR-2823.
VI-70 mg/L of WR-2823.
STRIPED BARS= 63 ng/ml of Ach.
OPEN BARS = 700 ng/ml of Ach.

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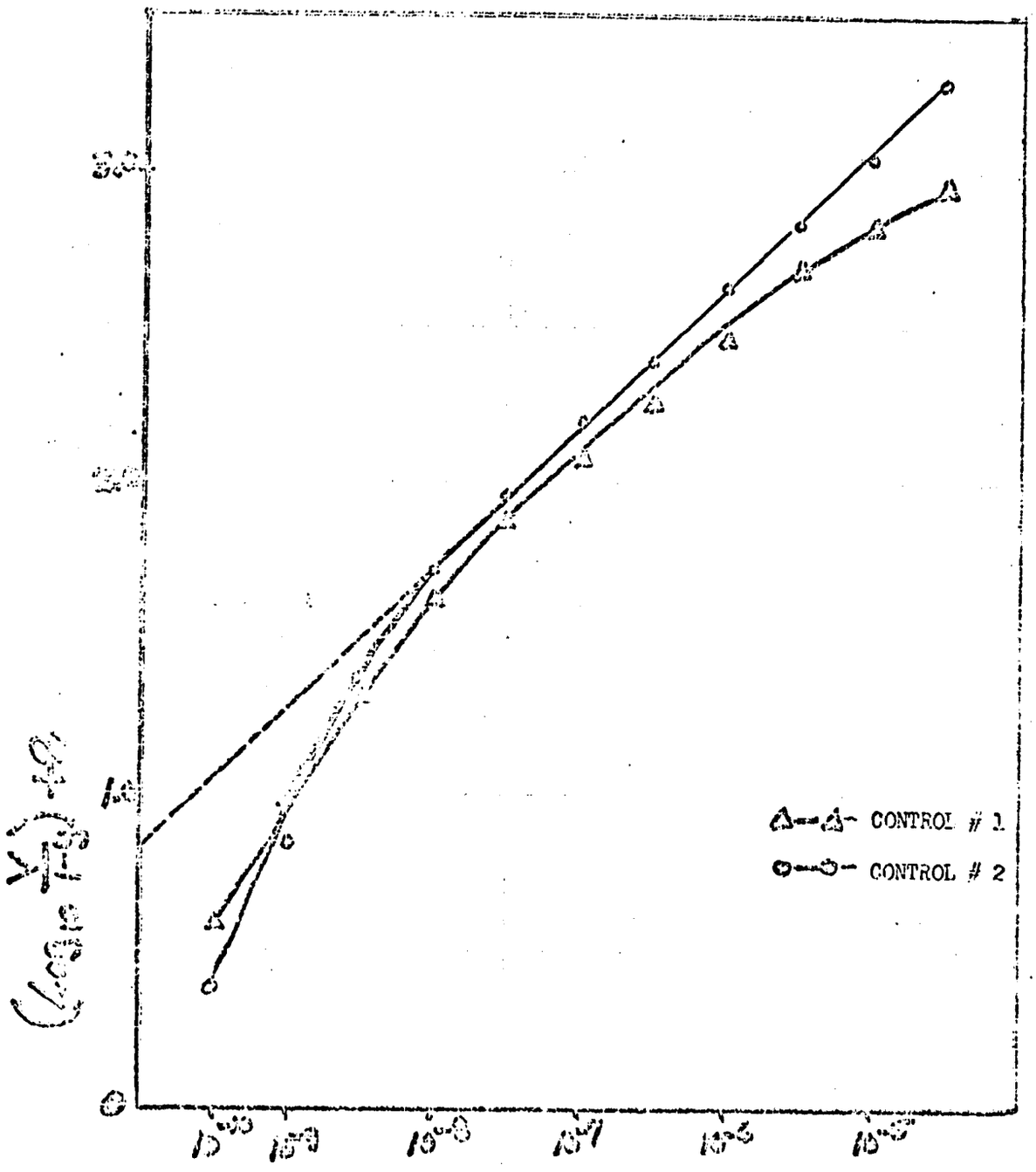
FIGURE A

LOGIT TRANSFORMATIONS OF CONTROL DOSE-RESPONSE CURVES FOR 1-NOREPINEPHRINE



MOLAR CONCENTRATION OF 1-NOREPINEPHRINE

H. Collaborative studies with the Division of Surgery, WRAIR, showed that WR-2823 was able to antagonize the deleterious effects on the microcirculation after 2 hours of hemorrhagic hypotension and the circulation improved even further after epinephrine infusion. We, in our own laboratory, are going to use the thiol derivative of WR-2823 (WR-1729) to determine its therapeutic usefulness in endotoxin shock and hemorrhagic hypotension.

I. Preliminary experiments in our laboratory indicate that ionizing radiation interfered with the alpha adrenergic receptor beginning about 2 hours after irradiation and lasting for at least 5 hours. This phenomenon will be studied in more detail this coming year.

J. Primarily as the result of Maj. Vick's interest in the effect of snake venom, a limited amount of work has been initiated using venoms as pharmacological tools. Maj. Vick has shown that heparin is effective in treating the lethal effects of Russell's Viper if given 30 minutes after injection of the venom.

II. Summary and Conclusions

The chemical synthesis program has now conclusively demonstrated that heterocyclic modification of chemical structures is possible while still retaining antiradiation action. The chemical program has therefore succeeded in introducing conventional features associated with most other drugs. It has also demonstrated that variations in the covering functions make for appreciable differences in activity. We now have at least thirty covering functions.

Prediction of Activity: As a result of synthesis of one covering function, it is not safe for an appreciable number of other covering functions. Therefore, the synthesis program alone is faced with a factorially large problem, if one considers variation in the substituents upon the nitrogen in conjunction with variations of substituents of covering functions.

The biological program data have been reconfirmed by outside workers. The production of sound test data requires a continuing high level professional interest. One of the active compounds has demonstrated a very unusual slope in that small doses of the agent give some radio-protection. Thus, for the first time we believe we have an agent which will offer significant protection to people without observable side effects.

The Pharmacology Program has progressed to the point that additional medicinal uses have been demonstrated for the antiradiation agents. These additional uses in other aspects of medicine will not only broaden interest in this class of compounds but make possible the participation in the program by a large number of specialists who would be otherwise unable to apply their specialties.

Papers Published

1. D. L. Klayman and P. T. McIntyre, J. Org. Chem., 33, 884 (1968); 2-Amino-2-thiazoline. IV. The Ring Opening of 2-Amino-2-Thiazolines and 2-Amino-2-selenazoline with Hydrogen Sulfide to Form Thiourea Derivatives.

2. D. L. Klayman and R. J. Shine, Anal. Chimica Acta, 41, 408 (1068); Categorization of N-Substituted Thioureas via Their S-Methyl Derivatives.

3. D. L. Klayman and R. J. Shine, Chemical Communications, 372 (1968); A new Synthesis of 1,1,3,3-Tetramethylselenourea.

William E. Rothe and David P. Jacobus, J. Med. Chem. 11, 366-368 (1968) Laboratory Evaluation of the Phototoxic Potency of Quinolinemethanols.

Kadhim N. Salman, Ph.D., Robert S. Rozman, Ph.D., Helmut F. Cascorbi,, and John C. Krantz, Jr. Am. J. of Vet Research 29, PP 165 (1968) Intravenous Administration of a New Volatile Anesthetic, 2,2-Dichloro-1, 1-Difluoroethyl Methyl Sulfide in Dogs and Monkeys.

Robert S. Rozman, et al. Biotransformation of Fluroxene. I. In Vivo Metabolism in Mice and Dogs. Biochem. Pharmacology, Vol. 16, PP 1237-48 (1967).

Papers Accepted for Publication

James A. Vick - The Effect of Vasodilator Therapy on Total Peripheral Resistance in Shock. Am. J. Physiology

A. Ferrari, LTC C. R. Angel and D. P. Jacobus - A New Concept for Automated Bio-Analyses for Drug Screening. I. Antiparasitic Drugs. Second Conference on Automation in Industrial Pharmaceutical Process and Quality Control. New York Academy of Sciences, March 1967.

Papers in Press

D. L. Klayman, J. J. Maul and G. W. A. Milne, J. Heterocyclic Chem.; 2-Amino-2-thiazoline. V. Phenylthioureido and Phenylureido Derivatives of 2-Thiazoline.

D. L. Klayman and G. W. A. Milne, Tetrahedron; 2-Amino-2-thiazoline. VI. The Formation of a Thiazolo-s-triazine from 2-Amino-2-thiazoline.

D. L. Klayman and R. J. Shine, Mechanisms of Reactions of Sulfur Compounds-1967; Organic Thiosulfates (chapter)

D. L. Klayman and R. J. Shine, Quarterly Reports on Sulfur Chemistry, to be published as Vol. 3, No. 4, (1968); The Chemistry of Organic Thiosulfates (Review of about 575 references).

Papers Submitted for Publication

James A. Vick, Clifford R. Roberts, Melvin H. Heiffer - Pharmacological Effect of Lethal Doses of Snake Venom.

James A. Vick, Karl H. Slotta - Identification of the Direct Lytic Factor from Cobra Venom as Cardiotoxin.

Vick, Klein, Taylor, DeGraaf, Roberts, Mahlandt, Remmele and Lincoln. Pathophysiological Effects of Staphylococcal Enterotoxin B.

Robert S. Rozman, Ph.D. and Arnold A. Kurland, M.D. - The Effect of Flurothyl and Electroshock on Pulmonary Diffusion.

Robert S. Rozman, David A. Blake, Francis J. Meyer and John C. Krantz, Jr. Anesthesia LXXV: Biotransformation of Fluroxene. I. In Vitro Metabolism In Mice.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. PROJECT CONTROL SYMBOL
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5. DATE OF RESUME	6. KIND OF RESUME	7. SECURITY	8. REGRADING	9. RELEASE LIMITATION	10. LEVEL OF RESUME
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C. IN-HOUSE	NA / NA / NA / NA	PRIOR 69	7	175
		CURRENT 69	7	175

19. GOVT. LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
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	TEL. 202-576-2211 TYPE DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
RADIOBIOLOGY RADIATION PROTECTION	NA

23. KEYWORDS

RADIATION PROTECTIVE AGENTS, DRUG TOLERANCE, CHEMOPROPHYLAXIS.

24.

(U) TECH OBJECTIVE - THE DEFINITION AND QUANTITATION OF CHEMICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHANGES OCCURRING AFTER ADMINISTRATION OF CHEMICAL COMPOUNDS THAT SHOW REDUCTION IN MAMMALIAN MORTALITY AFTER EXPOSURE TO IONIZING AND NEUTRON RADIATION.

(U) APPROACH- BASIC BIOCHEMICAL MECHANISMS OF RADIOPROTECTIVE DRUG ACTION AT THE CELLULAR, ORGAN, AND WHOLE BODY LEVELS ARE BEING EXPLORED IN BACTERIA, MAMMALIAN CELL CULTURES, AND SMALL MAMMALS. AN INTEGRATED MULTI-DISCIPLINED EFFORT INVOLVES CHEMISTRY, BIOCHEMISTRY, PHYSIOLOGY AND PHARMACOLOGY LEADING TO DEFINITION OF THE MECHANISM OF ACTION AMINOTHIOLS IN CELLULAR AND MAMMALIAN SYSTEMS. RADIOISOTOPIC TECHNIQUES FOR DISTRIBUTION, RETENTION AND EXCRETION OF RADIATION MODIFIERS ARE EMPLOYED WHERE APPLICABLE.

(U) PROGRESS - JUL 67 THRU JAN 68 ROLES AND EFFECTS OF THIOL AND AMINO GROUPS ARE EVALUATED BY PROTECTION STUDIES OF BACTERIA IRRADIATED AT 1 DEGREE C IN NITROGEN AND OXYGEN, ESR EFFECTS AND PROTECTION IN FROZEN BACTERIA (-196 DEGREES C), LYOPHILIZED PROTEINS, AND REACTIVITY OF COMPOUNDS WITH A MODEL FREE RADICAL, DPPH, AT 20 DEGREES C. PHYSIOLOGICAL AND NEUROLOGICAL RESPONSES AFTER ADMINISTRATION OF CHEMICAL RADIOPROTECTANTS ARE BEING STUDIED IN SMALL MAMMALS. DURATION OF EFFECTS STUDIES AND STUDIES OF WHOLE BODY DISTRIBUTION OF AMINOTHIOL COMPOUNDS ADMINISTERED TO RATS ARE CONTINUING. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORTS, 1 JULY 1967 - 30 JUNE 1968.

24.

TEXT NOT REPRODUCIBLE

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ 1. COMSEC OR COMSEC RELATED ☑ 2. NOT RELATED		AR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
1212[illegible]-5[illegible]	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (IN THOUSANDS)	36.

DD FORM 1 JAN 66 1490m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A025601A824, IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 01, Ionizing Radiation Injury, Prevention and Treatment

Work Unit 056, Protective effect of aminothiols against ionizing and neutron radiation

Investigators.
Principal: LTC Dorsey T. Mahin, MC
Associate: LTC Charles R. Angel, MSC; LTC LaWayne R. Stromberg, MC; LTC Merrill C. Johnson, MC; LTC Harold M. Swartz, MC; LTC Hollis Bivens, MC (MMAS Course); MAJ Richard D. Farris, VC; CPT Robert M. Donati, MC; CPT C. J. Klobukowski, MSC; Edmund S. Copeland, Ph.D.; Albert Einheber, Ph.D.; Stephen R. Heller, Ph.D.; Mary M. McLaughlin, MS; Earl C. Richardson, MS; Minnie H. Davis, BS; John I. Davis, BS; Robert E. Wren, BS.

Description.

The overall objectives of this work unit are (1) to determine the mechanism of radioprotective action of aminothiols through performance of studies utilizing a broad variety of methods; (2) to study absorption, fate, excretion, and duration of effect of selected compounds with a view towards their possible future use in humans; (3) to measure the effects of treatment with these drugs, either alone or in combination with radiation, on animal response to other types of trauma; and (4) to utilize the available drugs of this class to explore the basic mechanisms by which ionizing radiation damages biological systems. This work unit overlaps Work Unit 015, Mechanisms. Reports of studies under this work unit are sub-divided into categories of animal, chemical, bacterial, and tissue culture studies.

1. Animal studies.

The purpose of this subdivision is to measure and define some aspects of the interractions of protective drugs with recipient animals, but does not include pharmacologic effects which are the subject of study in the Division of Medicinal Chemistry. Studies have been designed to investigate the alteration of lethality induced by these drugs when their administration is combined with other forms of sublethal injury, as well as the interdependence of individual drugs in producing radioprotection.

2. Chemical studies.

In this subdivision, the purpose is to conduct studies which establish the precise chemical identity and purity of chemicals which have been tested and shown to be radioprotective. Other studies are designed

to elucidate the sites and nature of chemical bondings between chemical protectants and target molecules, and the relationship between these bonds and radioprotection.

3. Bacterial studies.

In the WRAIR Annual Report, 1966-67, bacterial studies were reported which established a close relationship between radioprotection and the free radical production of ionizing radiation. Further studies have been designed to examine the relationship between chemical structure and radioprotective activity. A number of compounds have been synthesized, and are currently being used in bacterial studies.

Other bacterial studies demonstrated that an aminothiol, MEA, protects against lethal damage produced by freezing bacteria in the presence of oxygen. (See this report, 015 Mechanisms). Features common to injury by freezing, drying, exposure to high partial pressures of oxygen and exposure to ionizing radiation are being investigated.

4. Tissue culture studies.

Further studies have been performed to improve the quantitative precision of replicate human lymphocyte cultures in vitro. Supervisory and technical personnel changes have severely hindered the application of this study model to studies of radiation injury mechanisms and radioprotection.

Progress.

1. Animal studies.

a. Effects of aminothiols on trauma resistance. (Dr. Einheber, Mr. Wren, CPT Klobukowski).

It was reported (WRAIR Annual Report, 1966-67) that pretreatment with several aminothiols increased mortality in mice subjected to Noble-Collip drum treatment (NCDT). The sublethal effects of aminothiol and NCDT treatment appeared to be additive. In aminothiol-treated mice, the period of decreased resistance to trauma corresponded to the period of maximum radioresistance. The a priori implication is that mechanisms of radioprotection and depression of trauma resistance are related. Therefore, it is important to determine whether these effects can be differentiated.

In previous studies, it was noted that hexobarbital pretreatment of mice protected them against the lethality of NCDT. Hexobarbital treatment might, therefore, counteract the harmful effects of aminothiol treatment. In order to distinguish between the effects of these two

treatments it is necessary to prove that hexobarbital does not abolish the radioprotective effects of treatment with aminothiols.

Experimental studies were designed to test the interactions of aminothiols and hexobarbital with NCDT. The results are summarized below:

1) The survival of mice subjected to ten minutes of NCDT was increased by pretreatment with hexobarbital (100 mg/kg), decreased by MEA (150 mg/kg), WR 2721C (600 mg/kg), or WR 638 (400 mg/kg), and virtually unaltered by WR 2721C (300 mg/kg).

2) As compared with mice given MEA (150 mg/kg), or WR 638 (400 mg/kg) alone, survival after ten minutes of NCDT was increased in mice given hexobarbital (100 mg/kg) in tandem with MEA (150 mg/kg) or with WR 638 (400 mg/kg).

3) In contrast, hexobarbital (100 mg/kg) given in tandem with WR 2721C (300 or 600 mg/kg) not only failed to increase post-NCDT survival, but actually decreased survival relative to that observed after WR 2721C (300 mg/kg) alone or no treatment.

4) Mice pretreated with MEA (150 mg/kg), WR 2721C (300 or 600 mg/kg), or hexobarbital (100 mg/kg) were protected against the lethal effects of whole-body irradiation. Hexobarbital was least effective, and 300 mg/kg of WR 2721C was somewhat more effective than 150 mg/kg of MEA.

5) MEA (150 mg/kg) or WR 2721C (600 mg/kg) was equally radioprotective when given alone or in tandem with hexobarbital (100 mg/kg) in advance of ordinarily lethal whole-body irradiation.

6) Treatment of mice with MEA (150 mg/kg) or WR 2721C (300 mg/kg) in tandem with hexobarbital (100 mg/kg) had little effect on the hexobarbital-induced sleeping time; MEA prolonged it about 30%.

7) When mice pretreated with hexobarbital (100 mg/kg) alone or in tandem with MEA (150 mg/kg) were subjected to ten minutes of NCDT (which they survived) their respective sleeping times were about 3 and 4 times greater than observed in similarly pretreated nontraumatized mice.

8) Treatment of mice with either hexobarbital (100 mg/kg), MEA (150 mg/kg), or WR 2721C (300 mg/kg), alone; or tandem treatment with hexobarbital and MEA, or hexobarbital and WR 2721C, resulted in temporally and quantitatively distinctive patterns of change in colonic temperature during six hours post-treatment. All treatments resulted in marked reductions in temperature after treatment. Arrangement of the data according to overall degree and duration of colonic temperature reduction gave the following decreasing order of effectiveness: hexobarbital and WR 2721C; hexobarbital and MEA; WR 2721C; hexobarbital; MEA.

9) Nontreated mice manifested significant and prolonged colonic temperature reductions after ten minutes of NCDT. Mice pretreated with WR 2721C (300 mg/kg), hexobarbital (100 mg/kg), or hexobarbital (100 mg/kg) and MEA (150 mg/kg) and subjected to ten minutes of NCDT (which they survived) showed greater and more prolonged colonic temperature reductions than occurred without injury. The patterns of change were distinctive for the different treatments. The treatment groups arranged in decreasing order of overall degree and duration of colonic temperature reduction elicited by NCDT, were: hexobarbital and MEA; WR 2721C; hexobarbital; nontreated.

The observations that: (a) hexobarbital protected against NCDT and conferred some radioprotection; (b) 300 mg/kg of WR 2721C did not significantly alter survival after NCDT and was highly radioprotective; and (c) hexobarbital in tandem with MEA overcame the deleterious influence of MEA on post-NCDT mortality and was as radioprotective as MEA alone, demonstrate that chemical radioprotection can be disassociated from susceptibility to trauma of this kind.

All chemical agents and NCDT caused significant reductions in colonic temperature. The rapidity of onset and the duration and degree of these changes revealed distinctive patterns for the different treatments. However, these changes did not consistently correlate with the influence of the treatments on mortality after NCDT or whole-body irradiation. Reduced body temperature could account, in part, for the marked prolongation of hexobarbital-sleeping time seen after NCDT.

Hexobarbital given in tandem with MEA or WR 638 had a beneficial effect on post-NCDT survival, but a harmful effect in tandem with WR 2721C. Compounds WR 638 and WR 2721C are aminothiophosphates and MEA is an aminothiol; therefore, this chemical difference alone does not appear to be related to the opposite effects of hexobarbital treatment.

b. Effects of aminothiols on wound healing in irradiated animals. (See this report, Task 015, Mechanisms).

c. Tissue distribution studies. (LTC Nahin, MAJ Farris).

Studies performed in the Division of Medicinal Chemistry showed a profound reduction of radioprotection in mice treated with a small dose of MEA 15 minutes after a radioprotective dose of WR 1607 (a chemical analog of MEA). Treatment with 5-15 mg/kg body weight (BW) of MEA (a very low dose compared to the usual radioprotective dose of 125-150 mg/kg BW) completely eliminated radioprotection in mice that had received 5 mg/kg BW of WR 1607 thirty minutes before irradiation. This finding suggested the possibility that MEA treatment with less-than-protective doses resulted in reduction of WR 1607 concentrations in vulnerable tissues at the time of irradiation.

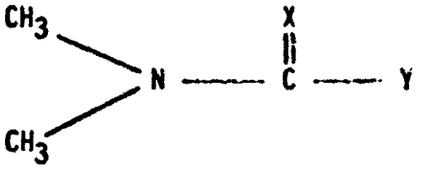
A study was performed in mice utilizing both ^{35}S tagged and nonradioactive forms of MEA and WR 1607. No significant alterations in organ content of the tagged protectants could be demonstrated to result from prior or subsequent treatment with either compound. Although the results of this study were inconclusive, no evidence was found to indicate that prior or subsequent treatment with one compound significantly influenced the tissue content of the other. For a variety of technical reasons, the study was believed unreliable and no attempt at interpretation appears warranted.

2. Chemical studies.

a. Nuclear magnetic resonance (NMR) studies on hindered rotation in selenocarbamates and thiocarbamates. (Dr. Heller).

In collaboration with Dr. D. L. Klayman, Division of Medicinal Chemistry, we have investigated the hindered rotation about the C-N bond in a number of substituted carbamates prepared by the Division of Biochemistry in connection with their antiradiation and antimalaria drug programs.

Proton nuclear magnetic resonance (NMR) spectroscopy is being used to study the bonding properties of compounds of the type shown below:



X = O, S or Se
Y = CH_3, S-CH_3, Cl, H, D or S-Na

The data obtained by high resolution and pulsed techniques are being evaluated by computer using programs that calculate the rate of rotation, activation energy, enthalpy, entropy and frequency factor. The pulsed NMR studies are being done in collaboration with Dr. T. Farrah of the National Bureau of Standards.

b. NMR studies of the binding of aminothiols. (Dr. Heller).

As a part of the program to investigate the mechanisms of action of aminothiols as radioprotective agents, a study of the binding of these agents with nucleic acids and derivatives was undertaken.

Nuclear magnetic resonance (NMR) spectroscopy was used as a tool for studying the association of MEA with these compounds. MEA (2-mercaptoethylamine) free base was used because in addition to being the fundamental compound in the aminothiol series, its proton NMR spectrum is relatively easy to interpret.

Since the purine (adenine and guanine) and prymidine (thymine, uracil, and cytosine) bases are only slightly soluble in water at neutral pH, no information could be obtained under physiologic conditions.

During NMR studies of interaction of the radioprotective drug MEA with purine and pyrimidine nucleosides in D_2O it was found that, in the presence of the MEA, a specific substitution of a deuteron for a proton occurred at the C-5 position. This reaction occurred only in nucleosides with a C-4 carbonyl group. It was found that this substitution would occur with any basic solution, including NaOH, and did not specifically require aminothiols.

Further studies are now in progress on the interaction between MEA and nucleotides and polynucleotides. These studies require measurement of the bonding conditions of phosphorus. Our instrument lacks the capability to detect phosphorus bonding and these NMR studies are being performed on the HA-100 spectrometer at the National Institutes of Health.

c. The reaction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with aminothiols. (Dr. Heller).

Previous studies here and elsewhere have suggested a relationship between the free radical scavenging and radioprotective properties of chemical compounds. To test this possible relationship, DPPH, which contains a relatively stable free radical, was reacted with a large group of radioprotective compounds (especially aminothiols) and chemically similar compounds. Methyl alcohol was chosen as the solvent because almost all of the materials used are readily soluble in it and many of its properties are similar to those of water. The reactions were conducted in deaerated solutions to eliminate oxygen effects. All reactions were run at 20.0°C.

Preliminary results indicate that MEA and its radioprotective analogs reacted as much as 1000 times faster than other nonprotective chemical analogs. Preliminary results also indicate that the reaction kinetics are complex. Simultaneous competing reactions are shown below:

$$RSH + DPPH \longrightarrow RS\cdot + HDPPH \qquad (1)$$

$$2\ RS\cdot \longrightarrow RSSR \qquad (2)$$

$$RS\cdot + DPPH \longrightarrow RSDPPH \qquad (3)$$

Reactions (1) and (2) indicate the simple radical reaction of DPPH with a thiol. Both the RSSR and HDPPH solutions are generally very lightly colored species (versus the dark purple color of a DPPH solution). Hence, colorimetric determinations of mixed solutions of these compounds reflect the DPPH concentration and permit direct measurement of the rate constant.

However, if reaction (3) occurs to any appreciable extent, the decrease in color of the solution is not directly a measurement of the DPPH reaction with thiols. In order to obtain a true rate constant for reactions (1) and (2), the relative and absolute concentrations of the two components must be experimentally varied so as to establish the conditions under which reaction (3) occurs to an appreciable extent.

To date, kinetic studies have been done on about 30 compounds; however, in practically all cases the extent of reaction (3) is not known.

d. Purity of radioprotective chemicals. (Dr. Lofberg).

Chemicals used in radioprotective studies are frequently impure. Since the nature, amount, and biological effects of impurities are usually unknown, reliable interpretation of chemical radioprotection data often is complicated and difficult, if not impossible. In order to overcome this handicap, a program was initiated to establish standards of purity, to develop methods for purity assay, and where necessary, to purify compounds used in radioprotective studies.

Most of the pertinent compounds fall into one of three major chemical categories: 1) aminothiols; 2) Bunte compounds, and 3) aminothiol-S-phosphates. Progress in this work unit is reported according to category.

1) Aminothiols.

(a) A comprehensive study was made of amperometric procedures for purity assay. While suitable in special cases, these procedures usually were not satisfactory.

(b) Colorimetric assay techniques were investigated. Many of the common methods such as the nitroprusside test, the fluoropyruvate method and Ellman's procedure, have value in specific applications. However, each has serious defects with respect to analysis of aminothiols in general. The most useful colorimetric method is a variation of the procedure (advocated by S. Akerfeldt) based upon the reaction of a thiol with 2-chloromercuri,6-nitrophenol in a two-phase solvent system. The loss of the 2-chloromercuri,6-nitrophenol from a 1,2-dichloroethane solution to a citrate solution of the aminothiol at pH 4 is directly proportional to the amount of aminothiol.

(c) Another method proposed by S. Akerfeldt for identification of aminothiols involved use of paper chromatography. This method was adapted to thin layer chromatography. This technique involves the reaction between a thiol and 2-chloromercuri,6-nitrophenol described in paragraph 1), (b) above. An aqueous citrate buffer solvent (pH 4) containing the reaction products was adjusted to pH 7.0-7.5 and spotted on Kodak K301-R plates. After drying, the plates were developed in ethanol-water mixtures.

Rf VALUES (AVERAGE OF FIVE RUNS)

Compound	100 % ETOH	3:1 ETOH:H_2O
2-aminoethanethiol hydrochloride	0.62 ± 0.01	0.73 ± 0.03
Reduced glutathione	Less than 0.1	0.31 ± 0.01
Cysteine hydrochloride	0.12 ± 0.02	0.66 ± 0.03

This procedure gave very satisfactory results in preliminary general testing and will be studied further.

(d) Gas chromatography also showed promise as a method for identification and quantitation of thiol compounds. It is necessary to make stable volatile derivatives of aminothiols prior to chromatography. The derivative selected was the trimethylsilyl compound formed by reaction with N,O-bis-(trimethylsilyl) acetimide in dimethylformamide solution. A dual-column, dual-flame gas chromatograph (F&M Model 810) was used. The 6% SE-30, "Chromosorb G" columns were operated at 170°C.

REPRESENTATIVE RESULTS ON GAS CHROMATOGRAPHY

Compound	Retention Time (Minutes)
2-aminoethanethiol hydrochloride	12.6
N,n-octyl 2-aminoethanethiol hydrochloride	22.8
N,n-decyl 2-aminoethanethiol hydrochloride	122.4

Mixtures of all three compounds listed above could be identified and quantitatively analyzed. Temperature programming will improve the procedure.

2) Bunte compounds.

Bunte compounds (R-SSO_3H) can be analyzed by variations of the procedure suggested by MacDonald. In this method, the compounds (as strong acids) were titrated in pyridine solution against tetrabutyl ammonium hydroxide. The end point could be determined potentiometrically or colorimetrically. Results of this method are not yet available.

3) Aminothiol-S-phosphates. Potential methods for compounds in this category are under study. Methods have not been selected or developed.

3. Bacterial studies.

a. Survival studies on lyophilized bacteria. (Dr. Copeland).

In order to obtain a viable test organism for use in dry-state radiation protection studies, the bacterial strains, E. coli B/r and E. coli B_{s-1} have been studied. It was found that, when lyophilization is carried out at -30°C and the lyophilized bacteria are not exposed to oxygen, survival levels in the range of 40 to 60 per cent can be achieved. Three procedures were studied: (1) lyophilization at -30°C with no exposure to oxygen, (2) lyophilization at 25°C with no exposure to oxygen, and (3) lyophilization at 25°C with exposure to oxygen after lyophilization. The results for E. coli B_{s-1} were 40%, 3%, 0% survival for conditions (1), (2) and (3), respectively; whereas E. coli B/r gave survival levels of 40%, 20% and 6%, respectively, for the three conditions.

b. Radiation protection studies with lyophilized bacteria. (Dr. Copeland).

Bacteria were lyophilized from aqueous suspensions with MEA-HCl and the resulting powder was irradiated *in vacuo* with appropriate doses of ^{60}Co gamma radiation. Irradiation was carried out at 77°K and the bacterial preparations were studied at this temperature using ESR spectroscopy. The samples were then pulse annealed at increasing temperatures and the transfer of radiation-induced free radicals from bacteria to MEA-HCl was followed with ESR spectroscopy. The samples were then resuspended in aqueous solution and plate counts were made on solid medium. Preliminary studies indicate that MEA-HCl provides a 2000-fold protection to E. coli B/r irradiated in the dry state. ESR studies on the same preparations indicated a 65 to 75% reduction in the E. coli free radical yield with a corresponding increase in free radicals localized on the protectant, MEA-HCl. Studies are in progress to quantitate further the transfer of radiation energy from biological test organisms to radioprotectants and to correlate this transfer with survival.

c. Radiation energy transfer studies in enzyme-model membrane systems. (Dr. Copeland).

Using electron spin resonance spectroscopy, studies are being conducted to follow the radiation energy transfer between sulfur-containing enzymes (ribonuclease and trypsin) and the modified dextran, Sephadex, as a function of the degree of penetration of the enzymes into the macromolecular matrix of Sephadex. Solid state molecular mixtures were prepared by lyophilizing enzyme solutions with suspensions of Sephadex of different pore size. Depending on the pore size, the enzyme

either penetrates into the matrix or remains on the surface. Preliminary results indicate that the degree of radiation energy transfer increases with increasing penetration. Transfer occurs from the matrix to the enzyme. This implies that enzymes imbedded in membrane matrices may be more radiosensitive than those absorbed to the surface.

d. Radiation protection studies with lyophilized enzymes. (Dr. Copeland).

Previous studies (European Journal of Biochemistry, 1:312, 1967; and Radiation Research, in press) of energy transfer between proteins and radioprotectants irradiated in the dry state are being extended. Attempts are being made to correlate energy transfer (observed by ESR spectroscopy) with the enzymatic activity observed after dissolving irradiated preparations in aqueous media.

e. Relation between free radical reduction and radiation protection by aminothiols. (LTC Swartz, Mr. Richardson, Dr. Copeland, Dr. Heller, Dr. Lofberg).

Work reported in the previous WRAIR Annual Report (1966-67) described a relationship between the ability of a compound to scavenge free radicals and its ability to protect bacteria from ionizing radiation. The results suggested a structure-function relationship. At present, there are no useful data on structure-function relationships. Results of such studies in mammals are equivocal because the form of the compound at the time of protection can not be known. Accordingly, structure-function studies using structural variations of the parent compound, beta-mercaptoethylamine, are being performed in bacteria. The first series of experiments with these compounds will investigate (1) the role of the thiol group; (2) the role of the amino group; and (3) the effect of the length of the carbon chain between SH and NH_2 groups.

Since many of the required compounds were unavailable commercially, it was necessary to synthesize these compounds. These and other (commercially available) compounds were tested for purity and, if necessary, purified (See Section 2, Chemical Studies, part d above).

Basic compound.

(1) $NH_2CH_2CH_2SH$

Thiol function modification.

(2) $NH_2CH_2CH_3$

(3) $(NH_2CH_2CH_2S)_2$

(4) $NH_2CH_2CH_2S\text{-}SO_2H$

(5) (6) (7) $NH_2(CH_2)_nS\text{-}SO_2H$ n = 3, 4 and 5

(8) $NH_2CH_2CH_2S\text{-}PO_3H_2$

Amino function modification.

(9) CH_3CH_2SH

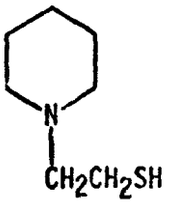
(10) $CH_3NHCH_2CH_2SH$

(11) $(CH_3)_2NCH_2CH_2SH$

(12) $\emptyset\text{-}NHCH_2CH_2SH$

(13) $(\emptyset)_2NCH_2CH_2SH$

(14)

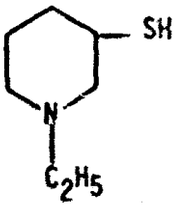


Variations of carbon chain length between SH and NH_2 groups.

(15) (16) (17) $NH_2(CH_2)_nSH$ n = 3, 4 and 5

Miscellaneous.

(18)



Several of these compounds have been prepared and analyzed in quantities sufficient for study and are being utilized as follows:

(a) Protection against killing of bacteria irradiated at 1°C in nitrogen and oxygen.

(b) ESR effects and protection against killing in irradiated lyophilized bacteria.

(c) ESR effects in lyophilized proteins.

(d) Reactivity with a model free radical, DPPH, at 20°C. (See Section 2, Chemical Studies, part b, above).

f. Protection studies of bacteria irradiated at 1°C in N_2 and O_2. (Mr. Richardson).

The experiments summarized below compare protection and toxicity factors for several aminothiol compounds. All of the compounds tested are structure-function variants of the parent compound, beta-mercapto-ethylamine ($NH_2CH_2CH_2$-SH).

A suspension of Escherichia coli B/r in phosphate buffer was prepared from a washed stationary culture that was grown in minimal media at 37°C for 18-24 hours. Various concentrations of the compounds to be studied were prepared in phosphate buffer and added to the bacterial suspension. Aliquots of these preparations were equilibrated with oxygen or nitrogen gas for three minutes prior to irradiation. Gassing was maintained during irradiation. Nitrogen gas was freed of O_2 contamination by passage over hot (400-450°C) copper turnings and through several pyro-gallol washings. Bacterial samples were exposed to a dose of 121 Krad irradiation in the Cobalt-60 Gammacell. This dose was chosen because lower doses were not sufficiently damaging to reveal relative protection. After irradiation, samples were plated on minimal media and, after 48 hours incubation at 37°C, scored for colony forming ability. During each experiment cell suspensions, compound solutions, and postirradiation diluting solutions were kept at ice bath temperatures.

Protection and toxicity data for each of the compounds tested are summarized below. The toxic factor was measured in irradiated cultures as the ratio of survivors treated with a compound to untreated survivors.

1) Beta-mercaptoethylamine (MEA).

This compound was evaluated at concentrations up to 2 molar. It was found to exhibit 50% toxicity at concentrations above 0.8 molar. At the lowest concentration (0.8 molar) affording maximum protection to anoxic cells, MEA produced a 10-fold enhancement of the protection given by anoxia alone. This concentration bestowed as much protection to O_2-treated cells as anoxia alone. At MEA concentrations near 1.4 molar, oxygen-treated cells were protected by a factor of 1000. There was no additional enhancement of protection in oxygen- or nitrogen-treated cells at MEA concentrations above 1.4 molar. At these higher concentrations (1.4 molar and greater) both oxygen- and nitrogen-treated cells had the same survival fraction.

2) $NH_2CH_2CH_3$.

This compound was tested at concentrations of 0, 0.5, 1.0 and 2.0 molar. It was found to be non-toxic in both oxygen- and nitrogen-

treated cells. However, there was some indication that toxicity occurred if the concentration of the compound was not reduced by dilution after irradiation. There was no protection in nitrogen-treated cells. A three to five-fold increase in protection was observed in oxygen-treated cells.

3) $(NH_2CH_2CH_2S)_2$.

This compound was tested at concentrations of 0, 0.1, 0.2, 0.4, 0.7, 0.9 and 1.0 molar. Toxicity was observed in both nitrogen- and oxygen-treated cells at concentrations above 0.4 molar and was as high as 70% in nitrogen and 90% in oxygen at 1 molar. At a concentration of 1.0 molar, the nitrogen-treated cells were protected by a factor of 10, and the oxygen-treated cells by a factor of 1000. In both cases the maximal protective concentration (known to be greater than 1.0 molar) was not tested. In view of the toxicity encountered, increasing the concentrations of the compound probably would not yield meaningful results.

4) $NH_2CH_2CH_2S \cdot SO$.

This compound was tested at concentrations of 0, 0.25, 0.5 and 1.0 molar. Toxicity was observed in both the oxygen- and nitrogen-treated cells at all concentrations tested. No protection was found for the nitrogen-treated cells. There was a ten-fold protection at 0.25 molar concentration in the oxygen-treated cells but sensitization occurred at higher concentrations.

5) CH_3CH_2SH.

This compound was tested at concentrations of 0, 2×10^{-4}, 2×10^{-3}, 1×10^{-2}, 2×10^{-2}, 5×10^{-2}, 1×10^{-1}, and 2×10^{-1} molar. The compound could not be tested at higher concentrations because of its limited solubility. Toxicity was observed in both nitrogen- and oxygen-treated cells at concentrations above at 1×10^{-2} molar. At concentrations greater than 1×10^{-2} molar a two-fold increase in protection was observed in nitrogen-treated cells. No protection was observed in oxygen-treated cells at any concentration tested.

6) $(CH_3)_2NH_2CH_2CH_2SH$.

This compound was tested at concentrations of 0, 0.5, 1.0 and 2.0 molar. No toxicity was observed in the oxygen- or nitrogen-treated cells. Little or no protection was observed in the nitrogen-treated cells but a 1000-fold increase in protection was observed at 1 molar concentration in oxygen-treated cells and produced the same survival observed in nitrogen-treated cells.

4. Quantitative human lymphocyte culture. (LTC Johnson, LTC Mahin, LTC Bivens, Mrs. Davis).

An experiment was designed to allow statistical evaluation of temporal changes in media content of glucose and lactic acid, and of cell concentration, among concurrently prepared cultures from four lymphocyte donors and among replicate cultures from these donors.

Values for the three culture intervals studied are tabulated below. The standard error is relatively low. Measured characteristics, as expected, are different for each individual donor; however, when various measurements are plotted against time in culture, similar patterns and absolute differences smaller than previously reported are demonstrated.

CELL COUNT AND GLUCOSE AND LACTATE CONTENT

IN DONOR LYMPHOCYTE CULTURES

Donor	Time (Hours) in Culture 0	48	72	96
		* CELLS/mm^3		
A	600	416 ± 17	599 ± 31	557 ± 27
B	600	398 ± 19	697 ± 38	702 ± 39
C	600	320 ± 9	596 ± 18	508 ± 21
D	600	540 ± 19	772 ± 26	739 ± 64
		* mgm % GLUCOSE		
A	112 ± 3	40 ± 1	12 ± 1	** 3 ± 1
B	111 ± 4	30 ± 2	** 3 ± 1	** 0 ± 0
C	114 ± 1	46 ± 2	14 ± 1	** 8 ± 1
D	113 ± 1	44 ± 1	** 9 ± 1	** 5 ± 2
		* mgm % LACTATE		
A	42 ± 1	79 ± 1	119 ± 2	131 ± 1
B	35 ± 1	81 ± 1	123 ± 2	129 ± 1
C	41 ± 1	73 ± 1	110 ± 4	131 ± 2
D	39 ± 1	80 ± 2	131 ± 5	136 ± 2

* Standard errors are indicated.
**Values below 10 mgm % are not accurate.

An analysis of variance was made from the cell count and lactic acid data. Analysis of glucose data was not done because many values were below 10 mgm percent, and could not be accurately measured. The variance in cell counts between study periods was 39.24 and between donors it was 11.87. The respective P values were .001 and .01. The variance between culture periods for lactic acid concentration was 369.30 and between donors it was 5.24. The difference between culture period F is significant with a P of .001 but the differences between donors were not significant at the .05% level.

A formula promulgated for hematological quality control and utilizing differences between paired determinations was used to generate coefficients of variation (C.V.) on cell count data. Coefficients relating to (1) precision associated with two counts from a single aliquot, (2) precisions associated with single counts on each of two aliquots from the same culture, and (3) precision associated with single counts on aliquots of two cultures from the same donor are given below. Twelve pairs of determinations were used to generate each C.V. Eight C.V. determinations were averaged to quantitate precision in the three areas of interest.

COEFFICIENT OF VARIATION

	Average	Range
(1) Two counts from one aliquot	9.1	7.6 - 10.3
(2) One count on each of two aliquots; same culture	9.8	4.3 - 14.9
(3) One count from each of two cultures; same donor	11.5	7.3 - 18.4

These results were interpreted to mean that counting precision was largely influenced by random variations in counts due to cell content of aliquots counted; electronic noise; and background particles (contaminants, etc.) in the diluting solution.

Two dose-response relationships were established utilizing the above characterized system. In the first experiment, cells were exposed to 0, 200, 400, 600, 800 and 1000 R. Cell counts were done at 48, 72 and 96 hours. Counts done at 96 hours showed the largest dose-response differences, presumably because of replication of undamaged lymphocytes. Averaged 96-hour cell counts were as follows: 0 R - 960, 200 R - 950, 400 R - 860, 600 R - 760, 800 R - 650, 1000 R - 600. Statistical analysis has not been completed on this work.

In a second experiment, chloroquine at three different concentrations was added to lymphocyte cultures. Differences in lactic acid and glucose content were most impressive at 96 hours and are shown below.

EFFECTS OF CHLOROQUINE ON

LACTIC ACID AND GLUCOSE CONTENT OF LYMPHOCYTE CULTURES

Chloroquine mgm %	Glucose	Lactic Acid
0	25	79
0.05	24	80
0.1	43	69
0.2	60	45

An analog of chloroquine without antimalarial activity did not affect glucose and lactic acid values. Statistical analysis of this work has not been completed but differences are large in relation to small differences between duplicate cultures.

Experiments are currently being done to establish toxicity and protective effects of aminothiols added to lymphocytes which are irradiated and cultured.

Summary and Conclusions.

During the past year several investigative methods and study models have been used to explore various aspects of the aminothiol group of radioprotective compounds. These studies are classified as mechanism of action studies and animal studies.

1. Mechanism of action studies.

In the Annual Report of 1966-67, evidence was presented and discussed concerning the mechanism(s) by which aminothiol treatment protects living organisms against the lethal effects of ionizing radiation. These included:

a. Induction of tissue anoxia through toxic pharmacological actions in animals.

b. Free radical scavenging in radiation damaged tissues.

c. Energy dissipation through preferential rupture of disulfide bonds between aminothiol and vulnerable biological molecules.

No animal studies have been performed in the Division of Nuclear Medicine to support or deny the first possible mechanism above.

During the past year, studies related to free radical scavenging and energy dissipitation have revealed that the interactions between biological tissues (molecules), ionizing radiation, and aminothiols are highly inter-related with oxygen effects. The relative lethality of oxygen in radiation resistant or sensitive strains of bacteria seems to be closely related to the radiation sensitivity. Furthermore, aminothiols protect against the lethal effects of oxygen under several conditions, such as freezing or freeze drying. Evidence is accumulating therefore that common mechanisms of biological damage exist in radiation injury, oxygen toxicity, freezing and drying. This relationship appears to be of fundamental importance, and studies are being conducted to define it more precisely.

We have initiated studies to examine in detail the relationship between chemical structure and biological function of these compounds. Synthesis of unavailable compounds has been initiated. Purity tests and purification methods for this family of compounds have been developed.

2. Animal studies.

In the Annual Report of 1966-67, evidence was presented that pre-treatment with aminothiols increased lethality of standardized trauma in mice. Studies have now demonstrated that simultaneous treatment with hexobarbital protects against lethality in aminothiol-treated, traumatized mice, but does not reduce radioprotection. These findings indicate that the deleterious effects of aminothiols in trauma can be differentiated from radioprotection. This interrelationship must be tested in other study models.

Studies of wound healing in irradiated rats have demonstrated that aminothiol treatment prior to irradiation produces a more normal wound healing pattern. Associated studies have demonstrated that wound healing is dependent upon bone marrow integrity. The beneficial effect of aminothiols in this model may depend upon their specific protection of bone marrow cells. Further studies are in progress.

Tissue culture studies have been severely hampered by personnel changes, but have continued. A carefully designed study has been performed to demonstrate that replicate human lymphocyte cultures can be made to produce nearly identical cell populations and lactic acid production at various times after initiation.

The system has been applied to radiation studies, and we have demonstrated a dose-response relationship between cell number at 96 hours of culture and radiation damage dosage delivered at initiation of culture.

In this tissue culture study model, chloroquine decreased glucose consumption and lactic acid production by replicating lymphocytes, but a chemical analog without antimalarial activity did not effect these measurements. Further studies are planned to explore the possible value of this model as a screening test for antimalarial drugs and the relationship between this chloroquine effect and antimalarial activity.

Publications.

1. Mahin, D. T., M. M. McLaughlin, C. R. Angel, R. D. Farris and K. T. Woodward. Tissue Distribution, Excretion and Duration of Radiation Protection of 2-(1-Decylamino) Ethanethiosulfuric Acid in Mice. Radiation Res. (Abstr.) 31: 566 (1967).

2. Swartz, H. M. and E. C. Richardson. Relation between Free-Radical Reduction and Survival in Irradiated E. coli B/r Treated with Aminothiols. Radiation Res. (Abstr.) 31: 547 (1967).

3. Swartz, H. M. and E. C. Richardson. A Correlation between Radiation-Induced Free Radicals and Survival in Microorganisms Exposed to Beta-Mercaptoethylamine under Oxygen or Nitrogen. Int. J. Rad. Biol. 12: 75-88 (1957).

PROJECT 3A63530 1D829
MALARIA PROPHYLAXIS

Task 01
Malaria Investigations

RESEARCH AND TECHNOLOGY RESUME

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. DOWNGRADING	8. RELEASE LIMITATION
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CC2600 BIOLOGY	07 66	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES ESTIMATE	PROFESSIONAL MAN-YRS	FUNDS (In Thousands)
C. IN-HOUSE	a. NUMBER NA	FY(PY) 68	3	100
	b. TYPE NA	FY(CY) 69	3	100
	c. DATE			
	d. AMOUNT NA			

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21. TECHNOLOGY UTILIZATION: FRACTIONATION OF ANTIGEN

22. COORDINATION: NA

23. KEYWORDS: MALARIA, PLASMODIUM, ANTIGEN, IMMUNITY, ERYTHROPHAGOCYTOSIS, AUTOIMMUNITY, DIAGNOSIS.

24.

(U) TECH OBJECTIVE - TO ISOLATE AND PURIFY VARIOUS PROTEIN ANTIGENS FROM PLASMODIUM BERGHEI AND P. KNOWLESI. TO CHARACTERIZE THESE SUBSTANCES IMMUNOCHEMICALLY, TO RELATE IMMUNOCHEMICAL CHARACTERISTS TO BIOLOGIC ACTIVITIES, SUCH AS PROTECTIVE IMMUNITY, DIAGNOSTIC SPECIFICITY, CROSS REACTIONS WITH NORMAL HOST TISSUE COMPONENTS, ETC.

(U) APPROACH- SEPARATE PARASITE PROTEINS BY PHYSICAL AND CHEMICAL MEANS. DETERMINE THE PRESENCE AND ACTIVITY OF METABOLIC ANTIGENS IN THE PLASMA OF ACUTELY INFECTED ANIMALS. ANALYZE THE FRACTIONATED PROTEINS WITH ANALYTICAL ULTRA-CENTRIFUGATION, POLYACRYLAMIDE GEL, ELECTROPHORESIS, IMMUNOELECTROPHORESIS, COMPLEMENT FIXATION, HEMAGGLUTINATION, AND FLUORESCENT ANTIBODY TESTS. STUDY ERYTHROPHAGOCYTOSIS IN SPLEEN SMEARS OF INFECTED AND CONTROL MONKEYS.

(U) PROGRESS - OCT 67 THRU JUN 68 THE P. BERGHEI ANTIGEN OBTAINED FROM THE FREEZE-THAW LYSATE OF INFECTED RAT OR MOUSE RED BLOOD COUNT IS OF GREATER THAN 65,000 MOLECULAR WT (ELUTES BEFORE HEMOGLOBIN ON SEPHADEX G-200 CHROMATOGRAPH) AND IS ANIONIC AT PHS 7.2 TO 8.2 AS DETERMINED BY GEAR CHROMATOGRAPHY AND PEVIKON BLOCK ELECTROPHORESIS. THE ANTIGEN AND/OR ANTIGENS ARE PRECIPITABLE IN AGAR WITH HYPERIMMUNE RAT ANTISERA AND CAN BE ATTACHED TO SHEEP RED BLOOD COUNT FOR USE IN THE STANDARD HEMOGLUTINATION TEST. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

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Project 3A63530ID029, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 406, Antigenic fractionation, serology of malaria

Investigators.
Principal: Elvio H. Sadun, Sc.D.
Associate: SFC Rufus W. Core; CPT Daniel J. Stechschulte, MC; Bruce T. Wellde, M.S.

Description.

The objective of this work unit is to isolate and purify various plasmodial antigens and to characterize them immunochemically in order to relate these characteristics to biological activities.

Progress.

1. A test for the mass diagnosis of human malaria using Plasmodium falciparum-parasitized erythrocyte lysates.

One of the most difficult problems in malaria control is that of recognizing asymptomatic patients with low grade parasitemias. For the past 50 years, many investigators have attempted to develop a practical diagnostic method for detecting malarial antibody when the parasites are so few that they cannot be readily found in thick blood films. This would be particularly important in 1) avoiding infections produced by blood transfusions through mass screening of potential blood donors, 2) preventing the reintroduction of malaria by nationals returning from malarious areas, international travelers in transit and servicemen returning from overseas military duty, and 3) screening of immigrants and migratory workers. In addition, knowledge of the antibody level in individuals or population groups exposed to malaria would be valuable in assessing the results of prophylactic and therapeutic measures.

Although several serologic technics have been developed since 1961, none of these is completely suitable for the purposes stated above. The most common shortcomings in existing methods are: 1) the lack of specificity and sensitivity which are related to the source and nature of antigen employed, 2) the relative unavailability of large quantities of human plasmodia, and 3) the requirements for costly and specialized equipment, expensive reagents, and well trained technical assistants.

When crude material obtained from infected organs or blood is used as antigen, a relatively low degree of specificity is to be expected. Elaborate technics for the systematic fractionation of crude parasite suspensions permitted the development of a highly specific hemagglutination

test and a complement fixation test. However, since large volumes of parasitized blood are needed to yield comparatively small quantities of purified antigen, it is unlikely that either of these tests for malaria could be applicable to mass screening of populations.

Perhaps the most practical and widely accepted test to measure circulating antibodies to malaria is the indirect fluorescent antibody technic (FAT). Using either Plasmodium vivax or P. falciparum as antigen, a reasonable degree of specificity can be achieved with the FAT in the laboratory diagnosis of human malaria. Although the FAT does not require a large amount of antigen, since individual parasites on a blood film are used to elicit the reaction, numerous slides containing sufficient numbers of parasites are necessary for the proper performance of the test. This poses an almost unsurmountable problem for laboratories which do not have ready access to infected patients.

The use of soluble antigens in an indirect fluorescent antibody (SAFA) test for the serodiagnosis of parasitic infections has been established in recent years. This technic obviates the need for maintaining the parasite in the laboratory where the tests are performed, permits the investigator to select and purify the antigen to be employed and provides a means of mechanical reading of the test.

Excretions and secretions (ES) of parasites have been used more commonly to produce acquired immunity than to diagnose infections. Sprent first used nondializable adult and larval ES antigens from Ascaris to study the anaphylactic sensitivity of guinea pigs infected with this worm. Sadun and Norman, using an ES antigen in the serologic diagnosis of trichinosis, concluded that this antigen conferred a greater sensitivity to the flocculation test than somatic antigens. Since then ES antigens have been employed successfully in the serologic diagnosis of several parasitic infections. These attempts lead Thorson to suggest that the "separation and exact characterization of either specific excretions or secretions or somatic antigens plus a concerted effort to compare exhaustively various components in all of the available serologic tests will lead to efficient tools for diagnosis." A considerable advantage provided by the use of ES antigens is the possibility of large collections of antigenic material. This advantage may remove what has been regarded heretofore as the main obstacle to the mass screening of populations for malaria.

With these considerations in mind, studies were set up to develop a test employing lysates of red blood cells obtained from splenectomized chimpanzees which had been experimentally infected with P. falciparum. A soluble antigen was obtained from this lysate in fractions separated by column chromatography in DEAE. This antigen conferred to a newly developed SAFA test a relatively high degree of sensitivity, specificity, and reproducibility.

Antigen preparation. Juvenile splenectomized chimpanzees (Pan satyrus) were infected by blood transfer with a drug resistant Malaysian (Camp.) strain of P. falciparum according to described methods. Approximately 400 ml of blood were withdrawn from each chimpanzee when parasitemia levels were higher than 20%. After a blood transfusion from uninfected donors, between 200 and 400 ml of blood were withdrawn again when a parasitemia of at least 20% was reached. By this method antigen sufficient for the performance of approximately 50,000 tests could be obtained from each experimentally infected chimpanzee. The blood obtained from P. falciparum- infected chimpanzees was collected in blood donor bags containing sufficient amounts of citrate glucose solution (ACD fluid), refrigerated at 4°C, centrifuged and the plasma removed. The packed cells were washed 3 times with chilled physiological saline and resuspended to 50% of the original volume. The 50% cell suspension in saline was frozen and thawed 3 times in a dry ice-alcohol bath. After centrifugation at 27,000 G for 30 minutes, the crude hemolysate was divided into several aliquots and stored at -70°C. Subsequently, this was dialyzed for 12-18 hours at 4°C with 0.01M phosphate buffer at pH 7.5. Chromatography of 5 ml of dialyzed lysate on diethylaminoethly (DEAE), Sephadex A-25 columns (2.5 x 47 cm) was performed by sequential elution with following phophate buffers: 0.01M pH 7.5, 0.04M pH 7.5, 0.08M ph 7.5, and 0.1M pH 6.5. Samples of 8-10 ml were collected at a rate of 1-1.5 ml per minute. The protein concentration of the eluate was determined by spectrophotometry at 280 mμ and protein peaks were determined by spectrophotometric reading of aliquots and pooled accordingly. Four distinct fractions were obtained and labeled consecutively from 1 to 4. Fraction 1 contained most of the hemoglobulin; fraction 2 contained only a trace of hemoglobulin, but it had little antigenic activity. Fraction 3 was antigenically active, but it produced more non-specific fluorescence than fraction 4. Therefore, fraction 4 was used in the test. The volume was estimated to contain 0.15 mg of protein and/or material absorbing light at 280 mμ per ml. Cellulose acetate filter paper discs, 4.7 cm in diameter and pore size of 0.45 μ, were soaked in the antigen for 1 minute, placed on blotting paper for a few seconds, dried at room temperature and then stored in a desiccant jar under vacuum until used. Antigen from normal chimpanzee erythrocyte lysate was prepared in the same manner.

Serum specimens. Human serum specimens from 581 selected individuals were tested. Of these sera 270 came from persons in whom malarial infection had been established by demonstrating P. falciparum, P. malariae, or P. vivax microscopically in thick blood smears. Of the sera from malarial infections 163 were obtained in a hyperendemic area in New Guinea, 53 were from Army veterans who contracted malaria in South Vietnam, and 54 were from volunteers who had been experimentally infected with P. falciparum or P. vivax 4-9 weeks prior to withdrawal of blood. The specificity of the reaction was determined with sera from individuals with proven viral, bacterial, or parasitic infection other than malaria. Normal control sera

were obtained from 112 healthy individuals who were undergoing physical examination as candidates for appointment to a military academy, from 16 human volunteers before exposure to malarial infection, and from 4 personnel of our laboratory.

Test procedure. Soluble antigen fluorescent antibody (SAFA) tests were conducted essentially as described previously. A negative control was diluted 1:10 with a Tris-Tween buffered saline (0.05M, 2-Amino-2-(hydroxymethyl)-1,3-propanodiol with 2% Tween 80 at pH 8.0 in 0.15M NaCl) and further diluted in serial two-fold dilutions to 1:80. The optimal dilution of conjugate was determined by box titrations. In most instances a 1:20 dilution of labeled antihuman globulin was used. Two-tenths ml of diluted sera was placed in the wells of a plastic tray and antigen discs (punched from the stored antigen-coated cellulose acetate paper, 7 mm diameter) were immersed in the serum for 45 minutes at approximately 60 rpm on a serologic slide rotator. The discs were then given 3 rinses of 10 minutes each in 0.05M Tris buffered saline before the addition of antiglobulin diluted 1:20 in 0.05M Tris-Tween buffered saline. The discs were rotated in this solution for 30 minutes at room temperature. They were again rinsed 3 times for 10 minutes in Tris buffered saline and then placed on a black masking tape at 1 cm intervals. Test results were read on the dial of a Model 111 Fluorometer (G. K. Turner Associates, Palo Alto, California) with a primary filter transmitting 254-420 mμ and a sharp cut secondary filter passing <520 mμ in combination with a 6.5% neutral density filter. On the basis of previous tests, arbitrary values were established for the interpretation of fluorometer dial readings. A reading of 8 or less was recorded as non-reactive. A reading of 9 or more was interpreted as reactive.

The results obtained with the SAFA test for malaria are summarized in table 1. The findings with sera from malaria patients illustrate the sensitivity of this procedure. The findings with sera from patients with other conditions and from healthy individuals provide an index of the specificity of the test. Positive reactions were observed with 7 of 13 leishmaniasis specimens, 4 of 12 trypanosomiasis specimens, and 3 of 9 hookworm specimens from patients living in areas where malaria is present. Positive reactions were also observed in 5 of 38 specimens from individuals with proven syphilis. Only one serum specimen from individuals with proven malaria failed to react in this test.

Table 1

Results Obtained in the Soluble Antigen Fluorescent Antibody Test for Malaria (581 specimens)

DIAGNOSTIC STATUS	NUMBER TESTED	NUMBER REACTING AT GIVEN TITER:				
		<10	10	20	40	>80
Falciparum malaria	148	1	12	17	23	95
Vivax malaria	94	0	5	9	9	71
Malariae malaria	28	0	0	2	1	25
TOTAL MALARIA	270	1	17	28	33	191
HEALTHY CONTROLS	132	129	3	0	0	0
INFECTIONS OTHER THAN MALARIA	179	156	9	8	4	2
Bacillary dysentery	4	4	0	0	0	0
Cholera	10	10	0	0	0	0
*Leprosy	8	6	0	2	0	0
Bacterial meningitis	16	16	0	0	0	0
Syphilis	38	33	5	0	0	0
Measles	9	9	0	0	0	0
Mumps	13	13	0	0	0	0
Amoebiasis	11	10	0	1	0	0
Toxoplasmosis	15	15	0	0	0	0
Filariasis	11	11	0	0	0	0
Schistosomiasis	10	9	1	0	0	0
*Leishmaniasis	13	6	1	4	0	2
*American trypanosomiasis	12	8	0	0	4	0
*Hookworm	9	6	2	1	0	0

*Obtained from areas where malaria is known to occur.

In order to obtain some information on the reproducibility of results, serum pools from infected and uninfected persons were divided into aliquots and tested at different times with the same lot of antigen and antiglobulin. As indicated in Table 2, the results were highly reproducible. None of the 23 specimens from proven infections gave negative readings. The degree of variability in titers never exceeded 2 fold. The antigen appeared to be stable for at least 6 months when frozen at -70°C and for at least 3 months on cellulose acetate filter paper discs kept at room temperature in a desiccator.

Table 2

Results of Repeated SAFA Tests on Three Sera with the Same Lot of Antigen

Serum	Number of Times Tested	Number of Times Given Titer Obtained				
		0	10	20	40	80
Infected High Titer	15	0	0	0	1	14
Infected Low Titer	12	0	3	8	1	0
Uninfected	27	0	0	0	0	0

Sera which reacted in the SAFA test with titers of 20 to >80 were selected for an absorption experiment to determine whether this is a true antigen-antibody reaction. A 0.1 ml aliquot of each undiluted serum specimen was incubated for 3 hours at room temperature and 12-18 hours at 4°C with 0.2 ml and 0.4 ml of the antigen. After absorption the sera were diluted with Tris-Tween buffered saline to a total volume of 1.0 ml to give a starting serum dilution of 1:10, and they were retested. The results (Table 3) indicate that sera absorbed with the homolysate either failed to react in the SAFA test or reacted with a marked reduction in titer. Conversely, incubation of sera with saline did not result in a detectable reduction in reactivity.

Table 3

Results of Absorption with *P. falciparum* Lysates

Serum Specimen	Amount of antigen used (ml)	Titer before absorption	Titer after absorption	Titer after incubation with saline
Infected (Pool No. 1)	0.2	>80	20	>80
Infected (Pool No. 2)	0.2	20	10	20
Normal Control	0.2	0	0	0
Infected (Pool No. 1)	0.4	>80	10	>80
Infected (Pool No. 2)	0.4	20	0	20
Normal Control	0.4	0	0	0

In order to determine whether the presence of iso-agglutinins in the lysate might account for some of the observed reactivity, anti-A, anti-B, and anti-Rh typing sera were tested against the lysate. Consistently negative results were obtained. Lysates from non-infected chimpanzee erythrocytes either failed to react or gave reactions at very low titers when tested against sera from persons with proven malaria.

The chief obstacle to large scale application of serodiagnosis in malaria has been the difficulty of obtaining sufficient quantities of malarial antigen. Although plasmodia from lower animals can be used to detect antibodies in human infections, their use as a source of antigen has resulted in marked loss of sensitivity or specificity. As a rule, large volumes of parasitized blood yield small quantities of somatic plasmodial antigen. However, this is not the case in the SAPA test employing infected red blood cell lysates which have been fractionated by sequential elution with chromatography on DEAE Sephadex A-25. This antigen may be a metabolic product of the parasites (excretions and secretions) or an altered host element which is released after the infected erythrocytes are lysed.

The results of the present studies using *P. falciparum*-parasitized erythrocyte lysates in the SAPA test indicate that this procedure possesses a high degree of sensitivity, specificity and reproducibility of results.

Whereas all but one of the specimens obtained from individuals with proven malarial infection reacted in this test at titers of 1:10 or higher, cross-reactions with sera from individuals with infections other than malaria were uncommon. Although some positive reactions were observed with sera from people with kala-azar and hookworm, these specimens were collected in Uganda, Brazil, and Panama where malaria also occurs. Five of 38 serum specimens from individuals with proven syphilis reacted at low titers although they were obtained from areas where malaria does not normally occur. These results were not surprising since cross-reactivity between malaria and syphilis has been reported repeatedly. This does not represent a serious obstacle to the utilization of this test for screening donors for blood transfusion banks, since people with a positive syphilis serology are not accepted as donors.

Our results of preliminary absorption studies indicate that the fluorescence of the lysates reacting with malarial antiserum no longer occurred when antibodies were removed. This is taken as suggestive evidence that the specific fluorescence is given by antigen-antibody precipitates resulting from a reaction between antibodies in the host serum and antigens released by the lysis of infected erythrocytes. Detailed studies are now being conducted in an attempt to characterize this antigen and to determine its ability to react in the hemagglutination test.

Although no precise information is as yet available on the time-course development of antibodies detected by this test, preliminary studies conducted in human volunteers infected with P. vivax or P. falciparum, either by blood passage or by sporozoite inoculations, indicated that the appearance of antibodies occurred at approximately the same time as patent parasitemias. These patients are being followed over a long period of time after infections were aborted by chemotherapeutic means to determine the course of antibody levels following parasitological cure.

Antigens prepared from cultures of human malarial parasites and from the serum of P. knowlesi-infected monkeys have been used in the complement fixation test. Torrey and Kahn and Corwin, et al., isolated serum antigens from the plasma of ducks infected with P. lophurae. Todorovic and his co-workers found that a serum-soluble antigen of Plasmodium gallinaceum cross-reacted widely with antisera from human, simian, rodent, and bird malarias in the latex agglutination test. Numerous attempts have been made to induce protection by immunizing animals with excretions, secretions and metabolic products of various parasites. In addition to the studies conducted with plasmodia, soluble serum antigens were found to engender protection against challenge in other hemotropic infections such as babesiosis and trypanosomiasis. Gray showed that sera from rats infected with trypanosomes contained soluble serum antigens which engendered protection against challenge. The use of ES antigens in the production of acquired immunity in helminths has also been reported. Studies are now being conducted to determine whether the lysates obtained from P. falciparum-infected erythrocytes are capable of stimulating protection against challenge with the homologous species.

The value of a serologic procedure is based not only on test specificity, sensitivity, and reproducibility, but also on the ease with which many specimens can be processed. The use of lysate antigen in the SAFA technic makes it possible to conduct a large number of tests with great economy of scarce parasite material. Preliminary observations indicate that approximately 50,000 screening tests can be performed with the amount of antigen normally collected from one infected chimpanzee. The use of a soluble antigen opens the way to further purification procedures which might increase the sensitivity and specificity of the test. Moreover, the SAFA test permits objective mechanical reading of results, which compensates for any nonspecific fluorescence contributed either by the serum or by free fluorescein in the conjugated antiglobulin. Since this test would lend itself to automation, one can envision the use of a simple apparatus to process numerous specimens semi-automatically in a relatively short time. If subsequent comprehensive studies support these observations and delineate clearly the relative efficiency of the test, the SAFA technic using P. falciparum-parasitized erythrocyte lysates may be well suited as a screening procedure in investigations of the serocpidemiology of malaria and for the mass screening of potential blood donors.

2. Detection of malarial antibodies by an indirect hemagglutination test employing a soluble antigen fractionated from the lysate of parasitized red blood cells. Antigen(s) fractionated by column chromatography and pevikon block electrophoresis from the freeze-thaw lysate of parasitized RBC's were used to detect malarial antibody in rats and humans. Extracts from normal RBC's prepared by the same methods showed no reactivity with either immune or normal sera. Ouchterlony studies with fractions from infected RBC's showed precipitin lines while preparations from non-parasitized RBC's did not. Antisera from P. berghei infected rats tested at intervals for 150 days after infection by indirect hemagglutination with P. berghei antigen showed a fluctuating antibody response when compared with the fluorescent antibody test using whole organisms (Fig. 1). The hemagglutination test was modified using the microtiter system, and antigen prepared from P. falciparum-parasitized chimpanzee cells was tested against sera from patients infected with malaria. Forty of 50 sera from patients with parasitologically proven malaria were positive at titers ranging from 1:20 - 1:10, 240. Normal human sera did not react above a titer of 1:20. No reactions were obtained with sera from patients with either syphilis, schistosomiasis, leishmaniasis or leprosy. Reactions were obtained with Onchocerca volvulus sera. However, these sera were obtained in areas where malaria is endemic.

GRAPHIC NOT REPRODUCIBLE

Summary and Conclusions.

1. A soluble antigen fluorescent antibody test was developed for the serologic diagnosis of human malaria by using Pladmodium falciparum-parasitized erythrocyte lysates from experimentally infected chimpanzees as antigen. The high degree of sensitivity, specificity, and reproducibility of the test, the objectivity of mechanical reading, the availability of large amounts of P. falciparum antigen and, the possibility of processing many specimens semi-automatically in a relatively short time suggest that this technic may be well suited as a screening procedure in investigating the sero-epidemiology of malaria and for the mass screening of potential blood donors.

2. An indirect hemagglutination test using parasitized RBC lysate as an antigen detected malarial antibody in rat and human sera. Antisera from rats showed a fluctuating antibody response when compared in the fluorescent antibody test using whole organisms as antigen. A hemagglutination test using the microtiter system and infected chimpanzee-blood lysate as antigen showed titers of 1:20 to 1:10240 with sera from human patients.

RESEARCH AND TECHNOLOGY RESUME

3. DATE OF RESUME	4. KIND OF RESUME	5. SECURITY	6. GRADING	7. CLASS & LIMITATION	8. COUNTRY OF PERFORMANCE
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20. GOVT. LAB/INSTALLATION/ACTIVITY	21. PERFORMING ORGANIZATION
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24.

(U) TECH OBJECTIVE - ISOLATION AND PURIFICATION OF MALARIA ANTIGENS AND INVESTIGATION OF IMMUNE RESPONSE. DEVELOPMENT OF METHODS FOR EVALUATING IMMUNOPATHOLOGIC RESPONSE TO INFECTION. ANTIGENS EVALUATED FOR DIAGNOSTIC ABILITY AND FOR IMMUNOGENIC PROPERTIES. IMMUNOPATHOLOGIC RESPONSE APPLIED TO STUDIES ON HOST PARASITE RELATIONSHIPS. THIS IS A CONTINUATION OF WORK INITIATED UNDER DA 040496, CODE NO. 61103011 3A03103011A91C 01 114.

(U) APPROACH- CF AND FA TECHNICS ARE USED IN CONJUNCTION WITH ANTIGEN PURIFICATION METHODS. OSMOTIC FRAGILITY OF ERYTHROCYTES AND C-PRIME LEVELS ARE USED AS CRITERIA FOR APPRAISING IMMUNOPATHOLOGIC RESPONSE IN HOST. TECHNICAL PROBLEMS INCLUDE LIMITED AVAILABILITY OF PARASITE MATERIAL AND SEPARATION OF PARASITES FROM BLOOD COMPONENTS.

(U) PROGRESS - OCT 67 THRU JUN 68 STUDIES ON A LYTIC FACTOR (LF) AND COMPLEMENT FIXING ANTIGENS OBTAINED FROM ERYTHROCYTE-FREE PLASMODIA HAVE BEEN CONTINUED. KINETIC STUDIES ON LF-INDUCED HEMOLYSIS OF ERYTHROCYTES OF VARIOUS ANIMAL SPECIES SHOWED CONSIDERABLE VARIATION OF SUSCEPTIBILITY TO LYSIS. HAMSTER, CHIMPANZEE AND MONKEY CELLS MOST SUSCEPTIBLE, HUMAN CELLS MODERATELY SUSCEPTIBLE, CHICKEN AND SHEEP CELLS QUITE RESISTANT TO LF ACTION. ISOTOPE-TAGGED HAMSTER ERYTHROCYTES BEING USED TO DETERMINE WHETHER CELL AGE INFLUENCES LF ACTIVITY AND ACCOUNT FOR FAILURE TO OBTAIN COMPLETE HEMOLYSIS. QUALITATIVE CHEMICAL TESTS INDICATED LIPIDS AND PROTEIN-LIKE MATERIAL MAJOR COMPONENTS OF LF. TLC ANALYSES OF LIPID FRACTION REVEALED TRIGLYCERIDES, CHOLESTEROL, FREE AND ESTERIFIED FATTY ACIDS, AND PHOSPHOLIPIDS. PROTEIN-LIKE COMPONENTS PROBABLY PEPTIDES BECAUSE OF LOW MW. FURTHER FRACTIONATION AND CHARACTERIZATION OF COMPONENTS IN PROGRESS. CF ANTIGENS FROM SIMIAN AND HUMAN PLASMODIA BEING USED TO EVALUATE SERA FROM IMMUNE INDIVIDUALS RESIDING IN HIGHLY ENDEMIC AREA, MANY HAVING MIXED INFECTIONS. CF ANTIGENS FROM P. KNOWLESI AND P. FALCIPARUM PARASITES PROVED HIGHLY EFFECTIVE FOR DEMONSTRATING AND/OR DIFFERENTIATING RECENT OR CURRENT VIVAX AND FALCIPARUM MALARIA. POTENTIAL OF P. FIELDI ANTIGEN FOR DETECTING P. MALARIAE INFECTION UNDER INVESTIGATION. STUDIES ON CROSS REACTIVITY PATTERNS OF VARIOUS PLASMODIA BEING CONTINUED IN EFFORT TO FURTHER EXTEND SERODIAGNOSTIC CAPABILITIES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 107, Malaria antigens

Investigators.
Principal: Earl H. Fife, Jr., M.S.
Associate: William M. Bigelow, B.S.; Albert E. von Doenhoff, Jr., B.S.

Description.

This work unit is concerned with the isolation and characterization of plasmodial antigens, studies on the antigenic structure of various plasmodia, investigations of serologic patterns developed during the course of infection, and elucidation of immune mechanisms associated with this disease. In vitro as well as in vivo methods are employed. In vitro methods are used in (1) development of procedures for separating malaria parasites from host blood components; (2) isolation, purification and identification of plasmodial antigens by physico-chemical and serologic methods; and (3) development, improvement and evaluation of serologic procedures for detection of antibodies and for following antibody patterns in infected hosts. In vivo studies include (1) the role of antigen and antibody in certain immunopathologic conditions associated with malaria infection; (2) production of specific antibodies to characterize experimental antigen fractions and to investigate the antigenic relationships of various species of Plasmodium; and (3) investigations on the immunogenicity of the purified antigen fractions with particular emphasis on their potential value as vaccines.

Progress.

1. Isolation and fractionation of serologically active malaria antigens. A new method for effectively separating malaria parasites from host erythrocyte components has been described in previous reports on this Work Unit (WRAIR Research & Development Reports, 1966, 1967). In this procedure, selective fragmentation of the red cell membrane is achieved by passing the parasitized cells through a French pressure cell under controlled pressures. During the present reporting period, the methodology has been further improved and the application of the technic extended for the isolation of additional species of Plasmodium. The standard procedure, including recent innovations, is as follows: Blood from experimentally infected animals showing 20-60 per cent parasitemia is collected in heparin and the erythrocytes washed three times in 0.9% NaCl solution to remove host plasma components. The packed, washed cells then are diluted in 0.9% saline to give a 20% suspension. The cells suspension is passed slowly through the French pressure cell at 1500-2000 psi and the effluent centrifuged at 50 rcf for 10 minutes to remove gross debris. The supernate containing the intact parasites then is centrifuged at 3,000 rcf for 5 minutes, and the parasite sediment washed three times in 0.9% saline to remove

residual red cell fragments. The washed parasites constitute the parent material for studies on plasmodial antigens and lytic factor. It is noteworthy that the plasmodia suffer little or no damage during this process. The parasites retain their infectivity for susceptible hosts and show no morphological abnormalities. It was previously suggested that this technic also might be employed for separating blood parasites other than malaria from host red cell components. This proved to be the case with Anaplasma marginale-infected cattle cells. Investigators at the USDA laboratories recently used the above technic for obtaining large numbers of erythrocyte-free Anaplasma. The complement fixing and hemagglutinating antigens prepared from this harvest were far superior to those prepared from harvests obtained by the usual methods employing chemical or hypotonic lysis, and led to the development of highly specific, sensitive serodiagnostic tests for anaplasmosis.

Current military operations in P. malariae endemic areas in the Far East have created the need for reliable serodiagnostic tests for P. malariae infection. Moreover, the occurrence of idiopathic splenomegaly among certain Vietnamese population groups and the possible role of P. malariae as the etiologic agent of this disease further emphasize the importance of developing this serodiagnostic capability. In view of the inability to obtain from human cases the quantities of parasites required for antigen production, efforts were made to select a simian Plasmodium that would give strong cross reactions with P. malariae antibodies, but show mimimum reactivity with P. vivax and P. falciparum antibodies. Collins et al (Am. J. Trop. Med. & Hyg., 15 : 11, 1966), using immunofluorescence technics, presented data suggesting that P. fieldi and/or P. brasilianum might fulfill these requirements. P. fieldi was selected for the initial studies because it could be cultivated in readily available splenectomized Rhesus monkeys. Use of P. brasilianum, on the other hand, would require special procurement of spider monkeys. Although the parasitemias in P. fieldi infections were relatively low (ranging 7-10%), the volume of parasites required for antigen fractionation eventually was obtained by pooling the harvests from several animals.

Initial experiments with P. fieldi-infected blood revealed that this species of Plasmodium was considerably more fragile than the P. knowlesi and P. falciparum employed in previous studies. Use of the standard pressure (1500-2000 psi) for freeing the parasites from the erythrocytes destroyed a large number of the plasmodia. Further investigations revealed that this problem could be overcome by reducing the pressure to 500-1000 psi. Under these latter conditions, the parasites showed normal morphology but some intact erythrocytes were present in the effluent from the pressure cell. As a result of these red cell contaminants, the crude parasite extract was unsatisfactory for use in CF tests. The red cell products, however, were effectively separated from the complement fixing antigens by filtration through a Sephadex G-200 gel column and the quality of the fractionated P. fieldi antigen appeared to be comparable to that of the purified P. knowlesi and P. falciparum antigens. The P. fieldi antigen gave strong reactions

in CF tests with homologous antiserum and was not anticomplementary at any concentration tested.

Certain features of the P. fieldi infection in splenectomized Rhesus monkeys were strikingly different from the clinical disease and pathology observed in infections with other malaria parasites. In P. fieldi infections, the parasitemias always were relatively low, rarely exceeding 10%. Nevertheless, the animals showed progressive debilitation and usually expired 14-20 days after infection. The hematologic picture also was unusual. All animals showed a marked monocytosis with a significant amount of phagocytosis of parasitized and non-parasitized erythrocytes by the monocytes. Moreover, the monkeys regularly developed a severe anemia during the terminal phase of the disease and the majority of parasitized cells showed unusual bizarre morphology at this time. The severe clinical disease and marked hematologic abnormalities associated with relatively low patent parasitemias suggest that P. fieldi infection in splenectomized Rhesus monkeys might provide a useful model for studying various aspects of host-parasite relationships in malaria in general.

The French pressure cell also was used to isolate P. berghei from the erythrocytes of experimentally infected mice. However, initial studies revealed that mouse erythrocytes were more resistant to mechanical damage than were monkey or chimpanzee red cells, and a considerable number of intact erythrocytes were present in the effluent from the pressure cell when the standard (1500-2000 psi) pressure was employed. Further studies revealed that this problem could be overcome by increasing the pressure to 2500 psi. Essentially all of the erythrocytes were ruptured under these conditions and there was no evidence of damage to the parasites. Complement fixing antigens prepared from these harvests reacted well with homologous antisera and were not anticomplementary.

2. Serodiagnostic tests for malaria. The potential value of purified P. knowlesi and P. falciparum antigens for the serodiagnosis of vivax and falciparum malaria respectively, was indicated in the preliminary evaluations summarized in the previous report on this Work Unit (WRAIR Research & Development Report, 1967). These antigens have been further evaluated in collaborative studies with members of the U.S. Army Medical Research Team (WRAIR) Vietnam. A preliminary evaluation of the potential of P. fieldi antigen for serodiagnosis of malariae malaria also was included.

These studies were conducted on two population groups residing in areas highly endemic for malaria. The first group consisted of Montagnard soldiers, many of whom regularly showed patent parasitemias of P. falciparum but no clinical disease. These subjects were selected to determine the incidence of malaria in an indigenous semi-immune population, to investigate the relation of the disease to combat operations, and to evaluate the patterns of antibody response in such a population receiving weekly suppressive doses of chloroquine. In these studies, 3-5 serum specimens were collected at 4-6 week intervals from each of

54 Montagnard soldiers. In all, a total of 224 specimens were tested. The observation period was designed to permit clinical, parasitological and serological examinations of these individuals just prior to deployment in combat operations immediately upon return to base camp, and a follow-up prior to redeployment to combat. The clinical, epidemiological and parasitological findings will be included in the Annual Research & Development Report prepared by the Medical Research Team. Although evaluation of all data has not been completed at this time, certain relationships between the parasitological and serological findings are noteworthy. These results are summarized in Tables 1 and 2. The superiority of the complement fixation test over thick blood film examination for appraising the malaria experience of an individual or a population group as a whole is clearly indicated. On the basis of CF test results, it is readily apparent that there was an exceedingly high incidence of P. falciparum infection among the group studied. Of the 54 individuals examined, 51 (94.4%) reacted with the falciparum antigen. Among these, 27 (50.0%) appeared to have only P. falciparum malaria whereas 24 (44.4%) showed evidence of mixed falciparum and vivax infections. One individual apparently had only P. vivax malaria and 2 presumably were not infected. These findings are in marked contrast to those obtained with the thick blood smears. With the latter method, P. falciparum was demonstrated in 10 (18.5%) of the subjects, P. vivax in 12 (22.2%) and mixed infections in 3 (5.6%). It should be noted that no malaria parasites were found in the smears on 29 (53.7%) of this group. Although antibodies were detected in many individuals whose thick smears contained no demonstrable malaria parasites, in no instance did an individual show parasites in the thick film and fail to give a reaction with the homologous antigen in the complement fixation test. As a point of further interest, it was observed that with one exception, all individuals showing only P. vivax on thick film examination had high antibody titers against the P. falciparum as well as P. knowlesi complement fixing antigens, indicating mixed falciparum and vivax infections. These findings raise a question concerning whether the asexual development of P. falciparum in a semi-immune individual is suppressed by superinfection with P. vivax. Studies are continuing to obtain further information along these lines. It is unlikely that the observed discrepancies between the thick blood films and the serologic tests are due to nonspecific reactions in the latter. Experience thus far has shown that there is very little cross reactivity between the knowlesi antigen and falciparum antibody or between the falciparum antigen and vivax antibody. Moreover, when reactions were observed with either or both antigens, titers well exceeding the cross reactivity level usually were observed. In view of the apparent limitations of thick blood film examination for demonstrating malaria parasites in individuals undergoing suppressive chemotherapy, a question is raised concerning the advisability of using the thick film alone for screening troops returning from malaria endemic areas. It is suggested that a combination of thick film examination and serologic testing would provide a more critical appraisal of the malaria status of such individuals.

Another group of sera from native Vietnamese was composed of speci-

Table 1
Results of Malaria Complement Fixation Tests
on 54 Montagnard Soldiers

Antigen	Number reacting with indicated antigen	
P. falciparum	27 (50.0%)	
P. knowlesi*	1 (1.9%)	
P. falciparum & P. knowlesi*	24 (44.4%)	2 (3.7%) of the subjects did not react with either antigen

*Used for detection of P. vivax antibody.

Table 2
Results of Malaria Thick Smears on
54 Montagnard Soldiers

Parasite	Number Positive	
P. falciparum	10 (18.5%)	
P. vivax	12 (22.2%)	
P. falciparum & P. vivax	3 (5.6%)	No parasites were found in smears on 29 (53.7%) subjects.

mens from individuals with idiopathic splenomegaly of unknown etiology. Kala azar was excluded on the basis of formol-gel tests and bone marrow aspirates. Since P. malariae was endemic in areas where the splenomegaly occurred, consideration was given to the possibility that the disease was similar to the P. malariae-induced "big spleen" syndrome observed in East Africa. Sera from 28 individuals with idiopathic splenomegaly were tested in complement fixation tests for malaria, using antigens prepared from P. knowlesi, P. falciparum and P. fieldi; on the basis of the experience of Collins et al, using immunofluorescence technics, it was anticipated that P. fieldi antigens could be used for detection of P. malariae antibodies. Results of tests with the P. fieldi antigen, however, were disappointing. Sera from 9 of these individuals contained vivax antibodies, and 21 showed reactivity with the falciparum antigen. On the other hand, only 2 reacted with the P. fieldi antigen. Two possible explanations could account for these findings. Either the purified P. fieldi was unsatisfactory for detecting P. malariae antibodies, or there was relatively little malariae infection among the group, and the idiopathic splenomegaly therefore was due to something other than P. malariae malaria. Although there was a high incidence of falciparum infection and some vivax malaria among the group, it is unlikely that either could have been the cause of the splenomegaly since the disease does not occur in other areas endemic for vivax and falciparum malaria. To resolve this question, efforts are being made to obtain sera from documented cases of P. malariae infection in order to precisely determine the efficacy of P. fieldi antigen for detecting malariae antibody. If subsequent studies indicate that the purified P. fieldi antigen does not fulfill the expectations based on the results of immunofluorescence tests, investigations will be initiated to determine the feasibility of using P. brasilianum antigens for the serodiagnosis of P. malariae infection.

3. Preservation of malaria parasites and antigens. The requirement for relatively large volumes of antigen for proposed comprehensive serological evaluations and the periodic availability of parasitized blood from experimentally infected animals have made it necessary to prepare and store parasite harvests and antigens for extended periods of time. Studies have been continued to gain further information concerning the optimal conditions for preservation and storage of these products.

a. Parasites. Previous studies showed that parasite harvests could be preserved for a number of months by storage in the frozen state at -60°C or by lyophilization in the presence of polyvinyl pyrollidone (PVP). However, critical experiments to determine the optimal conditions for preservation and storage were not conducted. Since PVP appeared to effectively stabilize the parasite material, the relative stability of parasites stored in 1%, 2% and 5% PVP at 4° and -60°C was investigated. In addition, lyophilization in the presence of the concentrations of PVP also was evaluated. For these studies, the desired concentrations of PVP were prepared in 0.9% NaCl solution and used to make 20% suspensions of the parasites. These suspensions then were stored at 4°, -60°C, or were lyophilized. It was observed that parasite harvests containing 2% PVP could be stored for more than 1 year without

deterioration of antigen components. Parasites stored with other concentrations of PVP, or at the higher temperature (4°C), deteriorated within a relatively short period of time. Parasites in 2% PVP also were very stable after lyophilization. However, difficulty was encountered in keeping the material frozen during the drying process, and parasites that thawed proved to be unsatisfactory for antigen preparation. The use of 2% PVP and storage at -60°C therefore was adopted as the standard procedure for preserving and storing parasite harvests.

b. Antigens. Incorporation of 2% PVP in 0.9% saline was necessary to stabilize the antigen during storage either in the frozen state (-60°C) or by lyophilization. In contrast to the experience with the parasite harvests, the antigens showed no tendency to thaw during lyophilization and the desiccated products were stable for more than one year. It was further noted that the sensitivity of lyophilized antigen consistently was greater than that of a fresh or frozen preparation. Comparison of fresh and lyophilized antigens in tests with homologous antiserum revealed that the lyophilized antigen consistently gave a two-fold or greater antibody titer than that obtained with the freshly prepared antigen. On the basis of these findings, incorporation of 2% PVP followed by lyophilization was adopted as the standard procedure for the preservation of malaria antigens.

4. Adaptation of the complement fixation test procedure to the Microtiter system. In view of the large numbers of tests involved in comprehensive serological evaluations and because of the expense and labor associated with the preparation of purified malaria antigens, studies were initiated to determine whether the standard complement fixation test procedure could be adapted to the Microtiter system. This seemed entirely feasible since the standard procedure was designed to use equal volumes of the 5 essential reagents in complement fixation. Results obtained with the standard volume test and the Microtiter procedure were compared in parallel tests on a number of malaria sera. No significant differences in sensitivity or reproducibility were observed. On the basis of these findings, the Microtiter technic was used in subsequent serological studies on malaria. The Microtiter system has two inherent advantages over the standard test tube method: 1) it reduces the reagent volume requirements by at least 75%, and 2) it greatly facilitates preparation of the numerous serum dilutions required in determining the antibody titers of large numbers of sera.

5. Isolation and characterization of a lytic component of P. knowlesi. During investigations on the fractionation of malaria antigens on Sephadex gel columns, it was observed that certain low molecular weight effluents from the columns possessed lytic properties. Preliminary observations on the physical properties and functional activity of this lytic factor (LF) have been presented in previous reports on this work unit (USAMC Research & Development Reports, 1966, 1967). In view of the possible role of the LF in the host-parasite relationships and pathology in malaria infections, studies have been continued to

further characterize the factor and investigate its functional activities. Basic improvements have been made in the technic for quantitative assay of LF activity. In the original assay procedure, a 20% suspension of sheep red cells was used as the indicator system for determining the lytic activity of a given LF preparation. Ancillary investigations, however, revealed that hamster erythrocytes were considerably more susceptible to LF-induced lysis than were sheep red cells. These findings suggested that hamster erythrocytes might provide a more sensitive measure of LF activity than could be obtained with the more resistant sheep cells. Subsequent experiments showed that this was the case, and the assay procedure accordingly was modified to use hamster rather than sheep erythrocytes as the indicator. Further studies on the optimal concentration of erythrocytes revealed that the sensitivity and reproducibility of the assay procedure was improved by using a 40% rather than 20% suspension of red cells. Details of the procedure currently used for quantitative assay of LF activity are as follows: First, a 40% suspension of the washed hamster red cells is prepared in 0.9% NaCl solution. The cells suspension then is standardized spectrophotometrically by adjusting the concentration of the suspension so that the lysate of a 1:200 dilution in distilled water gives an optical density of 0.500 ± 0.0100 at wavelength 540 mμ in the Beckman DU Spectrophotometer using a standard 1-cm cuvette. Standardized suspensions prepared in this manner contain approximately 1.8×10^9 erythrocytes per 0.4 ml. The standardized cells suspension is used to construct a hemoglobin curve to facilitate conversion of optical densities to number of cells lysed. LF assays are performed by combining 0.4 ml of the standardized cells suspension with an equal volume of LF and incubating at 37°C for 24 hours in stoppered tubes with periodic agitation. After incubation, the tubes are centrifuged at ca. 1300 ref for 10 minutes and the supernates transferred to another set of tubes. Each supernate then is diluted 1:100 in 0.9% saline and the optical densities determined with the Beckman DU Spectrophotometer at wavelengths 410, 540 and 575 mμ. Readings at 410 mμ are included to cover the possibility that the lysates are a mixture of methemoglobin and oxyhemoglobin rather than being composed primarily of oxyhemoglobin. The optical densities finally are compared with the standard hemoglobin curve and the number of cells lysed in each test is estimated.

The improved assay procedure has been used to further study the kinetics of LF-induced hemolysis. In general, the preliminary findings obtained with the original assay procedure have been corroborated with the more sensitive improved assay method. It has been demonstrated that the temperature and time of incubation as well as concentration of LF all influence the hemolytic activity of the factor. However, regardless of the temperature or LF concentration, little or no hemolysis occurred during the first 4 hours of incubation. On the other hand, hemolysis progressed in a linear fashion after the initial lag phase, and spontaneously terminated after incubation for 21 hours even though some intact red cells remained in the reaction mixture. These two phenomena, the initial lag phase and the spontaneous termination of lysis, are being further investigated. The pre-lytic lag phase suggests that a

"pre-conditioning" action of some nature must occur before lysis can take place. Arrangements are being made to determine whether electron photomicrographs of cells sampled during various periods of the lag phase will reveal damage to certain elements of the red cell membrane and provide an insight concerning the functional activity of the LF during the pre-lytic lag period.

It was originally postulated that the spontaneous termination of lysis after incubation for 21 hours was due to depletion of lytic factor in the test. However, this proved not to be the case. Unlysed cells remaining after the 21 hour incubation were washed and reincubated with fresh, active LF. No further hemolysis occurred. These findings indicated that the cessation of hemolysis was not due to depletion of the LF, but rather, suggested that the factor selectively lysed certain groups of the erythrocyte population. To test this hypothesis, young and old erythrocytes were specifically labeled with radioactive tracers and incubated with LF to determine whether one group was more susceptible than the other to LF-induced hemolysis. In these experiments, 10 young hamsters were inoculated intraperitoneally with Fe^{55}. The animals then were reinoculated 28 days later with Fe^{59}. Three days after the last injection, the animals were exsanguinated and the red cells used in the standard LF assay procedure. Under these conditions, the older cells will bear the Fe^{55} label and the newly formed cells will be tagged with the Fe^{59}. After incubation for 24 hours, the tests (with LF) and the controls (without LF) were centrifuged and the supernates examined in the scintillating counter to determine the relative levels of Fe^{55} and Fe^{59} in the tests and controls. LF-induced lysis resulted in a significant increase in the Fe^{55} count indicating that the older cells were more susceptible to action of the LF than were the newly formed cells. It is suggested, therefore, that the observed spontaneous cessation of hemolysis was due to depletion of the population of older cells in the test mixture. These radioactive tracer studies were conducted in collaboration with members of the Department of Hematology, WRAIR.

6. Physicochemical analyses of plasmodial lytic factor. Physicochemical analyses of the lytic factor obtained from malaria parasites have been continued. The findings to date are summarized as follows: Based on its filtration rate through a Sephadex G-200 gel column, the LF appears to have a molecular weight of less than 5000. Spectrophotometric analysis at wave length 280 mμ revealed an absorption band indicative of protein or protein-like component, and quantitative chemical tests (Lowry method) showed that the component, if protein, was present at a concentration of 35μg/ml. However, in view of the low molecular weight of the LF, it likely that this component consists of amino acids and/or small peptides rather than protein per se. A small amount of carbohydrate (12.5 μg/ml) was detected by the Shetlar and Masters technic. The principal component of the LF appeared to be lipid in character, with a relatively high cholesterol content. Thin layer chromatography of the LF has revealed the presence of a variety of lipids belonging to the following classes: phospholipids, free fatty acids, cholesterol, cholesteryl esters, and triglycerides. Studies are being continued to identify the individual components within these various classes and if possible, demonstrate a relationship of one or more of these components to the functional activity of the LF.

Summary and Conclusions.

1. The recently described method for separating plasmodia from erythrocytes by preferential fragmentation of the red cell membranes under controlled pressure has been successfully used for the isolation of a variety of species of Plasmodium. It was noted that the optimal pressure may vary for different parasites and hosts. Procedures now have been developed for the isolation of P. knowlesi and P. fieldi from Rhesus monkeys, P. falciparum from chimpanzees, and P. berghei from mice. Parasite harvests obtained in this manner have provided an excellent source of material for studies on plasmodial antigens and lytic factor.

2. The efficacy of P. knowlesi and P. falciparum antigens for the serodiagnosis of vivax and falciparum malaria respectively was evaluated with a large group of sera from individuals residing in an area highly endemic for malaria. Many of these semi-immune individuals had occult infection with negative thick blood smears. Nevertheless, essentially all of these individuals showed high antibody titers in the CF tests with P. falciparum antigen. In addition, mixed falciparum and vivax infections were revealed in a number of cases in which only a single species of Plasmodium, or no parasites at all, could be demonstrated in the thick film. The findings indicate that the CF test is far superior to the thick blood film technic for appraising the incidence of falciparum and vivax malaria in a semi-immune population residing in a highly endemic area and receiving suppressive chemotherapy. Attempts to use purified P. fieldi antigen for detecting P. malariae infection thus far have been unsuccessful even though P. fieldi parasites have been shown to give strong cross reactions with P. malariae antibodies in FA tests.

3. Studies on the optimal conditions for preservation and storage of parasite harvests and antigens have been continued. Optimal conditions for preserving the parasite harvests entailed preparation of a 20% parasite suspension in 2% PVP and storage in the frozen state at -60°C. No deterioration was noted after storage for more than one year. Lyophilization was contraindicated because the product tended to thaw during the drying process. Incorporation of 2% PVP followed by lyophilization proved to be the best method for preserving the purified antigens.

4. The standard complement fixation procedure was adapted to the Microtiter system. This effected a significant saving in the amount of antigen required for large scale serodiagnostic testing and greatly facilitated preparation of the serum dilutions required for antibody titrations.

5. Investigations on the functional activity of the plasmodial lytic factor (LF) and kinetics of the LF-induced hemolysis were continued. A more sensitive quantitative procedure for assaying LF activity was developed and reported in detail. Temperature and time of incubation as well as concentration influenced hemolytic activity of the LF. An initial

4-hour lag phase (without hemolysis) always occurred and was independent from temperature or LF concentration. Hemolysis spontaneously terminated after incubation for 21 hours even though intact cells remained in the mixture. Studies using young and old erythrocytes specifically tagged with Fe^{59} and Fe^{55} respectively revealed that the older cells were considerably more susceptible to LF-induced hemolysis than were the newly formed cells. It is suggested that the spontaneous cessation of lysis could be attributed to depletion of the population of the older cells in the system.

6. Studies on the physicochemical properties of the plasmodial LF were continued. The LF has a molecular weight of less than 5000 and contains some protein-like components, probably amino acids and/or small peptides. The principal component appears to be lipid in character and is comprised of phospholipids, free fatty acids, cholesterol, cholesteryl esters, and triglycerides.

Publications.

George, J.N., Wicker, D.J., Fogel, B.J., Shields, C.F., and Conrad, M.E. Erythrocytic Abnormalities in Experimental Malaria. Proc. Soc. Exper. Biol. & Med., 124 : 1086, 1967.

RESEARCH AND TECHNOLOGY WORK UNIT

1. DATE OF SUMMARY	2. KIND OF SUMMARY	3. SECURITY CLASSIFICATION	4. GOVT. ACCESSION NO.	5. AGENCY ACCESSION NO.	6. PROJECT, TASK, WORK UNIT NOS.
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18. GOVT LAB/INSTALLATION/ACTIVITY
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24. (U) TECH OBJECTIVE - BASIC BIOCHEMICAL ACTIVITIES OF MALARIAL PARASITES WILL BE STUDIED TO PROVIDE FURTHER DATA FOR EVALUATION OF DRUG-RESISTANCE OF PLASMODIUM FALCIPARUM. ANALOGUES OF ANTIMALARIAL DRUGS WILL BE SYNTHESIZED WHERE NOT AVAILABLE FOR BIOCHEMICAL STUDIES.

(U) APPROACH- PLASMODIUM BERGHEI WILL BE USED AS A TEST ORGANISM TO EVALUATE INTERMEDIARY METABOLISM OF THE MALARIAL PARASITE AND RESPONSES TO ANTIMALARIAL AGENTS. SPECIAL SYNTHESIS OF ADDITIONAL ANTIMALARIAL COMPOUNDS WILL BE DONE.

25. (U) PROGRESS - JUL 67 THRU JUN 68 PHOSPHOENOLPYRUVATE CARBOXYLASE FROM PLASMODIUM BERGHEI ISOLATED, STABILIZED, AND PARTIALLY PURIFIED. THE ENZYME IS STABILIZED BY ADDITION OF GLUCOSE AND VERSENE IN A PHOSPHATE BUFFER, AND PURIFIED BY AMMONIUM SULFATE FRACTIONATION. CHARACTERIZATION STUDIES ARE IN PROGRESS. TO TEST A HYPOTHESIS THAT RESISTANCE OF CERTAIN MALARIAL PARASITES TO CHLOROQUINE DEPENDS ON ABILITY TO RESTRICT CHLOROQUINE ACCUMULATION, 14-C-CHLOROQUINE HAS BEEN USED FOR PERFUSION TRANSPORT STUDIES. IN VITRO STUDIES USING FOUR ERYTHROCYTES HAVE SHOWN THAT AT PHYSIOLOGIC PH AND TEMPERATURE, THE ERYTHROCYTE MEMBRANE IS NOT A SIGNIFICANT BARRIER TO CHLOROQUINE ENTRY. A STUDY OF PHOTOTOXICITY HAS RESULTED IN SYNTHESIS OF A SERIES OF QUINOLINE DERIVATIVES WITH A VARIETY OF MODIFIED SIDECHAINS. PRELIMINARY EVALUATIONS IN THE ANTIMALARIAL TEST PROGRAM HAVE SHOWN THAT THE NON-PHOTOTOXIC AGENTS PRODUCED TO DATE HAVE DECREASED ANTIMALARIAL ACTIVITY. A CORRELATION OF STERIC FACTOR AND RING TESTING IS IN PROGRESS. FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A63530DD829 MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 108, Study of malaria and antimalaria therapy

Investigators.

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Description.

This investigation was designed to study the biochemical responses of the malarial parasite and to provide further insight into the role of a series of antimalarial agents in the prophylaxis of the disease. Further detailed study of the photosensitizing properties of the quinoline derivatives was a part of a study to develop a more effective antimalarial agent which does not carry some of the unwanted side effects.

Progress.

a. Study of carbon dioxide fixation by Plasmodia berghei. Carbon dioxide fixation in plasmodia is important for the formation of certain amino acids and supplying energy. It has been shown in this laboratory that this fixation occurs by the coupling of carbon dioxide with phosphoenolpyruvate to form oxaloacetate. Two enzymatic activities have been observed (Phosphoenolpyruvate Carboxylase and Phosphoenolpyruvate carboxykinase).

A several hundred fold purification of phosphoenolpyruvate carboxylase has been achieved by ammonium sulfate fractionation and ion exchange chromatography on cellulose phosphate. After chromatography this enzyme is relatively pure and stable under the proper conditions. It appears to be made up of subunits which interact with various substrates, ions, and drugs to effectively alter its enzymatic activity. Our next step will be to concentrate the enzyme after chromatography by ultrafiltration in order to study its physical properties and thereby gain an understanding of the control mechanism of this important enzymatic step.

b. Biochemical activity of antimalarial agents. For the first time, 1-methyl-3-nitro-1-nitrosoguanidine has been demonstrated to have antimalarial activity *in vitro*. Studies have been made to determine the uniqueness of it.

In vitro preincubation of 1-methyl-3-nitro-1-nitrosoguanidine (NMNG) with *Plasmodium berghei* has been found to completely destroy the infectivity of the plasmodia in mice. The ability of the plasmodia to infect healthy mice was completely destroyed when preincubated with 200, 400, or 500 µg of NMNG per 4×10^8 parasites. Preincubation with 50 or 100 µg of NMNG, however, showed an increased parasitemia in mice after the 6th and 8th day after inoculation, respectively. In the control group (no additions), however, parasitemia was observed as early as the 5th day after inoculation. The mice which showed parasitemia died shortly after the 8th or 9th day after inoculation. The mice which were inoculated with the plasmodia pretreated with 200, 400, or 500 µg of NMNG, on the other hand, remained healthy and showed no parasitemia for the duration of the experiment (30 days). Parasitemia is expressed as the number of infected blood cells per 500 uninfected blood cells.

Under the same incubation conditions, chloroquine base and quinine sulfate in much higher concentrations (3 mg and 0.5 mg, respectively), had no effect. None of the structurally similar available compounds or the anticancer compounds tested had any effect.

c. Protein synthesis by *Plasmodium berghei*. Although it is clear that malarial parasites contain significant amounts of DNA and RNA, surprisingly little information is available regarding protein synthesis in these organisms. Special interest concerns whether the parasite contains its own complement of tRNA's, simply cannibalizes these intermediates from the host cell, or if the parasite does utilize some or all of the host tRNA species, is a chemical modification required before these species can interact with the parasite mRNA and ribosomes?

A comparative study is being made of the aminoacyl-tRNA's of mouse red blood cells before and after infection with the malaria parasite *Plasmodium berghei*. The main technique used in this study is the reversed-phase column (RPC) chromatographic procedure of Weiss and Kelmers. Preliminary experiments done in this laboratory using rat liver tRNA charged with C^{14}-leucine show that at least 4 and possibly 5 subspecies of this tRNA can be satisfactorily separated by RPC. These results show the multiplicity of leucine-specific RNA in liver to be at least as great as that shown for *E. coli* (2,3). Preparations are presently in progress to cochromatograph C^{14}-leucyl-tRNA from noninfected mouse red blood cells with H^3-leucyl-tRNA from parasitized cells.

The two radioactivity profiles will be compared with an eye for the production of new tRNA subspecies or alterations in the amounts of the normally occurring isoacceptor tRNA's.

d. Intracellular chloroquine transport. New information concerning the ways by which erythrocytes and malarial parasites regulate intracellular chloroquine concentrations is a prerequisite for testing a hypothesis that chloroquine resistance in Plasmodium berghei, and possibly in Plasmodium falciparum, is conferred by a capability for keeping intracellular chloroquine concentrations low.

The ability to keep intracellular chloroquine concentrations low could be due to one or a combination of the following: (1) development of a more effective barrier against the movement of chloroquine into the cell; (2) development of a more effective process to move chloroquine out of the cell; (3) reduction of the concentration of a substance which binds chloroquine within the cell; or (4) development of a more effective process to metabolize and destroy chloroquine. Techniques are currently being devised to study these possibilities in vitro using mouse erythrocytes, normal and parasitized with Plasmodium berghei.

After preliminary studies to evaluate possible incubation media and methods of handling blood from normal mice and mice parasitized with Plasmodium berghei, the following standardized procedure was developed.

Young male mice of the Walter Reed strain are used throughout. On arrival at the animal quarters, each mouse is given an intraperitoneal injection of 5 mg of dl-α-tocopherol suspended with 0.005 ml of Tween 80 in 0.2 ml of the standard medium described in Table I. Alpha-tocopherol is given empirically because it stabilizes biological membranes against oxidants and because commercial rodent chow may not provide enough of this vitamin to meet abnormal stresses.

If parasitized blood is desired, randomly-selected mice are each injected intraperitoneally with 10^9 Plasmodium berghei parasites prepared as follows: One ml of heavily parasitized blood (95% parasitemia) is mixed with 49 ml of citrate-saline solution (7.4 g NaCl, 9.1 g sodium citrate monohydrate, and 0.6 g of citric acid per liter of aqueous solution); 0.1 ml of this suspension contains approximately 10^9 parasites. The parasitized mice are used for study after 6 days, when nearly all (80-95%) their erythrocytes contain parasites. Normal mice are studied after they have been acclimatized for at least 6 days. All mice are allowed commercial laboratory chow (C. L. Baking Co., Frederick, Maryland) and water ad libitum except for the night prior to study, when they are deprived of chow.

TABLE I

COMPOSITION OF STANDARD MEDIUM

Constituent	Concentration
NaCl	25 mM
KCl	4.8 mM
$MgSO_4 \cdot 7H_2O$	1.2 mM
Glucose	86 mM
Sodium Phosphate buffer*	50 mM

* The pH is adjusted to the desired value at 25°C and the pH values given below were measured at 25°C instead of at the incubation temperature.

To obtain blood, the mice are anesthetized with ether and bled through an axillary incision, allowing the blood to come in contact only with plastic or siliconized labware. Blood from several mice is pooled until a total volume of 6 ml has been added to 6 ml of standard medium (Table I) containing 7 mg of heparin. The blood and medium are gently mixed, and the cells are obtained by centrifugation and washed twice with 6-8 ml portions of fresh medium of appropriate pH. With each washing and centrifugation, the buffy coat is discarded. After the final washing, the cells are mixed with fresh medium of the proper pH to give an hematocrit of 20. This is the working suspension of erythrocytes.

An incubation period is begun by rapidly mixing 2 ml of working suspension with 6 ml of the medium to be studied. To evaluate chloroquine movement, the medium contains tracer chloroquine 3 ^{14}C (one mC/187 mg, obtained from New England Nuclear Corp.). The incubation is carried out under an atmosphere of room air; other conditions of incubation are listed separately below for each experiment. The incubation is terminated by centrifuging the mixture momentarily at 12,000 X g and separating the supernatant from erythrocytes. The time at which the centrifuge reaches 12,000 X g is taken as the end of incubation. Approximately 1.4 minutes are required for the centrifuge to reach 12,000 X g. Immediately upon removing the supernatant fluid, an hematocrit of the erythrocyte pellet is taken, after which the remainder of the pellet is weighed and brought up to 10 ml with 1 N NaOH. Then 2 ml of this solution is extracted twice

by shaking for 20 minutes with heptane containing 1.5 ml of isoamyl alcohol per 100 ml. Five ml of heptane is used for the first extraction and 4 ml is used for the second extraction. With this procedure, more than 99% of the chloroquine is recovered in the combined heptane fractions.

Samples of the heptane extract and of the supernatant fluid from incubation are counted in a liquid scintillation counter, using a xylene:dioxane:ethyoxyethanol solvent, so that the distribution of ^{14}C-chloroquine between erythrocytes and incubation medium can be calculated. The samples are counted for a minimum of 1000 counts and the counting rates are at least twice the background counting rate.

The data are expressed as a distribution ratio (D.R.):

$$\text{D.R.} = \frac{^{14}\text{C-Chloroquine per liter of erythrocyte water}}{\text{Concentration of } ^{14}\text{C-Chloroquine in medium}}$$

Erythrocyte water is defined as total water in the pellet, minus entrapped extracellular water. Total water is measured by drying the erythrocyte pellet to constant weight in an oven at 110°C; entrapped extracellular water is estimated by incubating with ^{14}C-inulin in the place of ^{14}C-chloroquine, and measuring the amount of entrapped ^{14}C-inulin. Calculations are based on the assumption that all of the inulin is extracellular and that the concentrations of inulin and of chloroquine are the same in entrapped extracellular fluid as in incubation medium. For normal erythrocytes, the mean of 31 determinations of total water was 69% of the wet cell weight; 13% of the wet cell weight consisted of entrapped extracellular water. As an additional monitor of uniformity from one erythrocyte pellet to another, the hematocrits of the pellets are routinely between 90 and 95%.

Results.

Data showing that chloroquine accumulation by the erythrocyte is rapid even at 0°C are given in Table II. The distribution ratio reaches a steady state within 7 minutes.

TABLE II

CHLOROQUINE ACCUMULATION BY NORMAL MOUSE ERYTHROCYTES

(Chloroquine concentration, 0.1 mM; temperature, 0-2°C; pH, 7.4)

Incubation Period	Distribution
Minutes	Ratio
1.9	1.0
7.3	2.1
11.6	1.9
17.0	1.9
26.4	1.8
36.6	1.9
55.6	1.9

At more physiologic temperatures, chloroquine accumulation was even more rapid, thus making measurements of initial rates at higher temperature impractical. Therefore, steady-state distribution ratios are given in the subsequent tables; the distribution ratio at 30 minutes of incubation is recorded as the steady-state value, but at least one other incubation period was included in each study to insure that a steady-state actually had been reached.

The effect of temperature on chloroquine distribution is shown in Table III. The maximum value for steady-state D.R. was reached at 42°C, with the D.R. at 48°C being less than the value at 42°C,

TABLE III

EFFECT OF TEMPERATURE ON CHLOROQUINE ACCUMULATION BY NORMAL MOUSE ERYTHROCYTES

(Chloroquine concentration, 0.1 mM; pH, 7.4)

Temperature (°C)	Distribution Ratio
0-2	1.9
22	3.9
32	9.0
42	9.8
48	7.5

The effect of pH on chloroquine accumulation is shown in Table IV. On graphing these data, a line with at least two distinct slopes is obtained. From pH 6.6 to pH 7.4 there is a slow increase in the distribution ratio, whereas from pH 7.4 on there is a rapid increase in steady-state distribution ratio. Although the values at 0-2°C are always less than corresponding values at 22°, the trends of the two sets of data are the same.

TABLE IV

EFFECT OF pH ON CHLOROQUINE ACCUMULATION

BY NORMAL MOUSE ERYTHROCYTES

(0.1 mM Chloroquine)

	Steady-State Distribution Ratio	
pH	0-2°C	22°C
6.6	0.5	2.0
7.0	---	3.0
7.4	1.9	3.8
7.6	---	6.0
7.8	---	10
8.2	8.0	11
8.4	---	14
8.6	---	17
8.9	---	18

In Table V is given the effect of nonradioactive chloroquine on the steady-state distribution ratio. Both at 0° and at 32° the distribution ratio falls as the concentration of chloroquine in the medium increases to 10 mM.

TABLE V

EFFECT OF NONRADIOACTIVE CHLOROQUINE

ON THE ACCUMULATION OF ^{14}C-CHLOROQUINE

BY NORMAL MOUSE ERYTHROCYTES

(pH 7.4)

Concentration	Distribution Ratio	
(mM)	0-2°C	32°C
0.05	1.5	8.5
0.1	1.8	9.0
0.2	1.7	---
0.5	1.6	8.4
1.0	1.7	7.1
3.0	---	7.3
4.0	1.3	---
5.0	1.3	4.6
7.0	1.3	---
7.5	---	4.5
10.0	1.2	3.7

At 0-2°C a complete time-course of chloroquine accumulation was measured at each concentration (cf. Table II) and indicated that the first incubation period of two minutes yields a reasonable approximation of initial rate. When the two-minute value for chloroquine accumulation was graphed against chloroquine concentration (graph not shown) a straight line was obtained indicating that the initial accumulation follows first-order kinetics with a rate constant of 0.25 min^{-1}. This is in distinct contrast to the decrease in steady-state distribution ratio with increasing chloroquine concentrations (Table V).

Discussion.

In discussing the foregoing results, the following three items have to be mentioned: (a) at the pH range under study, chloroquine carries either one or two protons and the interior of the erythrocyte is about 5-10 millivolts more negative than the outside; (b) chloroquine is known to bind to various proteins and, for example, might bind to hemoglobin, which carries a negative charge in the pH range under study; and (c) active transport of chloroquine into the erythrocyte against an electrochemical gradient might occur and could cause large distribution ratios. However, since the present data neither confirm nor exclude active transport of chloroquine, only the first two items need be considered now.

According to the Nernst equation, the charge on chloroquine and the relative negativity of the interior of the erythrocyte could produce a distribution ratio of about 2, at the most. Hence, the electrical gradient might be important in producing the low distribution ratios at 0-2°C, but it cannot account for the large distribution ratios at higher temperatures. On the other hand, binding of chloroquine to one or another protein could produce large distribution.

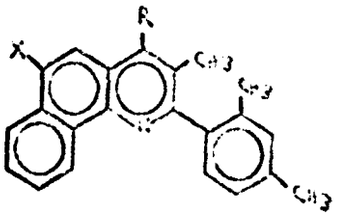
In support of chloroquine binding is the fall in steady-state distribution ratios as the concentration of nonradioactive chloroquine increases (Table V). This finding indicates that chloroquine interacts with a site in or on the erythrocyte and that this site can be made less available to radioactive chloroquine by increasing the concentration of nonradioactive chloroquine. The failure to saturate the process responsible for the initial movement of chloroquine into the erythrocyte at 0-2°C indicates that the binding site is on the interior of the cell. Furthermore, the initial rapid rate of chloroquine accumulation demonstrates that chloroquine easily penetrates the erythrocyte membrane even at 0°C; i.e., the normal erythrocyte membrane is not an effective barrier against the movement of chloroquine into the cell.

To explain the large pH and temperature effects, we suggest that chloroquine, carrying a single proton is preferentially bound and that the intracellular binding sites become more available as the temperature increases--probably due to conformational changes in the binding protein.

Further studies of chloroquine binding using ultrafiltration techniques are in progress, and complete studies of chloroquine accumulation by erythrocytes parasitized with Plasmodium berghei, sensitive and resistant to chloroquine, are planned.

e. Chemical study of antimalarial agents. A series of new compounds which are considered to be potential antimalarials are being synthesized to determine the chemical nature of potential phototoxic properties of these compounds.

Six new compounds (I-VI) of the benzo-[h]-quinoline-4-methanol type have been or are being synthesized. These compounds have been or will be submitted to Dr. Leo Rane, University of Miami, for the rodent antimalarial screen and to COL William E. Rothe, WRAIR, WRAMC, Washington, D.C., for phototoxicity evaluation.



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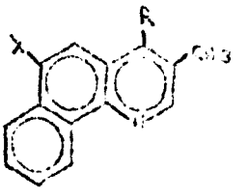

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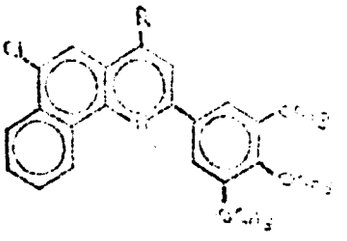
III IV



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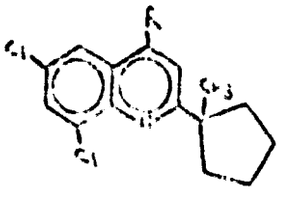
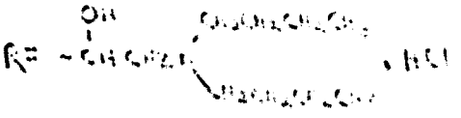


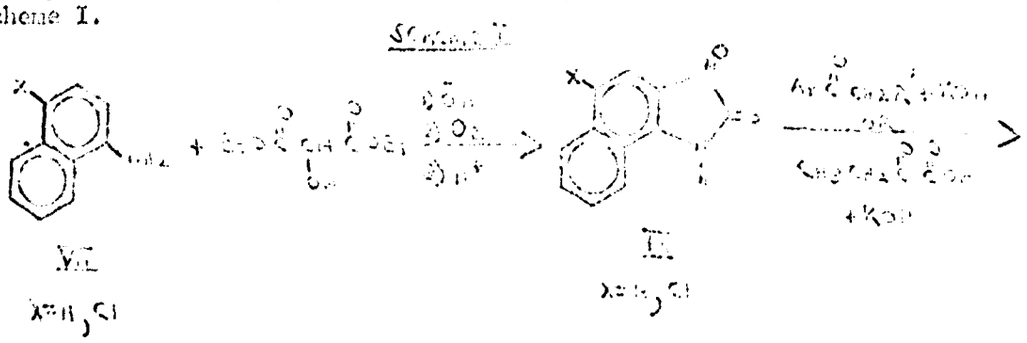
VI

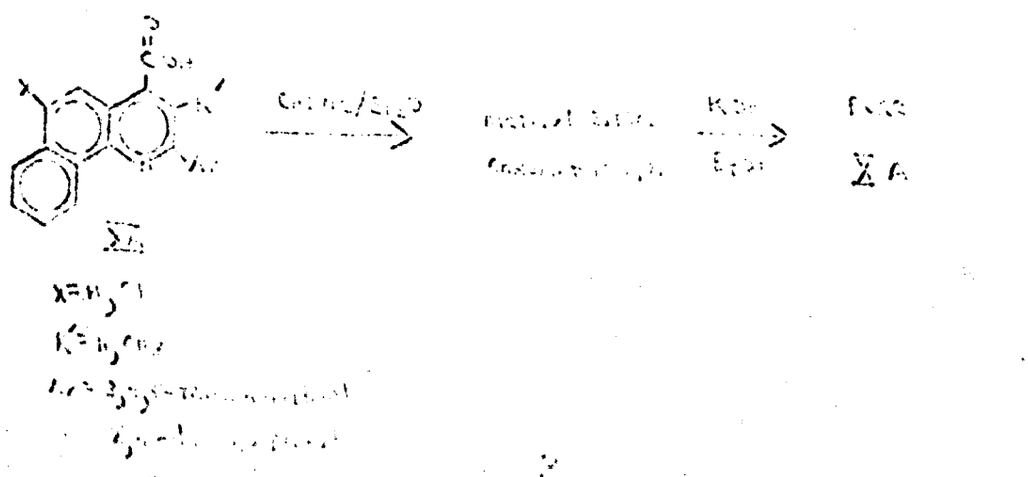


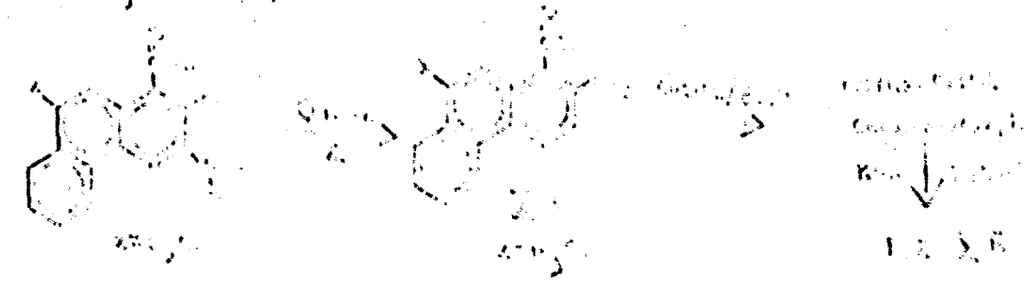
Compounds I, III, IV, and VI have been synthesized in 1.0 - 1.5 g quantities, and have been submitted for testing. Work is progressing on the synthesis of Compounds II and V.

The corresponding carboxylic acids (XA and XB) (R = $-CO_2H$) are the precursors of Compounds I-VI. The carboxylic acids (XA, XB) are made from the corresponding isatins (IX) and appropriately substituted arylmethyl ketones in a Pfitzinger condensation or from the isatin (IX) and an α-keto acid followed by selective decarboxylation. The crude carboxylic acids (XA, XB) so obtained are difficult to purify by conventional methods for use in the synthetic sequence. They are therefore converted to their methyl esters by treatment with ethereal diazomethane, chromatographed over alumina, and hydrolyzed back to the carboxylic acids (XA, XB) in ethanolic potassium hydroxide. The isatins (IX) are made from the corresponding 4-substituted or unsubstituted-1-naphthylamines (VII) and diethylketomalonate hydrate (VIII). The preparations are illustrated in Scheme I.

Scheme I

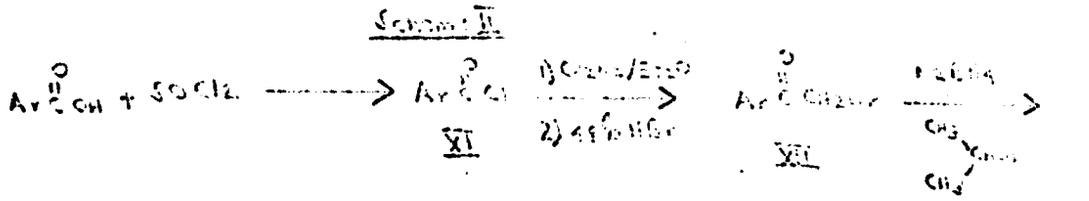


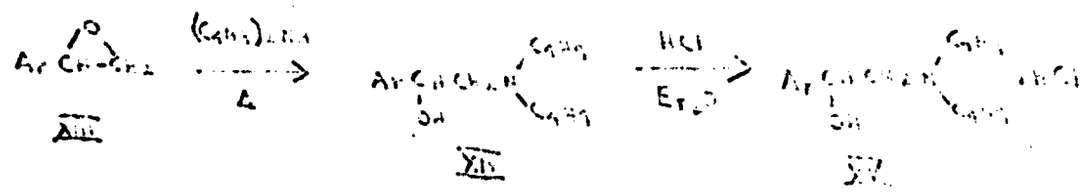


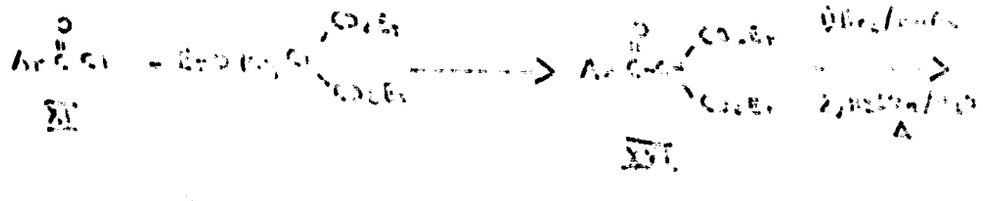


The carboxylic acids are converted to their corresponding α-(dibutylaminomethyl)-benzo-[h]-quinoline-4-methanols (XIV) by either of two routes (Schemes II or III). In Scheme II, the carboxylic acids are treated with thionyl chloride to give the corresponding acid chloride (XI). The acid chloride (XI) is then treated with ethereal diazomethane, followed by 48% HBr to give the α-bromomethyl ketone (XII). The α-bromomethyl ketone (XII) is treated with sodium borohydride in isopropyl alcohol to give the epoxide (XIII). The epoxide (XIII) is then opened with dibutylamine to give the desired α-(dibutylaminomethyl)-benzo-[h]-quinoline-4-methanol (XIV) as a thick syrup. Addition of ethereal HCl to this syrup gives the corresponding quinoline-4-methanol hydrochloride salt (XV) as a crystalline solid. These salts are submitted for testing. Compounds IV and VI were prepared by this route.

Scheme III differs from Scheme II in the preparation of the α-bromomethyl ketone (XII) from the acid chloride (XI). The acid chloride (XI) is treated with diethyl ethoxymagnesiummalonate followed by dilute aqueous acid to give the crude diethyl acylmalonate (XVI). This is then treated with bromine in glacial acetic acid followed by acid hydrolysis to give the α-bromomethyl ketone (XII). Compounds I and III were prepared by this route.

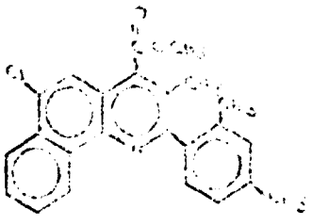




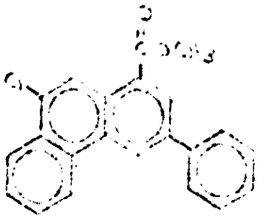


Preliminary results on compound I show that it has negligible phototoxicity but only minor antimalarial activity. Compound III shows negligible phototoxicity. Antimalarial and phototoxicity data for compounds III, IV, and VI have not been received yet.

The corresponding methyl esters of the carboxylic acid precursors (XA, XB) are also being submitted for phototoxicity evaluation to see if the aromatic benzo-[h]-quinoline ring system is responsible for the potential phototoxicity of the benzo-[h]-quinoline methanols. In this connection, compounds XVII and XVIII have been prepared and purified, and suitable crystals of each have been submitted to Dr. James Stewart, University of Maryland, for X-ray crystallographic analyses. It is hoped that complete three-dimensional drawings of these compounds can be obtained. After phototoxicity evaluations of these compounds, it is hoped that a relationship can be established between the degree of steric twisting of the 2-aryl group and the decrease in the amount of phototoxicity of the compound.



XVII



XVIII

The X-ray diffractometer data have been gathered for compound XVII and the data fed through the computer. The final stereo picture is almost complete. Preliminary X-ray work has been completed on compound XVIII.

f. Antimalarial drug levels. To support the study of antimalarial drugs by the analysis of biological fluids, antimalarial drug determinations continue to be performed on body fluids from malaria patients. Approximately 1,500 analyses of this nature have been performed during the past year. These data and all the data accumulated since the start of the program are now being evaluated in an effort to establish some correlation between drug levels and drug effectiveness. As reported previously, the normal range of plasma quinine concentrations are 5.0-10.0 µg/liter for 10 grain (648 mg) doses of quinine sulfate given every eight hours. However, the dose-response of the patient extremely ill with malaria is markedly

different from that of the patient who suffers a relapse and who displays milder symptoms. In the former, a peak plasma quinine level of 20.0-25.0 mg/liter was produced with 10-grain doses (every 8 hours), whereas the relapse malaria patients were able to tolerate a much higher dose (45 grains or 2.916 grams given every 8 hours for 2 days) for peak plasma quinine concentrations of not more than 15.0-18.0 mg/liter. In either case, clearance of the quinine is rapid as indicated in those patients where the drug was discontinued for a time because of extreme discomfort and/or severe side effects. Quinine was no longer detectable in the plasma, in these instances, 24-30 hours after the drug had been withdrawn.

What has been stated of quinine in regard to the very ill malaria patient applies to plasma pyrimethamine levels as well. The pyrimethamine concentrations were two to three times higher in the plasma of these patients. (The normal range for a plasma-pyrimethamine level is 0.4-1.0 mg/liter).

In conjunction with the field test of the diformyl derivative of 4,4'-diaminodiphenylsulfone (DDS), determinations have been performed on blood and urine of patients who have responded in the treatment with this new drug as well as some patients who have not responded to treatment. This study has been in progress only a short time, and no conclusions have been drawn. However, preliminary indications are that the diformyl derivative is rapidly converted to 4,4'-diaminodiphenylsulfone, and is detected as such in the blood and urine of those patients who have been treated successfully.

Summary and Conclusions.

Carbon dioxide fixation has been shown to be an important process in plasmodia. This occurs by coupling with phosphoenolpyruvate to form oxaloacetate. The enzyme phosphoenolpyruvate carboxylase has been purified and a study of its physical properties and the alteration of its physical and enzymatic properties thru interaction with other molecules is under way.

In vitro preincubation of 1-methyl-3-nitro-1-nitrosoguanidine with Plasmodium berghei destroys the infectivity of the plasmodia in mice. None of the structurally similar available compounds or the anticancer compounds tested had any effect.

Studies have shown that 4 and possibly 5 subspecies of leucyl-tRNA are present in the cytoplasm of rat liver cells.

At physiologic pH and temperature, the normal mouse erythrocyte membrane is not an effective barrier to chloroquine entry. Chloroquine is rapidly accumulated by the erythrocyte and the present studies indicate that this is due, at least in part, to intracellular binding. The ability

of the erythrocyte to accumulate chloroquine increases with increasing temperature from 0-42°C or with increasing pH from 6.6 to 8.9. The effect of changing pH indicates that chloroquine carrying a single proton is preferentially bound; and the effect of temperature suggests that binding sites are more available at higher temperatures, probably as the result of conformational changes in the binding protein.

Good synthetic methods for the preparation of several α-(dibutylamino-methyl)-benzo-[h]-quinoline-4-methanols as potential antimalarial agents have been developed. Compound 1, while being the first 2-aryl substituted quinoline compound to show no phototoxicity, had little antimalarial activity. It is possible that phototoxicity and antimalarial activity are linked in this series of compounds. An attempt is being made to identify the moiety of these compounds which is responsible for the phototoxicity.

In the evaluation of the quinine data, two variables have been determined which profoundly affect the drug levels. The patient very sick with malaria produces a vastly different drug response pattern from that of a patient whose condition is not so critical. Furthermore, considerable deviation in the day-to-day drug levels can be ascribed to the rapid clearance of the quinine whenever the drug was intermittently withdrawn.

Publications.

Siu, P. M. L. (1967), Carbon dioxide fixation in Plasmodia and the effect of some antimalarial drugs on the enzyme. Comp. Biochem. Physiol. 23, 785-795.

Dannadio, J. V., Jr., Whelton, A., and Kazyak, L. Quinine therapy and peritoneal dialysis in acute renal failure complicating malarial hemoglobinuria. Lancet 1: 375 (1968).

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. [illegible]
		DA 0[illegible]	[illegible]

5. DATE OF RESUME	6. PROJECT RESUME	7. REGRADING	8. RELEASE EDUCATION	9. [illegible]
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C. IN-HOUSE	NA	68	2	70
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21. GOVT. LAB/INSTALLATION ACTIVITY	22. PERFORMING ORGANIZATION
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23. TECHNOLOGY UTILIZATION	24. COORDINATION
PATHOPHYSIOLOGY, CLINICAL	NA

25. KEYWORDS

MALARIA, BLOOD, COAGULATION, TISSUE CULTURE.

26.

(U) TECH OBJECTIVE - BASIC STUDIES OF THE RELATIONSHIP BETWEEN THE PARASITE AND ERYTHROCYTE IN MALARIA.

(U) APPROACH- UTILIZATION OF A METHOD TO CULTURE MALARIAL PARASITES IN VITRO PERMITS STUDIES OF THE METABOLIC REQUIREMENTS OF MALARIAL PARASITES DURING THEIR INTRAERYTHROCYTIC LIFE CYCLE, STUDIES OF THE GROWTH OF PARASITES WITHIN RED BLOOD CELLS, AND THE EFFECT OF BOTH DRUGS AND CHANGES IN THE ERYTHROCYTIC MILIEU UPON PARASITIC GROWTH. IN VIVO STUDIES OF HEMATOLOGIC ABNORMALITIES INDUCED BY MALARIA AND THEIR EFFECT UPON THE DISEASE.

(U) PROGRESS - OCT 67 THRU JUN 68 REPARINIZATION OF MONKEYS WITH A LETHAL STRAIN OF P. KNOWLESI DECREASED THE MORBIDITY OF THE ILLNESS AND MARKEDLY REDUCED PARASITEMIA. STUDIES OF BOTH THE ANTIMALARIAL AND ANTICOAGULANT EFFECTS OF HEPARIN UPON MALARIA ARE IN PROGRESS. MALARIA PARASITES CONCENTRATE BOTH CHLOROQUINE AND QUININE. THESE DRUGS INHIBIT DNA SYNTHESIS BY THE PARASITE MORE THAN EITHER RNA OR PROTEIN SYNTHESIS. QUINOLINE METHANOL (WR300090A) PRODUCES GREATER INHIBITION OF DNA AND PROTEIN SYNTHESIS BY PARASITES THAN RNA SYNTHESIS. CHEMOPROPHYLACTIC DOSES OF CHLOROQUINE, PRIMAQUINE AND DDS CAUSE SIGNIFICANT METHEMOGLOBINEMIA IN METEROZYGOTES WITH NADH METHEMOGLOBIN REDUCTASE DEFICIENCY. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A063501B529 MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 109, Pathophysiology of malaria and antimalarial therapy

Investigators.
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Description.

Studies of host parasite relationships, metabolic requirements and growth of the parasite and the effects of antimalarial drugs upon the host and the parasite.

Progress.

Malaria is a group of parasitic diseases which cause clinical illness with pathologic changes in many body organs when parasites successfully invade and multiply in circulating red blood cells. Generally, the severity of illness is proportional to the number of parasitized erythrocytes and complications do not occur without either significant parasitemia or hemolysis. Despite the apparent importance of hemolysis in malaria, there have been relatively few recent studies of red cell abnormalities in this disease. Most investigators attribute the hemolysis of malaria to antibodies and support this hypothesis with the fact that specific malarial antibodies can be demonstrated in convalescent plasma and that huge doses of hyperimmune serum gamma globulin modify the severity of relapses. Yet, there is little direct evidence that these antibodies become fixed to erythrocytes to cause their premature destruction. In a study of 131 U. S. soldiers evacuated from Vietnam with drug resistant P. falciparum malaria, only four patients were found with a positive direct antiglobulin test. In three of these patients the positive Coombs' test seemed temporally related to the repeated administration of quinine for relapsed malaria and was associated with accelerated hemolysis even though antiquinine antibodies could not be demonstrated in the sera or red cell eluates from these patients. That this finding was an allergic phenomena was suggested by the occurrence of a dermatitis in two of these patients and a panagglutinin in quinine free red cell eluates of an additional patient. The fourth patient had a positive antiglobulin test with significant hemolysis which was unrelated to quinine therapy. The low incidence of a positive antiglobulin test suggested that antibody induced hemolysis was an unusual complication of malaria. Studies were performed in rats with Plasmodium berghei infection to assert in if serum antibodies were important in the etiology of

hemolysis. These investigations failed to show either antibodies adsorbed to red cell surfaces or an effect of antibodies upon the lifespan of circulating erythrocytes. The transfusion of normal animals with acute or convalescent sera from malarious animals had no effect upon the rate of red cell destruction of the recipient animal. Similarly, the incubation of normal or parasitized erythrocytes in either normal or acute and convalescent malarious sera failed to affect the rate of survival of these cells when they were subsequently transfused into normal recipient animals. Although convincing evidence is lacking that malarial antibodies significantly affect red blood cell lifespan, distinct morphologic and biophysical abnormalities have been observed in both parasitized and nonparasitized erythrocytes from malarious animals. Osmotic fragility studies have shown increased lysis of erythrocytes from malarious animals in hypotonic saline solutions. One population contained mature nonparasitized spherocytic cells which were easily lysed in hypotonic saline solutions. The other population was composed of parasitized erythrocytes and reticulocytes which had a greater rate of swelling in hypotonic saline solutions than was observed in similar centrifuged fractions from either normal blood or blood from animals with chemically induced hemolytic anemia. The presence of marked abnormalities in nonparasitized erythrocytes has been utilized to support the hypothesis that malarious anemia was induced by plasma transported antibodies. However, other possibilities exist which could explain this phenomenon. To investigate this paradox, we transfused normal and splenectomized monkeys with chromium^{51} labeled malarious blood and studied the rate of disappearance of both the parasites and the radiochromium from the circulating blood. These experiments indicated that the spleen removed parasites from infected erythrocytes and returned deparasitized cells into the circulation. This is similar to the pitting of other erythrocytic inclusion bodies from red blood cells which was previously reported by Crosby, and electron microscopic evidence which seems to show that the red blood cell containing an inclusion body is unable to pass through the splenic sinuses until both the inclusion body and the portion of the cell containing it is excised by the phagocytic processes of the spleen. Cells which endured this process would return to the circulation as smaller cells with an increased ratio of surface area to volume - a microspherocyte. This would provide an explanation for the adverse effects of splenectomy in malaria, the occurrence of spherocytes in the blood of animals with an intact spleen and the presence of many naked parasites in the spleen of malarious animals. On the contrary, splenectomized animals would have enhanced parasitemia, increased morbidity and mortality and few spherocytes in their circulating blood.

Thrombi in various phases of resolution can be found in the organs of patients dying with malaria. This could be attributed to the sludging of blood in smaller vessels except that the occluded

vessels are often accompanied by hemorrhage. The coexistence of both bleeding and thrombosis in malaria suggested that coagulation abnormalities observed similar to those described with the Schwartzman phenomenon. To study this hypothesis, coagulation tests were performed upon blood from U. S. soldiers evacuated from Vietnam with chloroquine-resistant Plasmodium falciparum malaria. Serial coagulation studies showed prompt coagulation defects in the blood from these patients during relapse and marked improvement in laboratory tests during convalescence. Two-thirds of the relapsed patients had thrombocytopenia. All of the patients had either a prolonged prothrombin time or partial thromboplastin time or both. Specific coagulation factor assays revealed that the defect was caused primarily by depletion of labile factor (V), stable factor (VII) antihemophilic globulin (VIII) and Stuart-Prower factor (X). Fibrinogen concentrations less than 200 mg/100 ml were found in blood specimens from one-third of relapsed patients. That fibrinogen determinations might be fallaciously elevated by circulating fibrinogen degradation products was suggested by the abnormal serial thrombin time and positive reactions to antifibrin and antifibrinogen antisera. These abnormalities in combination with a prolonged euglobulin lysis time suggested that evidences of fibrinolysis were caused primarily by the presence of split products in plasma rather than by increased plasminogen activation. The degree of abnormality of coagulation studies seemed related to the severity of illness in each patient. In human volunteer studies in which the subjects were infected with a more benign strain of falciparum malaria and were treated as soon as symptoms developed and parasites were identified in thick smears of the peripheral blood, only mild thrombocytopenia was observed. It was not possible to ascertain whether this was caused by decreased platelet production or was the earliest findings of accelerated intravascular coagulation. We concluded that this was an antibody-induced thrombocytopenia because the platelet depression was transient and known malarial antibodies persist in the plasma for months or years.

The abnormal coagulation tests in our patients with chloroquine-resistant Plasmodium falciparum malaria suggested that anticoagulants might provide a valuable therapy. Intravenous heparinization was utilized in several patients with severe coagulation defects and sufficient hemolysis to cause hemoglobinuria. Heparin therapy was followed by an increase in the platelet count and improvement in both first and second stage coagulation defects. Cessation of heparin therapy resulted in recurrence of thrombocytopenia and depletion of coagulation defects. Determination of the effects of heparinization in each of our patients was difficult because multiple drug therapy was used for treatment of their disease. Thus, animal studies were initiated to evaluate the usefulness of heparin therapy in the treatment of malaria.

A lethal Malayan strain of Plasmodium knowlesi was injected into rhesus (macaca mulatta) monkeys. This form of malaria usually kills the monkeys within 7 to 9 days. Heparin therapy prevented the depletion of coagulation factors and prolonged the lifespan of monkeys. In addition, there was marked suppression of parasitemia in the heparinized animals. Seven days after infection, nonheparinized monkeys had marked parasitemia; whereas, the heparinized animals had few parasites in their circulating red blood cells. Cessation of heparin therapy in the anticoagulated animals was followed by the development of marked parasitemia and death within 4 to 5 days. Thus, heparin therapy seemed to have an antimalarial effect in addition to acting as an anticoagulant. Unlike other drugs with antimalarial effects, heparin does not seem to be lethal to the parasite and probably acts indirectly by producing alterations in the host rather than by direct pharmacologic effects upon the parasite. Possibilities under consideration are that heparin affects red blood cell surfaces to make them less penetrable to parasites or that the decreased availability of phospholipids - an essential substrate for parasitic growth - in the plasma because of the stimulation of lipoprotein lipase by heparin might be responsible for the salutory effects of heparinization.

During previous years a method was devised in our laboratory which permitted the "in vitro" cultivation of malaria parasites throughout their intraerythrocytic life cycle. This system has proven efficacious for the cultivation of Plasmodium knowlesi, berghei and falciparum. P. knowlesi has been used for most studies because of the availability of biologic materials and the synchrony of this parasitemia. Using this system and the addition of radioisotopic precursors of DNA and RNA such as orotic-6-C^{14} and adenine-8-C^{14} or radiolabeled amino acids to test the system, DNA, RNA and protein syntheses were studied in parasites under a variety of conditions. In untreated culture systems, the incorporation of C^{14} labeled orotic acid and adenine into DNA followed a linear course during the ring and trophozoite stage of parasitemia. During the increase in nuclear material and division of the parasite in the immature schizont and segmenter stages of development, the incorporation of substrates became exponential. Throughout the entire intracrythrocytic stage of the parasite lifespan, RNA synthesis followed a linear rate of incorporation of precursors. Similarly, protein synthesis when studied by the incorporation of various radiolabeled amino acids showed a linear rate of incorporation into parasites. The addition of either chloroquine or quinine to the test systems caused greater inhibition of DNA synthesis by parasites than either RNA or protein synthesis. These antimalaria compounds were shown to be concentrated by the parasites and this would seem to explain their therapeutic specificity.

Studies with quinoline methanol (WR 30090A) showed much greater effects upon DNA and protein syntheses than upon the incorporation of radiolabeled precursors into DNA. Similar studies in test systems in which the supporting media was depleted of single selected amino acids showed that extracellular sources of l-isoleucine were essential for the growth of erythrocytic forms of P. knowlesi. Depletion of l-methionine caused a marked reduction in parasitic development. Significant but less marked inhibition of parasitic growth was observed in test systems depleted of l-cystine, l-tyrosine, l-arginine, l-glutamine, l-histidine and l-lysine indicating that P. knowlesi requires extracellular sources of these amino acids for optimal development. When the degree of inhibition of DNA synthesis in the absence of specific amino acids was correlated with the molar amounts of the same amino acids in adult hemoglobin, it was found that inhibition of DNA synthesis was most marked in the absence of those amino acids of which hemoglobin contained the least. This indicates that parasites are capable of utilizing amino acids from hemoglobin and suggests that certain amino acid analogs might be useful antimalarial drugs.

Summary and Conclusions

Investigation of the hemolytic disorder observed in malaria makes it difficult to attribute it to red blood cell antibodies. The alternative possibility that red cell lysis is caused by direct damage to the erythrocyte by the parasite was examined. The adverse effects of malaria upon nonparasitized red blood cells were explained by demonstration that the spleen pitted parasites from erythrocytes and returned the damaged deparasitized red cell to the circulation. This provides an explanation for the untoward effects of splenectomy in malarious patients and animals. Patients with P. falciparum malaria were shown to have accelerated intravascular coagulation. Preliminary studies in humans seemed to indicate that heparinization was an advantageous form of therapy for malaria. In animal studies heparin not only improved coagulation abnormalities but markedly reduced parasitemia. In vitro studies of the cultivation of malaria parasites were continued. The extracellular amino acid requirements of parasites were delineated. The effects of various antimalarial drugs upon DNA, RNA and protein syntheses by parasites were studied.

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(C) EFFECTS OF ACTION OF ANTIMALARIALS ON

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DA

CHLOROQUINE, ANTIMALARIALS, CHLOROQUINE, QUINACRINE, QUININE, NUCLEIC ACIDS, SUPERCOILED DNA, OPTICAL ROTATORY DISPERSION, MOLECULAR PHARMACOLOGY.

(U) TECH OBJECTIVE - ELUCIDATION OF THE PRIMARY MODES OF ACTION OF CHLOROQUINE, MEPACRINE, QUININE, AND OTHER ANTIMALARIALS AS THE MOLECULAR LEVEL. FROM THIS- EXPLANATION OF THE NATURE OF RESISTANCE TO ANTIMALARIAL DRUGS, RECOGNITION OF THE RELATIONSHIPS BETWEEN CHEMICAL STRUCTURES AND BIOLOGICAL ACTIONS OF SUCH DRUGS, DEVELOPMENT OF THEORETICAL PRINCIPLES FOR THE DESIGN OF IMPROVED ANTIMALARIAL DRUGS.

(U) APPROACH- EXPERIMENTAL STUDIES AT 3 LEVELS OF BIOLOGICAL ORGANIZATION- 1. MOLECULAR (BIOPHYSICAL STUDIES ON THE INTERACTION OF ANTIMALARIALS WITH THEIR PRIMARY MACROMOLECULAR SITES OF ACTION, ESPECIALLY DNA, 2. IN VITRO BIOCHEMICAL STUDIES ON THE ENZYMOLOGICAL CONSEQUENCES /INHIBITIONS/ OF SUCH INTERACTIONS, AND 3. BIOCHEMICAL, PHYSIOLOGICAL, AND PHARMACOLOGICAL STUDIES OF THE IN VIVO MANIFESTATIONS OF SUCH INHIBITIONS LEADING TO CYTOTOXICITY OR LETHALITY OF MICROORGANISMS EXPOSED TO ANTIMALARIALS.

(U) PROGRESS - OCT 67 THRU JUN 68 THE DIFFERENTIAL EFFECTS OF QUINACRINE ON THE REPLICATION OF THE BACTERIAL VIRUSES T2(D+) AND T 5 SUGGEST THAT THE DRUG ACTS PREFERENTIALLY ON DNA REPLICATION IN THOSE INSTANCES IN WHICH DNA ALTERNATES BETWEEN A CIRCULAR AND A SUPERCOILED MOLECULAR CONFIGURATION. BIOPHYSICAL STUDIES ON THE NATURE OF THE DNA-QUININE COMPLEX HAVE PROGRESSED TO THE POINT AT WHICH A STRUCTURAL MODEL IS SUGGESTED IN WHICH THE TERTIARY AMINO GROUP OF QUININE IS ATTRACTED TO PHOSPHATE RESIDUES OF DNA, THE QUINOLINE RING INTERCALATED BETWEEN BASE PAIRS IN DNA, AND THE HYDROXYL GROUP OF QUININE ATTACHED BY HYDROGEN BONDING TO THE AMINO GROUP OF 2 OF THE GUANINE CONSTITUENTS OF DNA. STUDIES OF DNA-LOCKED ANTIBODIES (COTTON EFFECTS) IN THE OPTICAL ROTARY DISPERSION SPECTRA OF ANTIMALARIALS AND DYES HAVE PROGRESSED TO A POINT AT WHICH, IN CORRELATION WITH OTHER OPTICAL MEASUREMENTS, THE FOLLOWING CONCLUSIONS ARE DRAWN- CHLOROQUINE INTERCALATES WITH DNA BY INTERCALATION, QUINACRINE AND METHYLENE BLUE INTERCALATE IN THE MANNER- I. BY INTERCALATION AND II. BY AGGREGATION, METHYL GREEN DOES NOT INTERCALATE BUT IS ATTACHED TO THE OUTSIDE OF THE DNA DOUBLE HELIX. SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT 1 JULY 1967 - 30 JUNE 1968.

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Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 110, Modes of action of antimalarials

Investigators.

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Description.

Scientific experimental studies in depth on the molecular biology, biochemistry, biophysics, microbial physiology and genetics of the actions of antimalarials and other chemotherapeutic drugs with a view to elucidating modes and mechanisms of drug action, explaining phenomena of drug resistance and offering conceptual guidance to both, improved methods of chemotherapy with existing drugs, as well as rational development of novel chemotherapeutic substances.

Progress.

1. Mode of Action of Quinine

(1) In order to extend *in vivo* studies on specific effects of quinine upon macromolecular biosyntheses in microorganisms, we have searched for a test organism more sensitive to the drug than *Bacillus megaterium* with which the original work had been carried out. A strain of *Micrococcus lysodeicticus* approached *B. megaterium*'s sensitivity to quinine, but numerous other bacteria, yeast and *Paramecium aurelia* were found to be insensitive to the drug. The unavailability of a sensitive test organism capable of growth in lifeless media constitutes an obstacle to extended study of quinine's mode of action. In a different approach, we have attempted to establish a specific concentration range in which quinine is bacteriostatic rather than bactericidal for *B. megaterium* with a view to investigating the biochemical basis for growth inhibition. It was, indeed, found that a narrow concentration range exists which is bacteriostatic. However, variations from experiment to experiment as concerns the bacteriostatic drug level have rendered this approach impractical.

(2) Spectrophotometric studies on the binding of quinine to DNA or to DNA-like double helices have shown: (a) DNA must be double-stranded for quinine to bind: single-stranded DNA is without influence on the drug's absorption spectrum. (b) The presence of guanine in DNA or in dGdC is essential to changes in the absorption spectrum of quinine; dIdC which differs from dGdC only by the absence of the amino group in

position 2 of the purine ring, is without effect. (c) High concentrations of urea and lower concentrations of inorganic cations reverse the DNA-induced changes in the quinine spectrum, indicating that quinine binds to DNA by hydrogen bonds, as well as by electrostatic attraction between oppositely charged ions (phosphates in DNA vs. the tertiary amino group in the quinuclidine moiety of quinine). (d) Spectrophotometric titration of quinine with DNA has produced preliminary results which show a low affinity constant of the quinine-DNA interaction and suggest that one drug molecule is bound per 25 base pairs of DNA. (e) Cupric ions (5×10^{-4}M) completely reverse DNA's effect on the absorption spectrum of quinine; this preliminary observation is of potential theoretical importance because of the unique manner in which Cu^{++} interacts with DNA. Studies are under way to finalize these data and to relate them to theoretically derivable binding parameters.

(3) Work on the relationship between the stabilization of DNA by quinine and the inhibition of the DNA polymerase reaction has been completed. Over a tenfold concentration range of the drug, the elevation of the median strand separation temperature of DNA by quinine is directly proportional to the quinine concentration. The logarithm of the elevation of this temperature ($\log \Delta T_m$) is directly proportional to the per cent inhibition of the DNA-polymerase reaction.

(4) Hydrodynamic studies of the intrinsic viscosity and sedimentation coefficient of the DNA-quinine complex by comparison to DNA alone have been completed. Quinine increases the intrinsic viscosity but decreases the sedimentation coefficient of DNA. Such findings are typical for substances that are intercalated (i.e., inserted) between the levels of base pairs of DNA.

(5) From our spectroscopic and hydrodynamic studies and from model-building experiments we derive a hypothetical model of the DNA-quinine complex as follows: the quinoline ring of the drug is intercalated between base pairs in DNA, the alcoholic hydroxyl group of quinine engages in hydrogen bond formation with the 2-amino group of guanine, and the quinuclidine moiety of the drug extends into the minor groove of the double helix and is electrostatically attracted with its tertiary amino group to phosphate groups of nucleotides adjacent to deoxyguanylic acid. Certain structure-activity relationships in the quinine series which had remained empirical can now be rationalized by the assumption that structural changes which are apt to influence the binding of quinine to DNA will also influence the antimalarial potency.

(6) Intercalation of biologically active substances into DNA is emerging as an important mechanism of action shared by an increasing number of experimental and clinical drugs. We are working, therefore, on additional tests of intercalation which are more direct and conclusive than hydrodynamic studies or classical spectroscopic measurements. The physical proximity of the bound drugs and DNA

bases makespossible transfer of energy from DNA to the drugs. That is, ultraviolet (UV) energy absorbed by DNA can be transferred to the drug and then appear as fluorescence or phosphorescence characteristic of the drug. Three classes of energy transfer are of interest: sensitized fluorescence is transfer from an excited singlet of DNA to a singlet of the drug; sensitized phosphorescence is transfer from a DNA triplet to a triplet of the drug; delayed fluorescence is transfer from a DNA triplet to a singlet of the drug. All of these effects have been observed in DNA complexed to the antimalarial quinacrine or its close chemical relatives. These effects have been interpreted as supporting the idea that quinacrine intercalates between adjacent DNA base pairs.

We are using fluorescence and phosphorescence to investigate the binding of other antimalarials with DNA. Interpretation of the experimental results requires knowledge of the energy of the singlet and triplet excited states of the drugs. These experiments are in progress. Thus far most of the work has been done on quinine. We have shown that, contrary to published reports, quinine does fluoresce at pH 7.0 (i.e.,physiological pH), although the fluorescence is reduced in intensity and shifted from visible to UV wavelengths. At room temperature and pH 7, UV was shown to cause photochemical changes in quinine. However, at 77°K, quinine appears to be unaffected by UV. Sensitized fluorescence experiments indicate that energy is transferred from DNA to quinine; however, the transfer efficiency is less than for quinacrine.

(7) All intercalation studies reported in the literature have been concerned with the interaction of intercalative drugs and DNA *in vitro* and have assumed that the results are directly applicable to the biological action of such drugs *in vivo*. Direct evidence of *in vivo* intercalation has been lacking. We have developed a novel procedure for testing the occurrence of intercalation of drugs into DNA in intact cells *in vivo*.

Ultraviolet light produces pyrimidine dimers in DNA *in vivo* and *in vitro*. We have investigated the reduction of dimerization by intercalating molecules. There are three non-trivial modes of this reduction: (1) physical blockage of dimer formation, (2) distortion of the DNA helix, and (3) transfer of energy from the DNA to the intercalant. Modes (1) and (2) are characterized by small values of β, the distance over which each intercalant is effective, while mode (3) predicts a large value of β (>10). Proflavine and acridine orange, both intercalating molecules, gave large values of β (about 20 and 50, respectively), thus implying that the mode of dimer reduction is by energy transfer.

If energy were transferred to the intercalant, it should be possible to determine whether the singlet or triplet energy state of the DNA donated this energy. Paramagnetic ions, known to quench

triplets, do not affect dimer yield; however, acetone, which can accept only singlet level energy from DNA, strongly decreases dimer yield. These studies confirm that energy transfer occurs from DNA to other molecules, and that the energy transfer occurs at the singlet level. They also provide evidence that the energetic precursor of the cyclobutane-type pyrimidine dimer is a singlet.

The reduction of dimers by intercalants is a very sensitive tool for detecting intercalation. Concentrations of the intercalant as low as 10^{-7} to 10^{-8}M produce detectable decreases in dimer yield. Work is in progress to investigate dimer reduction by intercalants, including important antimalarials, in vivo; these methods may provide the first direct test for intercalation in vivo.

2. Mode of Action of Quinacrine

(1) Our previous studies (Ciak & Hahn, Science 156, 655, 1967) have shown that the principal mode of antimicrobial action of quinacrine is an inhibition of DNA biosynthesis. However, a significant inhibition of protein biosynthesis was also reported (loc. cit.). We have found that single-stranded DNA alters the absorption spectrum of quinacrine and induces anomalous optical rotatory dispersion (Cotton Effects) in the ORD spectrum of the drug. These biophysical studies suggest that quinacrine also might influence biochemical processes, foremost protein biosynthesis, in which single-stranded nucleic acids are engaged in information transfer. This hypothesis is strengthened further by recent reports in the literature that proflavine, a simple diaminoacridine, inhibits in vitro amino acid incorporation through interfering with (1) the biosynthesis of amino acyl transfer-RNA, and (2) the transfer of amino acyl residues from these intermediate compounds into peptidic linkage.

In preliminary experiments we have shown that quinacrine, indeed, inhibits strongly the in vitro formation of polyphenylalanine in standard "incorporation systems" of the Nirenberg type. Inhibition is observed regardless of whether the experimental systems are supplied with phenylalanine and, therefore, must synthesize phenylalanyl transfer-RNA prior to polymerization, or with preformed phenylalanyl transfer-RNA. The codon recognition reaction, i.e., the binding of phenylalanyl transfer-RNA to the ribosome-poly U complex, is unaffected by quinacrine. It is anticipated that studies on the inhibition of protein synthesis by quinacrine will be completed at the time of the next Annual Progress Report.

(2) In continuation of studies of the effect of quinacrine on the replication of bacterial viruses, it has been found that the drug inhibits the intracellular formation of phage lambda much more strongly than that of phage T-5. The salient difference between these two phages is that T-5 possesses a linear DNA-chromosome while lambda has a circular DNA-chromosome which, in the course of replication, passes through a supercoiled ("closed circular") configuration. Recent

findings in two laboratories have established that intercalative drugs (ethidium bromide) produce configurational transitions between circular and supercoiled DNA. We conjecture that such transitions may also be caused by quinacrine and may be responsible for some of the selective biological effects of the drug.

3. Mode of Action of Chloroquine

(1) An unexplained feature of chloroquine's action has remained the effect which the drug has upon ribosomes. We reported earlier (Ciak & Hahn, Science, 151, 347, 1966) that chloroquine causes a destruction of ribosomes of sensitive organisms in vivo. More subtle effects of chloroquine upon the ribosomes of plasmodia have been noted by several electron microscopists. We have resumed studies of the breakdown phenomenon with the following preliminary results. The destruction of ribosomes of Bacillus megaterium, following the addition of chloroquine to mass cultures, is rapid, if not instantaneous. Chloramphenicol-induced bacteriostasis through arrest of protein biosynthesis, or stabilization of ribosomes by preexposure of the bacteria to streptomycin, do not protect ribosomes from chloroquine-induced destruction. Metabolically starved, viz., "resting," bacteria show some ribosome degradation even without chloroquine; addition of the drug increases this degradation. When the extent of this effect is naively considered the arithmetic difference between degradation with or without chloroquine, the effect of the drug seems to be slightly less than in actively metabolizing cultures. Observations, so far, suggest, however, that the phenomenon is a physical event occurring upon contact. Current efforts are directed toward reproducing chloroquine-induced ribosome breakdown in vitro. We are entertaining the working hypothesis that the drug replaces organic amines which are ribosome constituents and are instrumental in uniting the constituent macromolecules of the particles. Ion exchange on ribosomes is documented in the literature.

(2) A series of chloroquine derivatives with structural variations in the side chain is being made available by the Division of Medicinal Chemistry for structure activity studies. So far, the two isomeric butene-amines and the actylenamine compound have been compared to chloroquine itself as concerns the ability to stabilize double-stranded DNA to heat. The stabilizing activity increases in the order cis-en-amine<actylene-amine<trans-en-amine<chloroquine. This is the beginning of an investigation which attempts to relate chloroquine's binding to DNA to the antimalarial potencies of compounds of the chloroquine series.

4. Mode of Action of One Antimalarial Naphthoquinone

A series of 2-hydroxy-3-alkyl naphthoquinones are investigational antimalarial substances. We have carried out our work with one member of this set, 2-hydroxy-3-cyclohexylpropyl-1,4-naphthoquinone,

which is moderately soluble in water by comparison to other compounds of this series which are nearly insoluble. At the beginning of this study, there existed a working hypothesis which attempted to explain the antimicrobial effects of naphthoquinone as the result of an inhibition of electron transfer reactions in competition with coenzyme Q (a benzoquinone). It was known, however, that coenzyme Q reverses the inhibition, for example, of succinate oxidation, by naphthoquinones only partly and that this same limited reversal also is produced by quinones which are not electron carriers in the main respiratory pathway.

Growth of a test organism, Bacillus megaterium, was inhibited by the naphthoquinone at the extremely low concentration range of the order of 10^{-6}M. At 6×10^{-6}M, cultures escaped from inhibition after three doubling times and resumed growth; in contrast, at 8×10^{-6}M, the drug was markedly bactericidal. The naphthoquinone can exist in aqueous solution in an ionized and neutral form, depending upon the hydrogen ion concentration. A spectrophotometric titration yielded a pK value of 7, with the neutral form predominating at pH 6. At that pH the naphthoquinone was five times more potent as a growth inhibitor than at pH 7 at which 50 per cent are ionized. Oxygen consumption by resting B. megaterium metabolizing glycerol and reduction of the non-reversible indicator triphenyl tetrazolium chloride were inhibited by the drug.

These results are paradoxical. If the naphthoquinone was a reversible redox substance, serving as an alternate electron carrier, facultative anaerobes should grow in its presence. If the drug was an irreversible electron acceptor (an electron sink or trap) it would act as a bacteriostatic agent from which cultures should escape after the last molecule was reduced. Such may actually occur at 6×10^{-6}M. The inability of B. megaterium to overcome the only slightly higher concentration of 8×10^{-6}M and, foremost, the strong bactericidal effect at this concentration, suggests a third mode of action in which the naphthoquinone is first reduced and in its reduced and non-ionic form condenses covalently with some vitally important cellular constituent in a manner which can not be reversed. Pending the receipt of a quantity of radioactively labelled naphthoquinone, we intend to test this hypothesis. A precedent is the action of the antibiotic, Mitomycin C, which is enzymatically reduced in vivo to an active compound which binds covalently to DNA and, hence, kills bacteria. We do not infer, however, that reduced naphthoquinone must react necessarily with DNA.

5. Biophysical Studies on Complex Formation with DNA of Antimalarial and Related Compounds

(1) Absorption spectroscopy has shown that quinacrine (like acridine orange) and methylene blue, which only differs from acridine orange by the presence of a sulfur atom in the heterocyclic ring, form

complexes with double- and with single-stranded DNAs. The blue shift of the absorption spectra induced by DNA suggests the same bimodal binding which has been described for acridine orange: (a) intercalation binding at low drug concentrations, and (b) peripheral attachment accompanied by drug-drug interactions at higher concentrations.

(2) Spectropolarimetry has shown that whenever an electronic transition (indicated by an absorption maximum) in a drug or dye molecule is influenced by DNA, there will also be found a DNA-induced anomaly in the optical rotatory dispersion (ORD) spectrum, i.e., an induced Cotton Effect. This is the case for chloroquine which intercalates, for methylene blue and quinacrine which bind bimodally, and for methyl green which attaches to the periphery of the double-helix but does not intercalate.

(3) Measurements of flow dichroism of DNA-drug complexes have shown that methylene blue is, indeed, partly oriented perpendicular to the long axis of DNA, i.e., parallel to DNA's base pairs, while methyl green is oriented perpendicular to these base pairs.

6. Studies on Ribosomes and on Actions of Ribosomotropic Inhibitors of Protein Synthesis

(1) Immunological studies concerned with structure and function of ribosomes (see the preceding Annual Progress Report) have advanced to the publication of a first paper (see Publications below).

(2) Studies on the disassemblage of ribosomes by heat and on the stabilization of ribosomes to heat by streptomycin (see the preceding Annual Progress Report) have advanced to the submission of two manuscripts, one of which is in press in Biochemical & Biophysical Research Communications.

(3) Studies on the mode of action of tetracyclines are in progress on the basis of the following considerations. Tetracyclines have been reported to inhibit protein synthesis by interfering with the attachment of amino acyl transfer-RNAs to the ribosome-messenger-RNA complex. This was readily confirmed by us. One plausible mechanism of this interference would be for tetracyclines to interpose themselves physically between a messenger-RNA coding triplet in, or close to, the "reading frame" and the anticodon triplets of amino acylated transfer RNAs; this would amount to a direct inhibition of the reaction by which the genetic code is read and implemented. In model experiments we have shown that the formation of a double helix between polyuridylic and polyadenylic acids in the absence of Mg^{++} is not influenced by oxytetracycline, while the formation of a triple helix between these polymers in the presence of Mg^{++} is disturbed by the antibiotic. Tetracyclines bind Mg^{++} by chelation, but the significance of this property for inhibition of protein synthesis is not clear. Chelation of Mg^{++} or Mn^{++} by oxytetracycline produced in our hands

significant changes in the absorption and ORD spectra of the drug. This work is being continued with a view to testing directly and conclusively whether or not tetracyclines block the codon-anticodon reaction.

(4) Studies on the mechanism of action of chloramphenicol have progressed in two directions: (a) A thorough theoretical analysis of the problem of chloramphenicol's inhibition of individual acts of peptide bond synthesis has concluded that the drug is an inhibitor of the ribosome-integrated enzyme, peptide snythetase, and blocks the peptidyl recognition site of the enzyme in such a manner that the growing peptide chain cannot be transferred to the α-amino group of the next incoming amino acid. A paper reporting these studies is in press in Experientia. (b) It is being investigated if a preferential accumulation of dipeptides, known to occur in cell-free model systems of protein synthesis under the influence of chloramphenicol, also occurs in vivo in chloramphenicol-inhibited Lactobacilli. The outcome of this study might permit one to decide if chloramphenicol is, indeed, an inhibitor of peptidyl transfer or rather an inhibitor of the translocation reaction on the ribosomal particles.

(5) Studies on the action of chalcomycin on protein synthesis have been initiated. Chalcomycin is one of the macrolide antibiotics, but does not contain nitrogen. This interesting property limits greatly the possible modes of interaction of the drug molecule with biological sites of action. We found chalcomycin strongly inhibitory for Gram-positive bacteria (Bacillus cereus, Bacillus megaterium), but non-active against E. coli. Concentrations as low as 0.5 µg/ml of the antibiotic inhibit protein biosynthesis in the susceptible bacteria. This work is in an early stage.

Summary and Conclusions.

Quinine binds selectively to double-stranded DNA by intercalation of its quinoline ring, hydrogen bond formation with the amino group in position 2 of guanine, and ionic binding of the tertiary amino group to phosphate ion of DNA. Interruption of energy transfer in DNA by intercalated molecules produced quenching of fluorescence or phosphorescence and reduction in the extent of UV-induced dimerization of pyrimidines. Quinacrine binds to double- as well as to single-stranded DNA. Like proflavine, it has a secondary action on protein synthesis. Quinacrine inhibits the multiplication of phage lambda (having a circular chromosome), but not that of phage T-5 (having a linear chromosome). The breakdown of ribosomes under the influence of chloroquine is a physical phenomenon, occurring practically upon contact in vivo. Inhibition of cell-respiration by an antimalarial naphthoquinone explains insufficiently the cytocidal action of this type drug. Biophysical studies of complexes of DNA with drugs or dyes distinguish between (a) pure intercalation binding, (b) bimodal

intercalation and peripheral attachment and (c) purely peripheral attachment as modes of complex formation. Chloramphenicol may inhibit peptide bond synthesis by specific action upon the ribosomal peptide synthetase. Oxytetracyclines influence polyribonucleotide interactions in in vitro model studies of the codon-anticodon binding. Chalcomycin, a nitrogen-free macrolide antibiotic, is a potent inhibitor of growth and protein synthesis in Gram-positive bacteria.

Publications.

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Fred E. Hahn. Erythromycin and Oleandomycin. In Antibiotics: Mechanism of Action, Vol I. Springer-Verlag, Berlin-Heidelberg-New York, 1967. 378-386.

Betsy M. Sutherland, W.L. Carrier and R.B. Setlow. Pyrimidine Dimers in the DNA of Paramecium aurelia. Biophysical J., 8, 490, 1968.

B.M. Sutherland, W.L. Carrier and R.B. Setlow. Photoreactivation in vivo of Pyrimidine Dimers in Paramecium DNA. Science, 158, 1699-1700, 1967.

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A.D. Wolfe and F.E. Hahn. Effects of Oxytetracycline and Other Inhibitors of Protein Synthesis on tRNA Binding to Ribosomes During Polyphenylalanine Synthesis. Bact. Proc., 1968, p 114.

A.D. Wolfe. Degradation of Bacterial Ribosomes at a Constant Elevated Temperature. Fed. Proc., 27, 1968.

[illegible]

[illegible]

[illegible]

(U) FINE STRUCTURE - MALARIA PARASITES

[illegible]	17. CONTRACT/GRANT: NA	18. START DATE: 09 64	19. CUTOFF DATE: NA
20. FUNDING AGENCY: OTHER DA	21. PROJECT NUMBER: NA	22. PROJECT NUMBER: NA	23. FUNDING AGENCY: OTHER DA
24. PROJECT NUMBER: C. IN-HOUSE	25. NUMBER: NA	26. PROJECT NUMBER: 69	27. PROJECT NUMBER: 5
28. FUNDING AGENCY: 130	29. TYPE: NA	30. PROJECT NUMBER: 69	31. PROJECT NUMBER: 5
32. FUNDING AGENCY: 130	33. AMOUNT: NA		

34. GOVT. LAB INSTALLATION/ACTIVITY	35. PERFORMING ORGANIZATION
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	INVESTIGATORS PRINCIPAL: SPRINZ, COL. H.
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36. TECHNOLOGY UTILIZATION: MEDICINE	37. COORDINATION: NA

38. KEYWORDS: MALARIA, PLASMODIUM, ELECTRON MICROSCOPY, MOSQUITOES, CHLOROQUINE, MODE OF ACTION.

39.

(U) TECH OBJECTIVE - TO DEFINE THE FINE STRUCTURE OF VARIOUS STAGES OF SEVERAL MALARIA PARASITES DURING METABOLISM AND AS MODIFIED BY DRUG ACTION. THIS IS A CONTINUATION OF WORK INITIATED UNDER DA 0A6492, CODE NUMBER 61130011 3A013001A91C 01110.

(U) APPROACH- HIGH RESOLUTION ELECTRON MICROSCOPY IS EMPLOYED.

(U) PROGRESS - JUL 67 TO JUN 68 THE PIONEER IN-HOUSE EFFORT ON THE MORPHOLOGY OF SPOROZOITE DEVELOPMENT WAS COMPLETED. THE STUDY OF DRUG EFFECTS ON SPOROZOITE DEVELOPMENT IS CONTINUED UNDER CONTRACT. OTHER COMPLETED STUDIES INCLUDE- FINE STRUCTURE OF ASEXUAL STAGES OF PLASMODIUM ELONGATUM, THE PELLICULAR COMPLEX OF P. FALLAX, DEVELOPMENT OF EXOERYTHROCYTIC STAGES OF P. GALLINACEUM IN CHICK EMBRYO LIVER, COMPARISON OF A REPTILIAN PLASMODIUM WITH PARASITES OF WARM-BLOODED ANIMALS (AVIAN, RODENT, SIMIAN AND HUMAN), AND STUDIES OF DRUG EFFECTS SUCH AS, PRIMAQUINE EFFECT ON EXOERYTHROCYTIC STAGES AND CHLOROQUINE EFFECT ON ERYTHROCYTIC STAGES OF P. GALLINACEUM. ADDITIONAL WORK OF FINE STRUCTURE OF MALARIAL PARASITES AND OF DRUG EFFECTS ARE IN PROGRESS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

[illegible]	41.	42. DOD CODE: AR	43. PROJECT CODE: 1
44. [illegible]: AR 7[illegible]-5	45.	46. PARTICIPATION: NA	
47. [illegible]	48. SPECIAL EQUIPMENT		
49. [illegible]	50.		

[illegible]

Project 3A063530D829, MALARIA PROPHYLAXIS

Task 01 - Malaria Investigations

Work Unit 112, Fine Structure - Malaria Parasites

Investigators.
Principal: COL Helmuth Sprinz
Associate: CPT Roger Ladda
Masamichi Aikawa, M. D.

Description.

The fine structure of different stages of various plasmodia was studied with the aid of the electron microscope. Natural as well as experimental infections served as source of the parasites.

Progress.

1. A study of Plasmodium elongatum was completed and published (see Bibliography). This parasite which in other respects is similar to other avian plasmodia has a functioning cytostome in both the erythrocytic and the exoerythrocytic stages. Exoerythrocytic stages of P. elongatum in non-hemoglobin containing blood cell precursors were seen to ingest ribosome packed host cell cytoplasm. In contrast, the feeding mechanism of exoerythrocytic stages of P. fallax which were cultivated in an in vitro system is still not entirely resolved. Work subsequent to our original publication suggests that cytostome feeding may possibly be a factor.

2. The study of the pellicular complex of P. fallax (see Bibliography) was greatly enhanced by the use, for the first time, of the technique of negative staining and shadow casting. It was shown that the merozoite had a thin outer membrane, a labyrinthine, discontinuous inner membrane below which was a layer of microtubes. The inner membrane and microtubules presumably assure rigidity and mobility of the parasite.

3. The study of exoerythrocytic stages of P. gallinaceum (see Bibliography) was a first of its kind in that such stages had not been studied before in an in vivo system with the aid of the electron microscope. It was shown that in the chick embryo liver P. gallinaceum preferentially infects sinusoidal endothelium. As the parasite grows, the endothelial cell becomes an attenuated shell around the plasmodium while the hepatocytes remain essentially unaffected. The structure and development of the parasite was identical to that observed in the in vitro tissue culture system.

4. For the first time a reptilian malaria parasite was studied and compared with avian and mammalian plasmodia. The report by M. Aikawa and H. B. Jordan, entitled "Fine Structure of a Reptilian Malarial Parasite," has been accepted for publication by the Journal of Parasitology.

5. The following reports are in preparation:

a. A Comparative Study of the Fine Structure of Gametocytes and of the Exflagellation of Microgametocytes.

b. Experimental P. Falciparum Infection in Chimpanzees

c. P. Vivax Infection of the Owl Monkey

d. P. berghei Infection in Rodents

e. The Nature of Schüffner's Dots

Summary and Conclusions.

The work accomplished during the past year together with that of preceding years has established this department as the international leader in the field of fine structure of malarial parasites. This was recognized by the invitation extended by Prof. P. C. C. Garnham to Dr. M. Aikawa to present our findings at the forthcoming Eighth International Congress of Malariology in Teheran, Iran, 1968. In our investigations over the past several years we have been able to establish an inventory of the fine structure of reptilian, avian, rodent, simian and human malaria parasites. We have studied erythrocytic as well as exoerythrocytic and mosquito stages. Our work will increasingly be oriented toward the study of drug effects and host-immune responses on the parasite. Two of our associate investigators who have been trained under this project will continue to work independently in this field after their departure from the Institute.

Publications.

1. Aikawa, M., Huff, C. G., and Sprinz, H. Fine Structure of the Asexual Stages of Plasmodium elongatum. J. Cell Biol. 34:229-249, 1967.

2. Aikawa, M., Ultrastructure of the Pellicular Complex of Plasmodium fallax. J. Cell Biol., 35:103-113, 1967.

3. Terzakis, J. A., Sprinz, H., and Ward, R. A. The Transformation of the Plasmodium gallinaceum Oocyst in Aedes Aegypti Mosquitoes. J. Cell Biol., 34:311-326, 1967.

4. Terzakis, J. A. Substructure in an Epithelial Basal Lamina (Basement Membrane). J. Cell Biol., 35:273-278, 1967.

5. Aikawa, M., Huff, C. G., and Sprinz, H. Exoerythrocytic Stages of Plasmodium gallinaceum in Chick Embryo Liver as Observed Electron Microscopically. Am. J. Trop. Med. & Hyg., 17:156-169, 1968.

6. Terzakis, J. A. Uranyl Acetate, a Stain and a Fixative. J. Ultrastruct. Research. 22:168-184, 1968.

RESEARCH AND TECHNOLOGY WORK UNIT

1. AGENCY ACCESSION DA	2. AGENCY ACCESSION NO. DA OA633	3. CONTRACT/GRANT NO. USC-0-1063			
4. FISCAL YEAR 67	5. CHANGE YEAR 67	6. CLASSIFICATION U	7. SPECIFIC APPLICATION DC	8. LEVEL OF EFFORT A. BASIC RSCH	

9. COMPLETE REFERENCE(S)
63153011 3A053010B820 00 113

10. TITLE
* (U) MALARIA (THAILAND)

11. SCIENTIFIC DISCIPLINE(S) 61600 BIOLOGY	12. START DATE 06 66	13. EST. COMPL. DATE NA	14. FUNDING AGENCY OTHER DA
15. PRINCIPAL INVESTIGATOR(S) C. IN-HOUSE	16. RESOURCES: b. NUMBER NA c. TYPE NA	d. DATE NA e. AMOUNT NA	f. PROFESSIONAL MAN-YEARS
			g. FUNDING (In Thousands)
	FY 67	6C	6 685
	FY 68	6	6 685

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19. TECHNOLOGY UTILIZATION
MEDICINE

20. COORDINATION
NA

21. KEYWORDS
MALARIA, MOSQUITOES, PRIMATES, CHLOROQUINE.

22.
(U) TECH OBJECTIVE - TO CONDUCT FIELD AND LABORATORY STUDIES ON MALARIA WITH PRIMARY INTEREST IN CHLOROQUINE - REFRACTORY PLASMODIUM FALCIPARUM.

(U) APPROACH- A BALANCED LABORATORY STAFF IS MAINTAINED IN BANGKOK AUGMENTED, AS NECESSARY, BY TDY PERSONNEL.

(U) PROGRESS - JUL 67 THRU JUN 68 NO SIGNIFICANT CHANGES IN THE PATTERN OF INFECTION IN THE MONKEY HAVE BEEN OBSERVED. RELATIVELY LOW LEVELS OF PARASITEMIA ARE STILL BEING OBSERVED AFTER MANY SERIAL PASSAGES. MATURE GAMETOCYTES HAVE NOT BEEN OBSERVED. FOR THESE REASONS, STUDIES IN THIS ANIMAL ARE BEING PHASED OUT. FOR TECHNICAL REPORTS SEE, ANNUAL PROGRESS REPORT SEATO MEDICAL RESEARCH PROJECT, BANGKOK, THAILAND. 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

23. CATEGORY CODE 6C	24.	25. DAT CODE P	26. BUDGET CODE 1
27.	28. PROJECT NO. NA		
29.	30. SPECIAL HANDLING		
31.			

USAF REPLACES THIS ISSUE OF DOD R&D WORK UNIT WHICH MAY BE USED UNTIL EXHAUSTED. DD FORM 1498

RESEARCH AND TECHNOLOGY WORK UNIT

3. DATE OF ENTRY: 01 07 68	4. KIND OF ACTION: D. CHANGE 30 11 67	5. ACTION NO: 0 0	6. PRIOR ACTION: 03	7. ACTION DATE: 01 07 67	8. CLASSIFICATION: U

9. CONTROL NUMBER CODE: 63153011 3A035001A827 00 114

10. TITLE: (U) MALARIA PROGRAM SUPERVISION

12. SCIENTIFIC OR TECH AREA: 012100 ORGANIC CHEMISTRY	13. START DATE: 07 66	14. EST COMPLETION DATE: NA	15. FUNDING: OTHER DA
16. PERFORM METHOD: C. IN-HOUSE	17. CONTRACT/GRANT: a. NUMBER NA / b. TYPE NA / c. DATE NA / d. AMOUNT NA	18. RESOURCES ESTIMATE PROFESSIONAL MAN YEARS: FY 68: 6.4, FY 69: 6.0, PRIOR YRS: 4, TOTAL: 10.4	19. RESOURCES ESTIMATE TOTAL MAN YEARS: FY 68: 300, FY 69: 300, PRIOR YRS: 300, TOTAL: 600

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22. COORDINATION: NA

25. KEYWORDS
MALARIA, DRUGS, BIOLOGY, CHEMISTRY.

26.
(U) TECH OBJECTIVE - TO MANAGE, TO INTEGRATE, AND PROVIDE QUALITY CONTROL FOR THE DRUG RESEARCH PROGRAM ON MALARIA, BOTH IN-HOUSE AND BY CONTRACT.

(U) APPROACH- TO DEFINE AREAS REQUIRING INVESTIGATION, TO DEVELOPE SUITABLE CONTRACT PROPOSALS, TO FOLLOW PROGRESS BY CORRESPONDENCE + 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 + CONTRACT). TWO OUTSIDE ADVISORY GROUPS ARE UTILIZED.

(U) PROGRESS - OCT 67 THRU JUN 68 PRECLINICAL WORKUPS WERE COMPLETED ON SIX OPEN AND THREE COMMERCIAL DISCREET PRODUCTS. A NUMBER OF OPEN AGENTS HAVE BEEN FURTHER DEVELOPED AND WORKUPS ARE NEAR COMPLETION. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

27. [illegible] 28. [illegible] 29. DOD ESTAB: AA 30. BUDGET CODE: 1

31. [illegible]: NA 32. PARTICIPATION: NA

33. [illegible] 34. SPECIAL EVALUATION

35. [illegible]

Project 3A635301D829 Title: Malaria Prophylaxis

Task 01 Malaria Investigations

Work Unit 114 Malaria Program Supervision

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Project No. 3A635301D829 Title: Malaria Prophylaxis

Task 01 Malaria Investigations

Work Unit 114 Malaria Program Supervision

Progress

The Chemical Synthesis Program

The chemical synthesis program operated on a budget of 2.7 million dollars for FY-68. During this period there were 89 active synthesis contracts, 5 preparations laboratories contracts, 1 contract for the radioactive labeling of compounds and 1 for chemical analytical work. During FY-68 10 new synthesis contracts were started and 17 contracts terminated. An approximate breakdown in the contracts with respect to the type of organization and funds involved is as follows:

	Number of Contracts (%)	Funds (%)
Academic	54	31
Research House	22	33
Industry	24	36

During FY-68 there was a total of 2392 compounds including 1307 target compounds submitted from the synthesis program. As of the end of FY-68 the overall average cost per target compound was $2458. A total of 59 publications have been forthcoming from the chemical synthesis program during FY-68. Several accomplishments from the strictly chemical standpoint deserve special mention in that they make a substantial contribution to the synthesis program, i.e., the chemistry would be generally useful in the program, or are novel and make a contribution to the field of chemistry in general. The following accomplishments are selected for special mention:

1. A method of circumventing the multi-step synthesis of 4-quinoline methanols containing an α-piperidyl moiety was developed which involves the reaction of the cinchophen with α-pyridyllithium followed by hydrolysis to the ketone and selective hydrogenation of the pyridine ring.

2. An approach to the 4-quinolinemethanols which is especially useful for large scale synthesis because it avoids the use of explosive reagents and the very difficult separation of the desired intermediate bromoketones from poly bromination products has been developed. The method involves the reaction of the quinoline carboxylic acid chloride with diethylethoxymagnesium malonate to yield the ketomalonate; the latter is then brominated, the ester hydrolyzed, and the dicarboxylic acid decarboxylated to yield the bromo ketone.

3. A generally useful and convenient introduction of a dialkylaminoethanol moiety into aromatic nuclei was developed which involves the reaction of the aromatic aldehyde with dimethylsulfonium methylide to yield the epoxide which is subsequently opened by treatment with the appropriate amine.

4. An unequivocal synthesis of pteridines containing unlike substituents in the 6 and 7 positions has been developed. This is based upon elaboration of appropriate 5-, 6-, or 5,6- substituted 2-amino-3-cyanopyrazine-1-oxides and then effecting ring closure of the pyrimidino moiety to yield the pyrazine position of the molecule.

5. A convenient synthesis of bis(dialkylaminoalkyl) anilines was developed. This alkylation, as opposed to ordinary alkylation which stops at the monoalkyl stage, is accomplished by pretreatment of the aniline with methylmagnesium chloride to form the intermediate magnesium halide complex which is then smoothly alkylated.

6. An improved and versatile method for the introduction of aminohydroxy or aminoketo side chains on the quinazoline nucleus, the resulting compounds being febrifugine analogs, has been developed. It consists of reacting the quinazoline with an omegohaloalkylepoxide. The direction of the reaction, i.e., whether halide is displaced or the epoxide opened, can be controlled by the choice of solvent.

As of the end of FY-68 the two most active areas of the synthesis program are the aminoalcohols and the antifolic acid antagonists. At the beginning of the year the 2-phenyl-4-quinoline methanols were being vigorously pursued because of their high activity. Attempts were being made through molecular modification to overcome the problem of phototoxicity. This undesirable characteristic seemed inherent in the 2-phenyl compounds and, therefore, emphasis was shifted during the year to the synthesis of isomeric quinoline methanols, viz. the 2-, 3-, 5-, 6-, 7- and 8. Programs are now in progress for the synthesis of these compounds and they are just now beginning to come in. In the meantime the synthesis of aminoalcohols in the phenanthrene series has been emphasized because of the apparent lack of phototoxicity and good activity in the 9-isomers.

The phenanthrene work includes aminoalcohols of the 1-, 2-, 3-, and 4-types as well as the series of isomeric azaphenanthrene aminoalcohols. Also included in this general area is some work on the synthesis of quinoxaline, quinazoline and pyridine aminoalcohols. The latter class, when the pyridine moiety is appropriately substituted, looks extremely promising.

The other large effort in the synthesis program is in what can be classed as folic acid antagonists, a diverse group of compounds including pyrimidines, purines, pteridines, deazapteridines, triazines, biguanides, sulfones, and others. Excellent activity has shown up in the guanidinoamino pyrimidines and substantial activity in the 1,3-diamino benzo[f] quinazolines and these types are being pushed vigorously. Because of the enormous number of sulfones that have been obtained and screened and because of their limitations, synthesis work on these compounds has ceased. Within this area increased effort has been put on the synthesis of trimethoprim analogs. Synthesis of compounds to selectively inhibit folic reductase by virtue of proper hydrophobic and hydrophilic bonding, as per the postulates of B. R. Baker, is being pursued.

Work in the quinone area continues although the number of contracts is lower. This includes, in addition to the 8-amino-5,6-quinolinedione types, the 5-imino-7-amino-8-quinolines, naphthoquinones, the 3,4-diamino-1,2-naphthoquinones and the benzimidazole naphthoquinones. To date the best lead in the quinones is the 2-hydroxy-3-alkylnaphthoquinones and the problem here hinges on solubilization and absorption.

Work in the 4-aminoquinoline area continues at about the same level. Structural variations have been mainly in the side chain and include the incorporation of unusual amines, hydrazines and unsaturation. Some of these compounds are highly active in the mouse screen and are now being examined in the chloroquinine resistant strains.

The polyhalogen compounds have proved to be of increasing interest during FY-68. The lead of 1,4-bis-trichloromethylbenzene has stood up and this area is receiving increased emphasis.

The synthesis of pantothenic acid analogs has continued through FY-68. This area has been disappointing with respect to activity of compounds. The best of the compounds will be screened in monkeys but the area will probably be deemphasized.

With the exception of the original 3-piperonyl sydnone, the mesoionics have had little or no activity and this class of compounds is being phased out.

Because of the activity of the terephthalic hydroxamic acid, increased emphasis has been placed on the synthesis of analogs.

Work on the guanyl hydrazones and organo tin compounds was continued at about the same level. Synthesis of RC-12 analogs and the phenyl pentadieneoic acid amides has been phased out and the work on the isoquinoline analogs of the 4-aminoquinolines will probably be terminated because of lack of antimalarial activity in the compounds.

The No Dollar Agreement has continued to be a fertile source of compounds. On the order of 25,000 compounds were received by this method during FY-68 and several very interesting and active compounds were obtained. The bottling team collected 20,272 compounds from 119 individual sources.

Two technical conferences were held at WRAIR for the contractors working in the aminoalcohol and antifolic acid areas respectively. The purpose of this was to afford the contractors first hand knowledge of each other's work and to exchange technical information through discussion. Equally important, the conferences provided a forum for the exposition of new ideas with respect to types of compounds that might be useful in enhancing antimalarial activity or decreasing undesirable side effects.

DESCRIPTION

Both primary and secondary biological test systems were operated to screen chemical compounds for potential value as antimalarial drugs. All data were processed by computer. All compounds received were tested in at least one primary screen. Those compounds showing promise in the primary screen were further tested in other primary and secondary screens.

PROGRESS

A. Primary Screens

1. The Plasmodium berghei - mouse test operated at the University of Miami under the direction of Dr. Leo Rane received all drugs submitted for testing for antimalarial activity. A total of 40,465 compounds were tested. (This figure includes 1493 combinations of compounds). All compounds were tested at three levels, with all promising compounds being tested at six or more levels. A total of 636,525 mice were used during the fiscal year. Of the compounds tested, 1192 showed significant activity. Total throughput was reduced by a cessation of testing for a period of approximately three weeks during the movement of the test system into new facilities. The primary screen operated at Illinois Institute of Technology was discontinued, however, this system can be reactivated in a very short time should the need arise.

2. A blood induced chick test was reactivated by Dr. Rane at the University of Miami. Approximately 3000 compounds were tested, of which 400 were active.

3. A total of 11696 assays were run in the In Vitro system at the University of West Virginia under the direction of Dr. Leroy H. Saxe. This represents a total of 10565 drugs tested and 1131 retests of drugs showing activity. This system which utilized the Technicon autoanalyzer, measures glucose consumption and lactic acid and amino nitrogen production and activity is based on the degree to which candidate compounds inhibit these parameters. Of the drugs tested, 2532 (23.9%) inhibited at least one parameter by 20 - 50% and 785 (7.4%) inhibited at least one parameter by more than 50%. The maximum throughput of this system increased from 350 compounds per week at the beginning of the fiscal year to 550 compounds per week at the present time. Although the protocol has not been completed, work has begun on the techniques to add a fourth parameter to this test, the inhibition of DNA synthesis.

4. The P. gallinaceum mosquito operated Insect Control Research under Dr. Eugene Gerberg performed 28266 tests on 22000 compounds during the fiscal year. During this period the throughput of this test increased from 600 tests per month to 3000 tests per month. Beginning 1 September 1967 all active compounds were retested to confirm activity. To date, 866 compounds have been confirmed as active by two or more tests. An attempt was made to develop a membrane feeder for mosquitoes in order to eliminate the difficult task of feeding mosquitoes on birds. A membrane

was developed; however, accessory equipment is bulky and difficult to operate. Effort now will be directed to improving this system.

B. Secondary Screens

1. University of Cincinnati

During the reporting period, 559 compounds were submitted to Dr. C. C. Smith at the University of Cincinnati (Christ Hospital Institute of Medical Research) for determination of folic acid inhibition. A total of 2054 assays were conducted on these compounds to determine both the degree of inhibition and the reversibility of the action.

2. IIT Secondary Test System

Three new tests were put into operation during the reporting period for IIT. These tests were a blood induced P. gallinaceum - chick test that is similar to the A-1 test utilized by Coatney and Sebrell, a sporozoite P. gallinaceum - chick test similar to the A-2 test by Coatney and Sebrell, and a sporozoite induced P. berghei - mouse test utilizing the Yoeli strain. (See inclosures 1, 2 and 3). As with the existing secondary systems, evaluation is based on levels of parasitemia rather than mortality of animals. Tests conducted during the reporting period and number found active are as follows:

Secondary (Oral and subcutaneous)
Tested 150
Active 22

Resistant strains
Chloroquine
Tested 140
Active 32

Triazine
Tested 160
Active 62

DDS
Tested 120
Active 28

Chick tests
Tested 290
Active 97

Present maximum capacity of the secondary screen is 10 compounds per week. A total of 30 compounds per week can be run in the three resistant strains.

A total of 12 compounds per week can be tested in systems using chicks.

3. Cryopreservation of Parasites

Illinois Institute of Technology acted as a repository for various strains of malaria, providing these parasites to other test systems when requested. At the present time normal P. berghei and P. gallinaceum are banked in cryopreservation. Strains of P. berghei are banked, which are resistant to triazine, diamino-diphenyl-sulfone, pyrimethamine, and chloroquine. There are three chloroquine resistant strains including the Yoeli strain.

4. Insectory

The insectory facilities were expanded to accomodate a colony of Anopheles stephensi mosquitoes. This colony has been successfully established and is being utilized.

SUMMARY AND CONCLUSIONS

The system of biological screens, all under contract, has been expanded to include secondary test systems utilizing Plasmodium gallinaceum and patterned after those used by Coatney. A secondary mouse system was also added using the mosquito to transmit the parasite. The throughput of primary screens was increased. The primary screen at Illinois Institute of Technology was discontinued, but can be put back into operation of short notice should the need arise.

IIT Blood Induced Chick Test

Eight day old white Rock chicks are infected with Plasmodium gallinaceum from seven day old donor chicks in a slight modification of the A-1 test used by Coatney. Infection is produced by intraperitoneal injection of 10^8 parasitized red blood cells. Total drug doses of 640, 160 and 40 mg/kg are given twice daily for four days. Five birds are used in each test (one drug at one level). Untreated controls and treated controls are run with each group of tests. Quinine and chloroquine are used as control drugs. Drugs are given orally and the initial treatment is given before infection to all but untreated controls. Slides for parasitemia determinations are made on days 6, 10 and 28 post infection. Survivors are sacrificed on day 28. In untreated birds, the parasitemia reaches a peak on the fourth to the seventh day with 60 - 100% of red cells parasitized. The death pattern is diphasic with peaks at 8 - 10 days and 20 - 22 days. Death occurs in over 90% of untreated controls. The criterion for assessing activity is the count of parasitized cells on day six (day one is considered to be the day of infection). A drug is considered effective if the average day six parasitemia count of the treated group is not greater than 25% of untreated controls. For parasitemia determination, the number of parasitized cells, unparasitized cells and free merozoites in 200 red blood cells is recorded. In calculation of percent parasitemia each free merozoite is considered as a single parasitized cell.

Incl 1

IIT Sporozoite Induced Chick Test

Eight day old white Rock chicks are infected with Plasmodium gallinaceum by the intraperitoneal introduction of sporozoites from infected mosquitoes. The injection is equivalent to two infected mosquitoes. Oral drug doses at 640, 160 and 40 mg/kg are given twice daily for four days beginning on the day of infection. The initial drug treatment is given before injection to all but the untreated control groups. Slides for parasitemia determination are made on days 7, 10, 14 and 28 post infection. Survivors are sacrificed on day 28. Drug activity is assessed by a count of the parasitized cells on day 10 (day 1 is considered to be the day of infection). A drug is considered significantly active if the 10 day parasitemia for treated groups is 50% or less than that of the untreated controls. For parasitemia determination, the numbers of parasitized cells, unparasitized cells and free merozoites in 200 red blood cells are recorded. In calculation of percent parasitemia, each free merozoite is considered as a single parasitized cell.

Incl 2

IIT Sporozoite Mouse Test

One to two day old mosquitoes (A. stephensi) are fed on anesthetized donor mice in a controlled room temperature of 24° C. Mosquitoes are raised at 26° C. and transferred to the 24° feeding room three hours before feeding. The mosquitoes are incubated at 24° for 14 days and a representative number are dissected for examination of infection. Experimental mice are injected intraperitoneally with a suspension of the equivalent of five mosquitoes. Predetermined drug doses are administered to the infected mice and blood slides are made on days 4, 8, 15 and 30 to check drug activity. A compound is considered active if there is a decrease in parasitemia from day 4 to day 8 or if the 15 day parasitemia of the treated animals is 50% less than that of the controls.

Incl 3

Pharmacology Malaria Program

During the past year the Pharmacology Department supervised the contractors responsible for their pre-clinical studies of the antimalarial drugs scheduled for clinical trial, the contracts responsible for the metabolism of these agents, and provided the first line of contact with the three clinical centers involved in Phase I and Phase II drug trials. There are nine primary contracts concerned with the preparation of safety information, five primarily concerned with the metabolism of the candidate compounds, one with the detailed formulation and one responsible for an independent check on the purity and components of both the raw drug and the formulated final product. Not counting the clinical centers, these contracts spent approximately 1.3 million last year, in addition to approximately 3/4 of a million dollars expended in the clinical effort. The Pharmacology Advisory Committee met twice.

During the year, the Pharmacology Department prepared nine investigational new drugs as well as eleven supplements. Details on each of these drugs are available in the appropriate IND. A general technique was developed for the handling of folic acid antagonists based on a standardized system in the beagle for examining reversibility of toxic phenomenon through the systemic administration of folinic acid. The development of the radiometabolism techniques provided a clue to the significance of the lead WR-17206, 1,4-bis-trichlormethylbenzene. This compound appears to load into body fat and therefore offers the possibility of long-lived depot-like action by mouth. This is the second instance of the procedure in the malarial program to radiolabel a drug as soon as the decision has been made to sponsor a clinical trial. Information important to the subsequent development of the class of chemicals is then available sufficiently early as to influence the subsequent synthesis and biological testing program. The definitive assays for phototoxicity have been conducted within WRAIR using the normal albino mouse. This system is supplemented by a contract using a hairless mouse. This is the only malarial test system in which the definitive data are obtained internally. It is maintained because of the importance of the phototoxic side-effect for one of the major components of the malarial program, namely, the quinoline-methanols. This phototoxicity testing has been supplemented by a major contract involving the evaluation of likely compounds for phototoxic effects in swine. During the past year, this program, in conjunction with the chemistry synthesis program, established definitively that the phototoxicity is due to the 2-phenyl substituent in the 4-methanol systems, the key compound being 2-phenylquinine which is highly phototoxic. Methanol systems (quinine-like) which are not phototoxic have been developed. These compounds have excellent activity in the mouse screening program and appear to meet, at least, some of the criteria which stimulated the original emphasis upon this phase of the program. The microorganism system for the assay of folic acid antagonism by appropriate compounds has screened approximately 2,000 materials for their activity in S. fecalis, L. casei, and P. servicei. Some new structures of remarkable potency have been found.

Interesting work has also been carried out using strains which have been made resistant to either chlorguanide or trimethoprim. Candidate compounds are then examined for their patters of cross-resistance with these two strains. The benzo-f-quinazolines and the 2-anilinoquinazolines represent classes of chemicals which either are not cross-resistant or which become more effective than they are against the appropriate starting strains of microorganisms. A member from each of these two classes has been selected for clinical trial.

The detailed reports from the three clinical centers will provide the specific information on the past year's activity. The major contribution from the University of Chicago has been the unequivocal demonstration of the gametocidal and sporontocidal action of primaquine when administered on a weekly basis. The administration of this drug on such a schedule means that U. S. personnel do not spread malaria as they travel. This finding applies to the drug-resistant strains of Southeast Asia but is expected to apply to all strains. Further studies are in progress. Basic studies in this facility have included enzyme deficiencies especially glucose phosphate dehydrogenaze and glutathione reductase in populations in the Genetics Clinic at the University of Chicago, the prison at Statesville, and upon bloods furnished from the WRAIR team in Vietnam. The Phase I trial on the naphthaquinone has also been completed.

The University of Missouri has completed work on the combination of kelfizina and trimethoprim, a combination which looks sufficiently promising to merit field trial. Additional work has also been conducted on the mechanism of folic acid action by folic acid antagonists in man. Trimethoprim has been found to be very weak in reducing resistance to itself or to other folic acid antagonists. Trimethoprim retains its original action against pyrimethamine resistant strains; however, it is possible to use a pteridine (2,4,7-triamino orthotolyl pteridine) to produce cross-resistance to trimethoprim. The basic research associated with this laboratory primarily involves the influence of photo-periodicity upon the course of the P. berghei infection, including the biological niche (reticulocytes and bond marrow) in which the berghei grows.

The University of Maryland has been primarily involved in the evaluatic of a sulfone derivative (WR-6798) as a possible replacement on a once-a-week basis for the daily sulfone capsule now being administered in Vietnam. At the present time, this agent still appears to be a possibility for such replacement. The basic work associated with this clinical facility has involved the evaluation of various mosquitoes for their ability to transmit malaria and the provision of blood samples for immunologic assay.

During the past year there was some minor expansion of facilities, primarily as the result of internal rearrangements of the facilities at the University of Missouri. Further expansion is expected during the forthcoming year at the University of Maryland when their state-approved hospital ward is constructed.

The current treatment for falciparum malaria primarily uses quinine and pyrimethamine. Major Blount treated 2,003 consecutive cases with 14 days of quinine and 150 milligrams of pyrimethamine with no failures. Colonel Conte has treated 1,000 consecutive cases with 10 days of quinine, 150 milligrams of pyrimethamine and continues daily Dapsone with less than one percent recurrence. A serious development, however, has occurred in that the clinical center operated by Dr. Powell has reported the development of a quinine-resistant version of the Malayan camp strain which is not controlled by extensive courses of quinine and pyrimethamine. The development of this super-resistant strain is a further indication of the serious problems faced in the support of troops operating in the tropics, especially in those areas in which quinoline resistance is widespread. At the present time, considering the agents for field trial and the number of different structural analogs scheduled for clinical evaluation, we hope that the malarial program will develop the variety of agents so that suitable alternatives will be available in case of resistance to any one line of therapy.

Summary and Conclusions.

The Malarial Program is developing a number of leads, some of which have reached the clinical level. These leads fall roughly into three general classes:

A. Compounds related to quinine but possessing significantly more activity than quinine.

B. Compounds related to known folic acid antagonists but possessing activity against strenge resistance to the established compounds.

C. New structural leads primarily discovered as a result of random screening. Because of the variety of drugs under development, the program managers believe that the Malarial Program will produce a variety of clinically useful drugs sufficiently different that the preventive medical officer will be able to "select the right drug for the right region at the right time." We feel that such selection is essential considering the bewildering rapidity with which multiple drug resistance has appeared on two continents.

RESEARCH AND TECHNOLOGY RESUME

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(U) FIELD STUDIES ON CONTROL OF MOSQUITOES OF SOUTHEAST ASIA

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202-576-3719 TYPE DA

MOSQUITO CONTROL

NA

KEYWORDS:
MOSQUITOES, DEET, INSECTICIDES, INSECT REPELLENTS, MOSQUITO CONTROL, AEDES.

(U) TECH OBJECTIVE - TO DETERMINE THE EFFECTIVENESS OF VARIOUS STANDARD AND NEWLY DEVELOPED INSECTICIDES AND REPELLENTS AGAINST MOSQUITOES AND OTHER ARTHROPODS IN NATURE, UNDER FIELD CONDITIONS IN SOUTHEAST ASIA, WITH PARTICULAR EMPHASIS ON ANOPHELES AND OTHER VECTOR SPECIES.

(U) APPROACH- MATERIALS TO BE TESTED ARE SELECTED BY THE UNITED STATES DEPARTMENT OF AGRICULTURE LABORATORY IN GAINESVILLE, FLORIDA, ASSISTED BY LAB TO DEVELOP MILITARY INSECTICIDES AND REPELLENTS. MATERIALS SELECTED FOR FIELD TRIAL ARE THOSE WHICH SHOW PROMISE IN SCREENING TESTS, OR STANDARD MATERIALS BEING TESTED AGAINST IMPORTANT VECTOR SPECIES. TESTS ARE CONDUCTED IN CONJUNCTION WITH MILITARY UNITS IN SOUTHEAST ASIA. INSECTICIDES ARE APPLIED AS FOGS AND OLSTS, AND IN VARIOUS FORMULATIONS AGAINST LARVAE IN THE LABORATORY AND FIELD. REPELLENTS ARE TESTED BY SKIN AND CLOTHING APPLICATION USING LOCAL VOLUNTEERS.

(U) PROGRESS - OCT 67 THRU MAY 68 A VILLAGE SCALE TRIAL OF AEDES AEGYPTI CONTROL WAS CONDUCTED ON KOH SAMUI ISLAND, THAILAND IN OCTOBER AND NOVEMBER. APPLICATION OF MALATHION FOG FOR ADULT CONTROL AND THE USE OF 1.0 PPM OF ABATE AS EMULSION CONCENTRATE IN POTABLE AND OTHER STORED WATER CAUSED A RAPID AND PROLONGED DECREASE IN AEDES AEGYPTI, THE DOMESTIC VECTOR OF DENGUE VIRUS ON THE ISLAND. THERE WAS NO APPRECIABLE EFFECT ON THE RELATED SPECIES, AEDES ALBOPICTUS, FROM WHICH VIRUS HAS ALSO BEEN ISOLATED. DETAILED OBSERVATIONS WERE ALSO MADE ON THE BIOLOGIES OF THE TWO SPECIES ON THE ISLAND, AND ON THE INTER-SPECIFIC COMPETITION IN PREPARATION FOR AN ISLAND-WIDE ATTEMPT TO REDUCE THE AEGYPTI POPULATION FOR A PROLONGED PERIOD. THIS WILL PERMIT AN ASSESSMENT OF THE ROLES OF THE TWO SPECIES IN DENGUE HEMORRHAGIC FEVER TRANSMISSIONS IN THE AREA. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORTS, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

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Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 122, Field Studies on Control of Mosquitoes of Southeast Asia

Investigators
Principal: LTC John E. Scanlon, MSC
Associate: Dr. G.A. Mount*, CPT M.A. Sullivan

Description

Materials for field study are selected jointly with the Laboratory of Insects Affecting Man and Animals, United States Department of Agriculture, Gainesville, Florida. The testing is accomplished chiefly in Thailand, in cooperation with the US Army Medical Component-SEATO, and Thai military and civilian health authorities. Insecticides are applied as larvicides, and adulticides and observations are made on the effectiveness of repellents on skin, clothing and other materials.

Progress

During 1967 attention of the field studies on mosquito control was shifted entirely to a study of the control of the dengue vectors, Aedes aegypti and A. albopictus. This was due largely to the availability of an excellent site for such studies, and to worldwide interest in the aegypti problem. In addition, suitable sites for large scale study of insecticidal control of Anopheles vectors of malaria in Thailand have not been detected as yet. The search for such sites is continuing, and the Anopheles tests will be scheduled when a suitable area is available. It is still believed desirable to test the larvicide Abate against jungle populations of Anopheles with a back-pack or similar small power equipment.

The dengue vector control tests grew out of work on aegypti control in potable water containers, reported in 1965 and 1966. Of the insecticides tested in Bangkok in those years, Abate (trade name, American Cyanamid Co.) showed great promise from two aspects: its effectiveness for long periods against aegypti at extremely low dosages, and its very mammalian toxicity. Both WHO and the US Public Health Service have approved its use in drinking water at a rate of 1.0 ppm with treatments repeated monthly if necessary. Small scale trials have been undertaken in the Caribbean, but under somewhat artificial conditions. The field trial reported here appears to be the first village scale trial of the insecticide. The study site, Koh Samui Island in the Gulf of Thailand, was chosen because of the extensive work done there on the ecology and epidemiology of dengue and dengue vectors by the appropriate departments

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of the US Army Medical Component-SEATO in the past several years. During those surveys it was determined that dengue virus appears on the island each year during the rainy season, and that Aedes aegypti and A. albopictus are present in large numbers during the epidemic period. Thus, the island offers an unusually favorable site for determination of the relative roles of these two species in transmission of the virus, and for assessment of control measures against them.

Preliminary mosquito studies showed that aegypti biting took place almost entirely indoors, while albopictus rarely if ever entered houses to bite. In the immediate vicinity of houses one might be subjected to the attack of both species, but outdoor biting by aegypti dropped off rapidly just a few meters from the houses, and even where it did occur the level of biting was so small compared to indoor biting that it could be considered negligible. On the other hand, no matter how far one went from the village attacks by albopictus occurred. Similarly, larval habitats of aegypti were almost entirely indoors, or on the periphery of houses, in artificial containers. The albopictus larvae were also found to some extent in artificial containers, and in a very few instances in indoor containers. In a small proportion of cases (8 per cent of all containers) larvae of the two species were found together. However, the general appearance was one of separation. It was particularly striking to note the essential absence of aegypti from natural containers such as bamboo stumps, palm bracts and similar habitats which were heavily populated by albopictus larvae. Our initial observations strengthened our belief that it would be possible to exert extreme insecticidal pressure against both larval and adult stages of aegypti with very little effect on the albopictus population. Our interests were generally as follows to determine:

1. The feasibility of aegypti control for a period in excess of the incubation period of dengue virus (tentatively set at 10 days) as a measure to interrupt an epidemic.

2. Effect of such control on the albopictus population, particularly whether that species could utilize what had been aegypti breeding habitats after insecticidal pressure was removed and before the reappearance of aegypti in large numbers.

3. The rate of reinfestation by aegypti.

Initial work by SEATO personnel consisted of the surveying and mapping of two villages to serve as experimental and control site and the determination of larval and biting levels in each village. The initial period of this project consisted of demonstrations of the techniques to be employed for the local residents, and additional observations on the mosquito populations. The pre-operational survey methods of indoor and outdoor biting collections and larval surveys of randomly selected houses in the villages were supplemented by placement of larval breeding traps, consisting of bamboo sections and several complete surveys of all containers in the treated village for larval infestation.

Efforts to control the mosquitoes consisted of two phases. One was dispersing malathion insecticide fog as a space treatment for adult mosquito kill. This work was done with a hand-carried pulse jet fogger (Swing fog). The malathion was diluted 2% (w/v) in diesel oil and dispersed at a rate of 5.5 gallons per hour. Fogging was done everyday between 0700 and 1100 hours for one week and then every other day for an additional period of three weeks. Most of the fogging was accomplished by drifting the fog through the village at 100-200 ft. intervals. This technique worked well as long as there was sufficient wind to drift the fog through the open houses. During the last week of fogging there was little or no wind, so the fog generator was operated for a few seconds inside of each house. The latter technique gave excellent coverage inside of and in the area immediately surrounding each house. The amount of malathion-fuel oil solution used varied from 3-4 gallons per fogging of the entire village. Bo Phut was estimated to be 20-25 acres in size.

The second phase of the insecticide treatment consisted of applying Abate insecticide to the household water containers. Most of the containers were earthenware, but some were made of concrete, metal and wood. The application was made by first diluting the insecticide concentrate in water and then spraying the mixture into the water containers. Small orchid sprayers were calibrated so that one pump would deliver the proper dosage of insecticide to 5 gal. of water. This application technique worked well except for treatment of large cisterns in which case the insecticide mixture was measured and then poured directly into the water. Initially a decision had been made to treat the water at a dosage of 0.5 ppm. After water containers had been treated in 15 houses it was discovered that one child which had been exposed to the treated water was ill. There was much doubt on our part that the insecticide had been responsible for the sickness. Nevertheless, treatment was discontinued for several days. The remainder of the water in the village was then treated at a dosage of 0.25 ppm. No more sickness that could be connected with the insecticide treatments was noted.

A total of 725 water containers were treated in the village and 363 (50)% of these contained mosquito larvae prior to treatment. Most of the larvae were observed to be Aedes aegypti. There was a total of 142 houses in the village giving an average of about 5 water containers per house.

Weekly surveys for mosquito breeding were made of the entire village to observe the effects of the fogging and water treatment combination. These observations revealed that no breeding was found in water containers treated with a dosage of 0.25 - 0.5 ppm of Abate for a period of two weeks.

Aedes aegypti larvae were found in treated water jars 2 weeks after treatment. A bioassay was then conducted on 14 water samples from the village. The results indicated that only 44% of the samples contained sufficient Abate to kill mosquito larvae. A decision was then made to retreat the entire village and on 7 and 8 November the village was retreated at a dosage of 1 ppm. No mosquito larvae were found in any of the treated containers for an additional 2 weeks at which time the insecticide team departed from Koh Samui.

Additional mosquito sampling in the village of Bo Phut has also confirmed the excellent control of Aedes aegypti by the use of malathion and Abate insecticides. Biweekly biting collections dropped to zero within 9 days after insecticide application and have remained at this level. Weekly inspections of 22 untreated bamboo water cups inside of houses revealed no Aedes aegypti breeding for a period of one month following insecticide treatment.

Results of larval control measures are presented in Figure 1, through 3 January, almost three months from initiation of the control and over two months after the last treatment. The difference in population level between the treated and control villages is striking, and it illustrates the unusual length of control period due to Abate treatment, even under considerable pressure of water use. There was little or no evidence of effect on the albopictus population except for the small portion of the population which occurred in and around houses, which is to be expected from the mode of application of the larvicide. Results of the control as measured by indoor biting are presented in Figure 2. Again, there was a marked and dramatic drop in aegypti population in the treated village, persisting until mid-January. There was a drop in the control village as well, indicating that some of the depression in the treated village might have been due to weather conditions during the period. However, the difference in population levels was sufficient to show the effect of the insecticidal treatment.

There was no appreciable effect on the albopictus population away from the immediate vicinity of the villages, and sufficient numbers infiltrated the edges of the check areas continuously from untreated breeding sites in the coconut plantations to keep up the biting population. There was no evidence of albopictus moving into breeding niches previously occupied by aegypti, even at the end of the effective period for the insecticide when aegypti were beginning to appear. One begins to suspect that albopictus never was a domestic species in the sense that aegypti is, and that aegypti occupied an essentially unoccupied niche when it first appeared on the scene in SE Asia some centuries ago. As urbanization, even on a small scale, proceeds in SE Asia there seems little doubt that aegypti will extend its range- at least until basic changes in sanitary engineering occur.

There was no opportunity to observe possible changes in the dengue attack rates during the field study due to the small population involved. However, in 1968 the study will be extended to the entire island, which has an extent of approximately 86 square miles and a population in excess of 30,000.

Summary and Conclusions

A combined Abate larvicide and malathion adulticide attack was used on populations of Aedes aegypti on Koh Samui Island in the Gulf of Thailand. The island population has been subjected to dengue attack for

several years, including cases of hemorrhagic dengue. The effect of the insecticidal attack on the aegypti was dramatic and long lasting as measured by several criteria. As expected, the methods employed had little effect on Aedes albopictus, the other important vector known from the area, because that species is primarily sylvan and not as accessible to insecticidal attack as aegypti. A larger control study, covering the entire inhabited portion of the island, is planned for 1968 and preliminary surveys are underway. Additional searches are also in progress to find suitable areas for assessment of insecticidal control on Anopheles populations in jungle areas of Thailand.

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Figure 1 -- Larval control

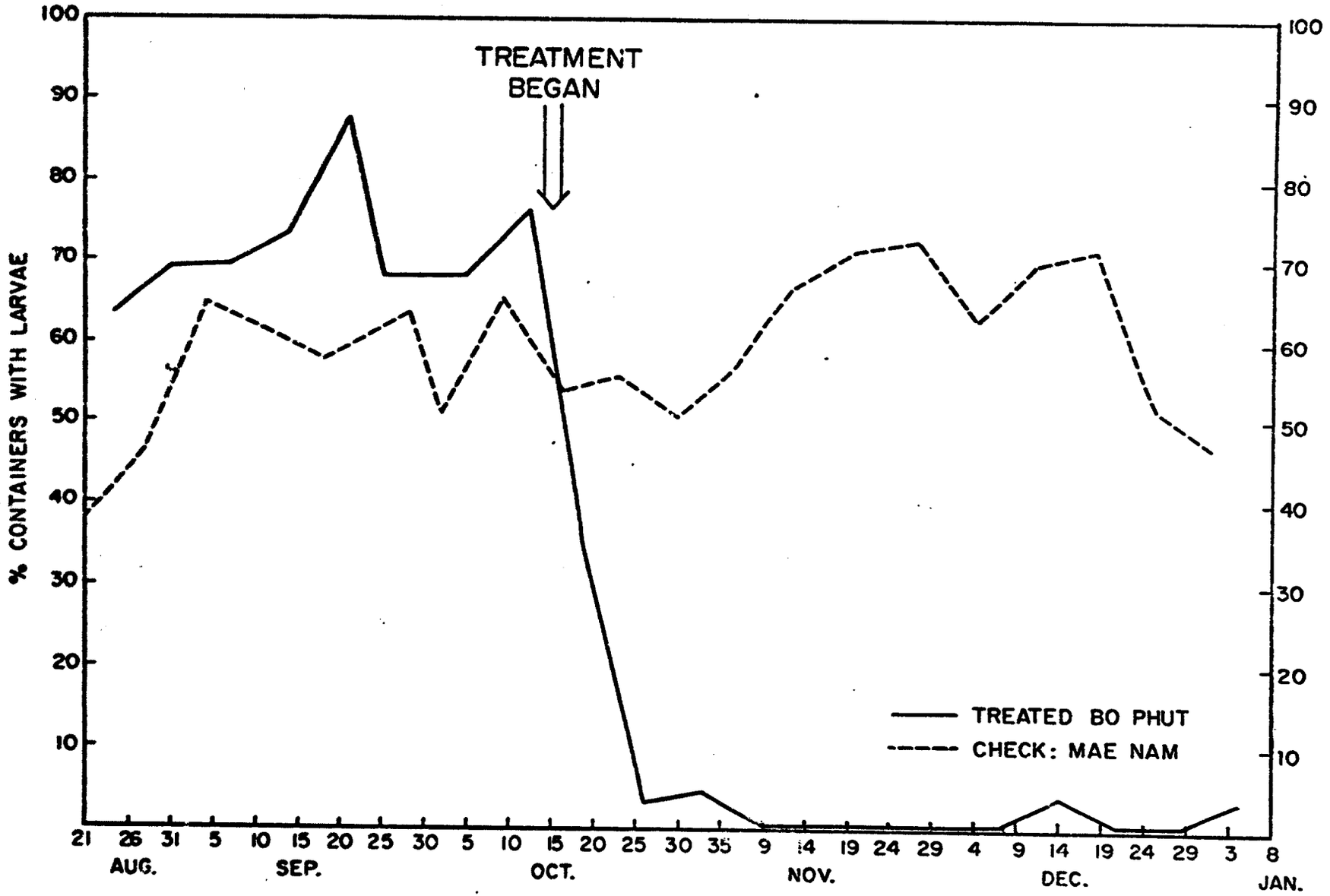
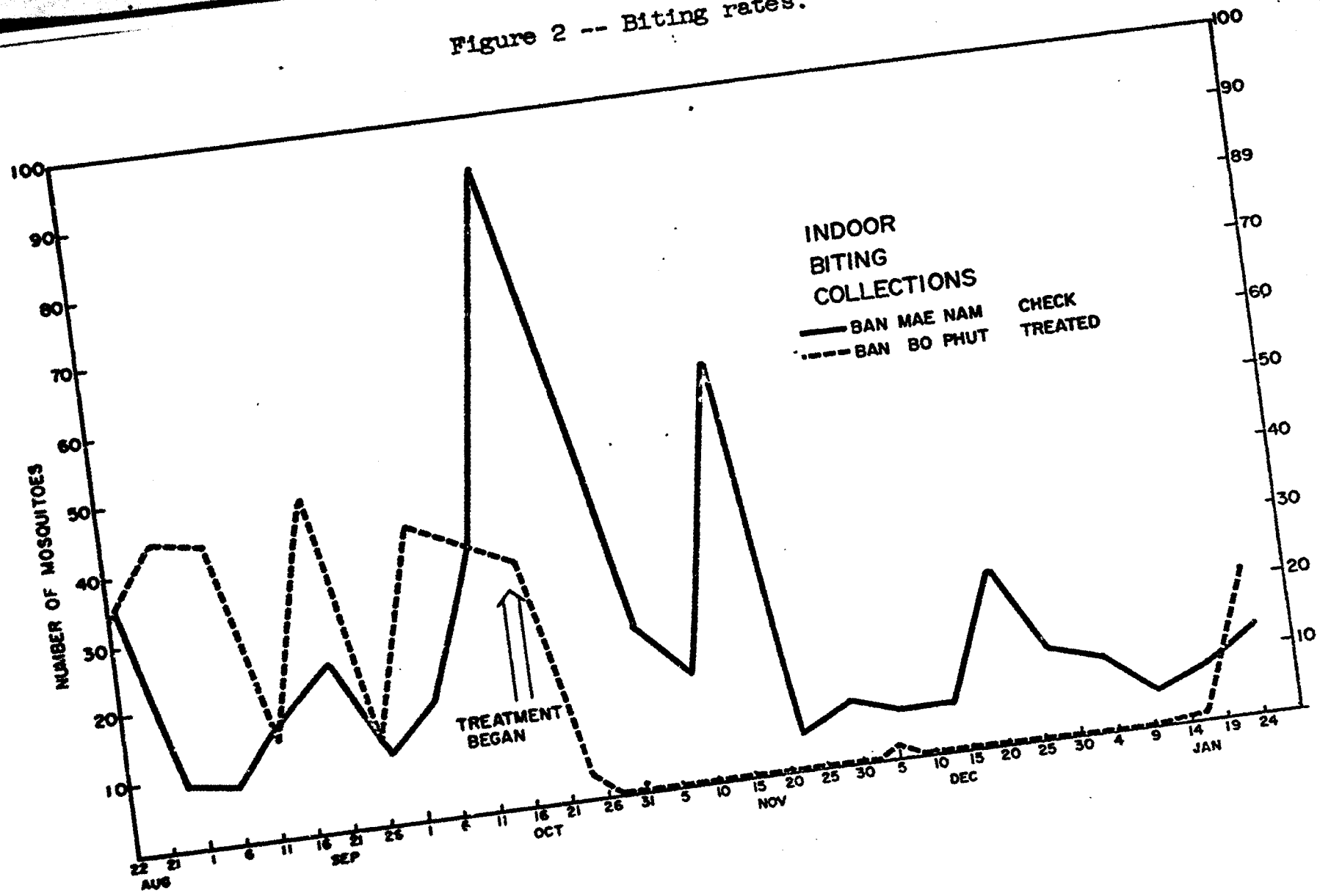


Figure 2 -- Biting rates.



RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
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4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 30 11 67	U U	NA	DC	A. WORK UNIT

10. CURRENT NUMBER/CODE: 63153011 3A635301D029 00 123

10A. PRIOR NUMBER/CODE:

11. TITLE: (U) COLONIZATION OF ANOPHELINE VECTORS 09

12. SCIENTIFIC OR TECH. AREA: 2600 BIOLOGY

13. START DATE: 07 65

14. CRIT. COMPL. DATE: NA

15. FUNDING AGENCY: OTHER DA

16. PROCURE. METHOD: C. IN-HOUSE

17. CONTRACT/GRANT: NA

a. DATE: NA

b. NUMBER: NA

c. TYPE: NA

d. AMOUNT: NA

18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (in thousands)
PRIOR FY 68	1	35
CURRENT FY 69	1	35

19. GOV'T. LAB INSTALLATION/ACTIVITY

NAME: WALTER REED ARMY INST OF RES

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RESP. INDIV: MERONEY, COL W. H.

TEL: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: WALTER REED ARMY INST OF RES

ADDRESS: DIV OF CD AND I
WASHINGTON D C 20012

INVESTIGATORS PRINCIPAL: WARD, DR. R. A.

ASSOCIATE: MOORE, CPT C. G.

TEL: 202-576-3719 TYPE: DA

21. TECHNOLOGY UTILIZATION: LABORATORY COLONIZATION

22. COORDINATION: NA

23. KEYWORDS: ANOPHELES, BALABACENSIS, COLONIZE, MOSQUITOES, VECTORS.

24.

(U) TECH OBJECTIVE - ESTABLISHMENT OF LABORATORY COLONIES OF ANOPHELINE MALARIA VECTORS. THESE COLONIES WILL BE USED FOR STUDIES OF COMPARATIVE SUSCEPTIBILITY TO PRIMATE AND OTHER MALARIAS, GENETIC AND PHYSIOLOGIC STUDIES, AND AS A SOURCE OF MATERIAL FOR OTHER IN-HOUSE AND CONTRACT RESEARCH PROGRAMS.

(U) APPROACH- MOSQUITOES ARE COLLECTED BY WALTER REED ARMY INSTITUTE OF RESEARCH OR SEATO MEDICAL RESEARCH LABORATORY FIELD TEAMS AND FORWARDED TO WASHINGTON, D. C., FOR LABORATORY STUDY AND COLONIZATION TRIALS. VARIOUS REARING PROCEDURES ARE DEVELOPED AND TESTED TO FIND OPTIMAL PROCEDURES FOR EACH SPECIES.

(U) PROGRESS - OCT 67 THRU MAY 68 ANOPHELES STEPHENSI, QUADRIMACULATUS AND BALABACENSIS ARE BEING MAINTAINED IN CULTURE, THE LATTER BY FORCED INSEMINATION TECHNIQUES. A SELF MATING COLONY OF A. BALABACENSIS WAS MAINTAINED FOR NINE GENERATIONS UNTIL FAILURE OF CLIMATE CONTROL SYSTEM IN REARING ROOM PRODUCED EXCESSIVE MORTALITY. SMALL SHIPMENTS OF A. DURENI, VECTOR OF P. BERGHEI, WERE RECEIVED FROM THE CONGO FOR ATTEMPTED COLONIZATION. ADDITIONAL COLONIES OF A. STEPHENSI HAVE BEEN ESTABLISHED FROM IRAN AND PAKISTAN FOR COMPARATIVE STUDIES. FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

25. CLASSIFICATION RESULTS	26.	27. QTR CODE	28. BUDGET CODE
[illegible]		NA	1

29. PARTICIPATION: NA

30. SPECIAL EQUIPMENT

31.

1.99 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 25 inclusive of the NASA Form 1122)

Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 123, Colonization of Anopheline Vectors

Investigators
Principal: CPT Louis C. Rutledge, MSC
Associate: LTC J.E. Scanlon, MSC, R.A. Ward, Ph.D., CPT C.G. Moore

Description

Colonies of Anopheles species from various parts of the world are established and maintained for several purposes. These include biological studies of the mosquitoes concerned; provision of material for malaria transmission, evaluation of antimalarial compounds and Anopheles control experiments. Material is supplied to other departments of WRAIR, to contractors, and to other agencies.

Progress

Laboratory colonies of Anopheles stephensi (India strain), A. balabacensis and A. quadrimaculatus have been continued. Production varies according to demand, available manpower and supervisory time, condition of environmental control systems, and vigor of the colonies. Usual weekly production approximates 15,000 A. stephensi, 1000 A. b. balabacensis and and 1000 A. quadrimaculatus. Production of A. b. balabacensis is time-consuming because of the necessity for individual forced-mating, but the species is excellent material for material transmission studies because of its longevity and high susceptibility to the human and simian malarias. Further attempts at selection of a self-mating strain were made. A self-mating colony persisted at low levels for 8 generations and produced only sterile eggs for the ninth generation. As shown elsewhere in this report, A. quadrimaculatus is relatively non-susceptible to malarial infection and for this reason the colony is being continued for possible future research on the basis of non-susceptibility.

Colonies of Anopheles maculatus and A. freeborni were lost during a period of failure of installed air-conditioning equipment in the insectary. Installation of new controls should minimize the effects of future failures.

Initial steps were taken to colonize Anopheles dureni, the natural vector of Plasmodium berghei. This mosquito is found in the remnant forest galleries of the Congo area and is of interest because of its close relationship to malarias of small wild animals and its predilection for forest galleries with a lower mean temperature than surrounding habitats in the Congo. It appears that this temperature relationship enters into its relationship with the parasites, since some strains of P. berghei must be maintained at lower than usual incubation temperatures during the sporogonous cycle. A collaborator at Lubumbashi was able to

collect large numbers of resting females for shipment to WRAIR. Arrangements for air shipment, however, proved to be quite difficult, with several changes of planes required. Only the first shipment was received in good condition and while eggs were obtained from some of these females the number was not sufficient to establish a colony. A small shipment of Anopheles farauti, the most important vector in much of the Pacific area, was also received from the Solomon Islands, but the numbers received were also too small to permit colonization. These occurrences reenforce the view that the best method for colonization (as was true of balabacensis) is to establish the colony at the site and ship aliquots of the established colony to WRAIR until it is established here. A colony of A. sundaicus from the London School of Hygiene and Tropical Medicine languished for five generations before extinction due primarily to poor survival in the larval stage (approximately 30%) and early adult mortality (within two weeks).

Since Anopheles stephensi is an important vector of human malaria with a range extending from the United Arab Republic across Southern Asia to Eastern Thailand, two new geographic strains of this mosquito (from Iran and Pakistan) have been established in the insectary for laboratory comparison with the long-established India strain. In addition to differences in susceptibility to P. cynomolgi, differences in egg structure, adult longevity, and mating response have thus far been studied.

The eggs of the Pakistan strain are smaller (141.9±2.1 X 464.3±2.3 microns) and have fewer float ridges (12.2±0.1) than those of the India strain (161.8±3.1 X 519.6±2.7 microns, 15.0±0.1 float ridges; those of Iran strain are intermediate (145.3±2.3 X 509.4±3.6 microns, 12.4±0.2 float ridges). The median lifetime under similar laboratory conditions is considerably shorter in adults of the Iran strain. In a controlled trial, median lifetimes were 10 and 13 days for Iran females and males, 17 and 26 days for India females and males, and 21 and 24 days for Pakistan females and males. Intensive care is required for maintenance of the Iran strain. Under ordinary laboratory conditions nearly 100% of India and Iran females are inseminated by males. However, it was found that only ⅓ of the Pakistan females became inseminated in the insectary room where rearing was begun; this room is maintained at 80°F. and 80% R.H. with 16 hours of light. Experiments have shown that 80% insemination is achieved in another room, which is maintained at 75°F. and 75% R.H. with 12 hours of light. Dissection of males reared and held under the former conditions revealed that spermatogenesis proceeded normally, indicating that a behavioral requirement is involved.

Summary and Conclusions

Strains of Anopheles stephensi from Pakistan and Iran have been introduced into the departmental insectary. Intraspecific differences in this species have been found in respect to egg size and morphology and adult behavior and longevity. These differences may account for the extremely wide distribution of the species over a variety of ecologic conditions.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. REPORT CONTROL SYMBOL
			DA CA6514	CSGRD-103

5. DATE OF RESUME	6. KIND OF RESUME	7. SECURITY	8. REGRADING	9. RELEASE LIMITATION	10. LEVEL OF RESUME
01 07 68	D. CHANGE 30 11 67	U U	NA	DC	A. WORK UNIT

10a. CURRENT NUMBER CODE: 63153011 346353010829 00 124

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) GENETIC ASPECTS OF SPOROGENY IN MOSQUITOES 09

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY	07 65	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (in thousands)
C. IN-HOUSE	a. DATE NA; b. NUMBER NA; c. TYPE NA; d. AMOUNT NA	PRIOR FY 68	1	35
		SUPPLEMENT 69	1	35

19. GOV'T. LAB/INSTALLATION/ACTIVITY

NAME: WALTER REED ARMY INST OF RES
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TEL: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: WALTER REED ARMY INST OF RES
ADDRESS: DIV OF CD AND I
WASHINGTON D C 20012

INVESTIGATORS PRINCIPAL: WARD, DR. R. A.
ASSOCIATE: MOORE, CPT C. G.
TEL: 202-576-3719 TYPE: DA

21. TECHNOLOGY UTILIZATION: GENETICS
IMPROVEMENT OF DRUG TEST SYS

22. COORDINATION: NA

23. KEYWORDS: AEDES, ANOPHELES, GENETICS, MOSQUITOES, PLASMODIUM, SUSCEPTIBILITY.

24.

(U) TECH OBJECTIVE - BASIC STUDIES ARE CONDUCTED ON THE INFECTIVITY OF MALARIAL PARASITES TO VARIOUS MOSQUITO VECTORS OF HUMAN AND ANIMAL MALARIAS. THE RESULTS OF THESE STUDIES ARE APPLIED TO EPIDEMIOLOGICAL STUDIES AND THE DEVELOPMENT OF TEST SYSTEMS FOR THE EVALUATION OF ANTIMALARIAL ACTIVITY OF DRUGS.

(U) APPROACH- THE SPOROGONOUS CYCLE OF SPECIES AND STRAINS OF MALARIAL PARASITES (HUMAN, SIMIAN, RODENT AND AVIAN) IS STUDIED IN VARIOUS MOSQUITO HOST SYSTEMS WITH VARIATIONS IN MINTRINSIC AND EXTRINSIC CONDITIONS. VARIATIONS IN SUSCEPTIBILITY OF MOSQUITOES TO MALARIAL INFECTION RELATED TO GEOGRAPHIC RACES OF MOSQUITOES, GAMETOCYTE MATURITY IN VARIOUS HOST SYSTEMS AND GENETIC SELECTIONS WITHIN THE MOSQUITO ARE STUDIED.

(U) PROGRESS - OCT 67 THRU MAY 68 STRAINS OF ANOPHELES STEPHENSI FROM INDIA, PAKISTAN AND IRAN ARE BEING COMPARED IN RESPECT TO SUSCEPTIBILITY TO P. CYNOMOLGI (B) INFECTION. GENETIC SELECTION OF AN INDIAN ISOLATE OF A. STEPHENSI IS BEING MADE FOR HIGH AND LOW SUSCEPTIBILITY TO SIMIAN MALARIA. SIXTEEN FEEDING TRIALS OF ANOPHELES STEPHENSI AND A. BALABACENSIS HAVE BEEN MADE ON AOTUS MONKEYS INFECTED WITH A CHIMP-PASSAGED P. FALCIPARUM STRAIN. DESPITE THE PRESENCE OF VIRTUALLY MATURE GAMETOCYTES IN EACH FEED, THE SPOROGONOUS CYCLE HAS NOT BEEN ESTABLISHED. P. BERGHEI YOELI HAS BEEN MAINTAINED BY CONSECUTIVE MOSQUITO PASSAGE FOR APPROXIMATELY ONE YEAR. FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

GRAPHIC NOT REPRODUCIBLE

27. COMMUNICATIONS SECURITY	28.	29. DSG CODE	30. PRESET CODE
☐ 1. COMPLETED ☒ 2. NOT COMPLETED		E2	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. ASSOCIATING AGENCY	34. SPECIAL EQUIPMENT

35. EXT. FUNDS TO BE USED: 36.

FORM 1 JAN 65 1199m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Item 1 to 25 of Form 1199)

Project 3A6353010D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 124, Genetic aspects of sporogony in mosquitoes

Investigators
Principal: Ronald A. Ward, Ph.D.
Associate: CPT Chester G. Moore, MSC and CPT Louis C. Rutledge, MSC

Description

Basic studies are conducted on the infectivity of malarial parasites to various mosquito vectors of human, simian and rodent malarias. Special emphasis is placed on the roles of genetic and environmental factors in mosquito susceptibility. The results of these studies are applied in epidemiological studies and the development of test systems for evaluation of antimalarial drugs.

Progress

1. Transmission of human malaria in monkeys

Sixteen attempts were made to infect mosquitoes with Plasmodium falciparum (Camp strain) in six splenectomized owl monkeys, Aotus trivirgatus, which had been inoculated with infected blood from either chimpanzees or other owl monkeys. Four feeding trials were made with Anopheles balabacensis and 12 with Anopheles stephensi. At the time of the feeding, gametocytes were observed in the peripheral circulation at densities between 50-5,000/mm^3. Some resembled the immature, spindle-shaped falciparum gametocytes found in experimental infections of gibbons and chimpanzees while others approached mature forms but were conspicuously broader in outline with more concisely defined pigment granules.

Exflagellation of microgametocytes was observed in stained films of blood from mosquito midguts prepared within 30 minutes of the feed. These exflagellating microgametocytes were elongate rather than circular or oval as seen in mosquitoes infected on man. These forms persisted in the mosquito gut for at least 24 hours after a feed. This type of aberrant exflagellation has been reported in mosquitoes fed on human cases.

Ookinetes could not be observed in the contents of mosquito stomachs. Within 30 minutes after feeding peculiar aculeate structures were observed in the midgut. These were still present 24 hours after feeding. The significance of these unnatural forms is not known.

Oocysts were not seen in the 639 mosquitoes which were dissected 3 to 8 days after feeding. Both mosquito species are known to be susceptible to falciparum malaria from experimental feedings of sub-colonies of the WRAIR colonies on human cases in Thailand. It may be concluded that either this strain of P. falciparum has lost its ability to infect mosquitoes in the course of numerous serial blood passages or that the owl monkey is an

unsuitable host for the production of infective gametocytes. Future experiments will utilize sporozoite-induced infections to determine the cause of gametocyte immaturity.

2. Transmission of simian malaria

a. Gametocyte infectivity as related to course of infection

A sporozoite-passaged isolate of Plasmodium cynomolgi (B) was obtained from the U.S. Public Health Service, Chamblee, Georgia, for experimental transmissions in rhesus monkeys (Macaca mulatta). Experimental feeds of Anopheles stephensi (India strain) were made on three monkeys throughout the course of their infections at approximately the same time daily. The periods of greatest infectivity to mosquitoes do not usually coincide with peak gametocyte levels but may either precede or follow them. An index of the infectivity of gametocytes to mosquitoes (oocysts/mosquito per no. gametocytes mm^3 blood) has been found to vary by a factor of 5×10^4. Experiments to determine whether there is any relation between fluorescent antibody level and oocyst counts are in progress. Statistically, these monkeys have not been consistent in being "good" or "poor" gametocyte carriers.

b. Variation in oocyst counts in mosquitoes

The number of oocysts produced in individual mosquitoes of the same species and strain fed simultaneously on the same host varies greatly. In most instances, some mosquitoes are refractory to infection while others in the same group may contain as many as 520 oocysts. For example, on 5 April 1968, monkey E-218, day 8 oocyst counts of 29 mosquitoes ranged from 4-699, with the counts varying by a factor of 175. It is known that much of this variation is attributable to variations in the size of the blood meal ingested, the physiological state of the mosquito (age, nutrition, etc.) and chance variation in the numbers of viable gametocytes ingested. In order to determine how much of the variability is due to genetic factors, sub-strains of the India strain of An. stephensi are being selected from the eggs of individuals developing few and many oocysts. This experiment is in the second generation of selection.

Five species and strains of anophelines are being compared with respect to their susceptibility to infection with P. cynomolgi (Table 1). On the basis of the ratios of the mean oocyst counts in these experiments the Iran strain of An. stephensi has been 22.4 times more susceptible than the India strain; An. balabacensis 3.5 times more susceptible; the Pakistan strain of An. stephensi 1.8 times more susceptible and An. quadrimaculatus 0.2 times as susceptible.

c. Rhythmic infectivity in simian malaria

The 1967 annual report indicated that mosquitoes which were fed on infected monkeys in the afternoon developed a greater number of oocysts than those fed in the morning. To examine this in further detail a controlled experiment was conducted with two splenectomized monkeys infected

with 10^5 P. cynomolgi sporozoites from An. stephensi. Cages of An. stephensi (India strain) were fed at four hour intervals on these monkeys for a period of $3\frac{1}{2}$ days beginning on day 16 of the infection. Parasite counts were made at the time of each feeding. Mosquitoes were maintained in an insectary at 27 ± 1°C with a photoperiod of 14 hours light and 10 hours dark with a simulated dawn and dusk period. There was no regulation of photoperiod in the monkey holding room except for normal illumination of about 12 hours of light and dark. Oocyst counts were made from engorged mosquitoes 8 to 9 days after the infective feed. The results are summarized in Table 2. Monkey A-95 showed higher oocyst counts at 1600 hours daily while monkey A-93 showed no significant change at this time, possibly due to the overall low infection level. These results are similar to those observed by Hawking et al., 1966, Lancet, pp. 422-426.

3. Transmission of rodent malaria

A new strain of P. berghei yoelii was received from Professor Garnham in London. This strain differs from the 17 X strain in that it is highly virulent and is chloroquine sensitive. The new strain was passaged through An. stephensi and Bagg mice for several generations and then stored at -65°C. For routine passage work this strain is not as satisfactory as the 17 X strain as parasitemia increases so rapidly that there are few days available for mosquito feeds at appropriate gametocyte levels.

The major effort of the rodent malaria program has been providing infected mosquitoes for tissue culture and immunological studies. 800 mice were inoculated with sporozoites or infected blood. Of these, 150 served as donors for feeding by approximately 20,000 An. stephensi. Several comparative tests with An. balabacensis indicated that this species is probably refractory to P. berghei yoelii infection.

Observations on factors responsible for heavy infections indicate that parasitemia in the mouse is the primary determinant with P.b. yoelii. Infections are obtained most consistently when parasitemia is between 2-4% and when gametocyte counts are 0.5-1.0%. Efforts to implicate other factors have been largely negative. Day of feeding has no consistent relation to infectivity except as it relates to change in parasitemia. A slightly larger percentage of infections was obtained from sporozoite induced infections (Table 3).

Summary and Conclusions

Plasmodium falciparum gametocytes from Aotus trivirgatus have not produced oocyst infections in either Anopheles balabacensis or An. stephensi mosquitoes. Exflagellation and ookinete formation, when observed, were atypical. There does not appear to be a close relationship between gametocyte level of an infected rhesus monkey (P. cynomolgi) and the ability to infect mosquitoes. In general, mosquitoes may show highest oocyst counts prior to or just after the peak gametocyte level has been achieved. Selection experiments with the P. cynomolgi - An. stephensi model have been

started to determine the role of genetic factors in the susceptibility of Anopheles to malarial infection. Comparative infection studies indicate that an Iran strain of An. stephensi showed greater malarial oocyst development than other strains of the species from India and Pakistan and species as An. balabacensis and An. quadrimaculatus. Mosquito feeds conducted at 4 hour intervals over a 3½ day period indicated that higher oocyst counts may be obtained in mosquitoes feed at 1600 hrs. than at other times of the day. This is probably related to the level of gametocyte maturity coinciding with the normal biting cycle of certain anopheline vectors. These studies should be replicated with animals showing higher initial infectivity levels as only one monkey of the two used was suitable. Transmission of P. berghei yoelii continued.

Publications

1. Cadigan, F.C., Jr., Ward, R.A. and S. Puhomchareon. 1968. Transient infection of the gibbon with Plasmodium vivax malaria. Trans. Roy. Soc. Trop. Med. Hyg. 62:295-296.

2. Rutledge, L.C., Gould, D.J., Cadigan, F.C., and V. Chaicumpa. 1968. Human malaria in non-human primates: experimental mosquito transmission and infection. Mosquito News, 28:46-49.

3. Terzakis, J.A., Sprintz, N. and R.A. Ward. 1967. The transformation of the Plasmodium gallinaceum oocyst in Aedes aegypti mosquitoes. J. Cell Biol. 34:311-326.

TABLE 1

Comparison of day 8 mean oocyst counts in paired feedings of anopheline species and strains on monkeys infected with *Plasmodium cynomolgi*

Mosquito compared	No. of trials in which mean oocyst count for *An. stephensi* (India strain) was:		P
	More	Less	
A. stephensi (Pakistan)	1	4	n.s.
A. stephensi (Iran)	0	2	n.s.
A. balabacensis	1	8	0.005
A. quadrimaculatus	4	0	0.025

TABLE 2

Effect of time of feeding on infection of *Anopheles stephensi* by *Plasmodium cynomolgi*, combined data for 72 hours

Monkey No.		Time of feeding (Hours)					
		0400	0800	1200	1600	2000	2400
A-95	x oocysts	1.56	2.36	2.57	13.18	3.87	1.15
	% infected*	24.2	44.1	69.5	67.5	45.0	20.8
A-93	x oocysts	3.13	1.87	4.24	3.62	1.38	1.96
	% infected*	21.0	43.8	50.0	40.2	26.7	46.4

* Percentages have been converted to ϕ ($p = \sin^2 \phi$)

TABLE 3

Effect of day of feeding and source of mouse infection on frequency of positive mosquito infections, P. berghei yoelii--An. stephensi system. All feeds made when parasitemia was between 2 and 4%.

Source of Mouse Infection		Day of feeding 3-4	5-6	7-8	9-10	11--	Total
Sporozoites	No.	---	11/13	6/7	14/16	1/4	32/40
	%	---	85	86	88	25	80
Infected blood	No.	3/4	26/36	24/34	3/7	6/9	62/90
	%	75	72	71	43	67	69

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. NAVY CONTROL SYMBOL
			DA CA6515	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 30 11 67	U U	NA	DC	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 63153011 3A6353010829 00 125

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) TAXONOMY AND ECOLOGY OF MOSQUITOES OF SOUTHEAST ASIA 69

12. SCIENTIFIC OR TECH AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY	07 65	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	19. PROFESSIONAL MAN-YEARS	20. FUNDS (IN THOUSANDS)
C. IN-HOUSE	a. DATE NA; b. NUMBER NA; c. TYPE NA; d. AMOUNT NA	PRIOR 68	1	35
		CURRENT 69	1	35

14. GOV'T. LAB INSTALLATION/ACTIVITY

NAME: WALTER REED ARMY INST OF RES
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RESP. INDIV.: MERONEY, COL W. F.
TEL: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: WALTER REED ARMY INST OF RES
ADDRESS: DIV OF CD AND I
WASHINGTON D C 20012

INVESTIGATORS PRINCIPAL: SCANLON, LTC J. E.
ASSOCIATE
TEL: 202-576-3719 TYPE: DA

21. TECHNOLOGY UTILIZATION: TAXONOMY
ECOLOGY

22. COORDINATION: NA

23. KEYWORDS

ANOPHELES, ECOLOGY, MOSQUITOES, TAXONOMY, VECTORS.

24.

(U) TECH OBJECTIVE - TAXONOMIC REVISION AND ECOLOGICAL INVESTIGATION OF THE ANOPHELES VECTORS OF MALARIA IN SOUTHEAST ASIA. TO COMPILE DATA ON THE DISTRIBUTION, ABUDANCE, HABITS, DISEASE TRANSMISSION POTENTIAL AND OTHER ASPECTS OF ANOPHELES BIOLOGY, AND TO PRODUCE MONOGRAPHS, KEYS, AND OTHER AIDS FOR UNITS IN THE FIELD.

(U) APPROACH- MOSQUITOES ARE COLLECTED BY COOPERATING MILITARY AND CIVILIAN ORGANIZATIONS IN SOUTHEAST ASIA AND FORWARDED TO A COMBINED WALTER REED ARMY INSTITUTE OF RESEARCH-SMITHSONIAN INSTITUTION TEAM AT THE UNITED STATES NATIONAL MUSEUM. DEFINITIVE IDENTIFICATIONS ARE MADE THERE AND COLLECTION DATA DEALING WITH BIOLOGY AND DISTRIBUTION TABULATED FOR LATER MACHINE PROCESSING. KEYS AND OTHER IDENTIFICATION AIDS ARE PRODUCED FOR FIELD UNITS, AND FOR LATER PUBLICATION. SPECIMENS ARE ALSO IDENTIFIED FROM OLDER COLLECTIONS AT VARIOUS MUSEUMS, AND ECOLOGICAL AND DISEASE DATA ABSTRACTED FROM PUBLISHED-LITERATURE.

(U) PROGRESS - OCT 67 THRU MAY 68 ADDITIONS WERE MADE TO THE ILLUSTRATIONS OF THAILAND ANOPHELES AND TO THE TEXT OF THE MONOGRAPH. INITIAL SPECIES FROM THE PHILIPPINES WERE ILLUSTRATED AND THE FIRST LARGE COLLECTION FROM THE EASTERN PORTION OF INDIA AND THE FOOTHILLS OF THE HIMALAYAN RANGE WAS RECEIVED FOR EXAMINATION. THE ANOPHELES FAUNA OF VIETNAM HAS BEEN REEXAMINED IN PREPARATION FOR A SECOND REVISION OF IDENTIFICATION KEYS. DETAILED STUDY OF THE MINIMUS SPECIES GROUP IN THAILAND ARE IN PROGRESS WITH LOCAL COOPERATION, RESULTING THUS FAR IN THE REMOVAL OF SEVERAL SPECIES FROM THE FAUNAL LIST BY CONSOLIDATION. ONE ADDITIONAL SPECIES WAS FOUND BREEDING IN CRAB HOLES - A HITHERTO UNEXPLORED ECOLOGICAL NICHE IN THAILAND. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25. COMMUNICATIONS SECURITY	26.	27. DOD CODE	28. BUDGET CODE
☐ COMPLEX OR COMPLEX RELATED ☒ NOT RELATED		EP	1

29. MISSION OBJECTIVE	30. PARTICIPATION
NA	NA

31. REQUESTING AGENCY	32. SPECIAL EQUIPMENT

33. EST. FUNDS (IN THOUSANDS)	34.

FORM 1 JAN 66 1490m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 25 identical to NASA Form 1122)

Project 3A635301D829 MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 125, Taxonomy and ecology of mosquitoes of SE Asia

Investigators
Principal: LTC J.E. Scanlon, MSC
Associate: Dr. B. de Meillon*, MSG E. Peyton, CPT J. Reinert and Dr. Y.M. Huang*

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 for later collation with published data on the ecology of the various species. Studies under this work unit are performed in conjunction with the Smithsonian Institution under contract MD-2672.

Progress

1. Field studies

Collections of some members of the hyrcanus group were made in South Thailand which appear to differ from the previously known species. These are still under investigation. No further field observations were possible on the important leucosphyrus group in 1967, since the field area, Koh Samui Island, appears to have none of these species. The only other Anopheles detected in numbers on the island, An. sundaicus, is an important malaria vector in SE Asia, but is sporadic in distribution and association with infections and appeared to be unimportant on Koh Samui. Arrangements were made for field collection of members of the Myzomyia group, including sibling rearings of minimus and associated species in the group. Standard morphological taxonomy has probably reached its limit in delineating the members of the group without access to sibling rearings.

Collections continued by SEATO personnel in Thailand, and by cooperating individuals in other parts of SE Asia, including Malaysia, India, Cambodia, Taiwan and the Philippines. Staff members did not participate in the field studies to as great an extent as in the previous year, but one trip was made to Indonesia to obtain assistance in further field studies. Local conditions did not permit collections during the period of discussions.

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2. Museum studies

a. General. During the year 45 collections consisting of 14,080 mounted adults and 10,376 slide preparations were received from 26 different military and civilian sources. The bulk of the material came from the SEATO Laboratory and a collaborator at the University of Malaysia. Small lots for determination or confirmation were received from Army and Navy units in Vietnam and Taiwan.

b. Anopheles. Several additional species were added to the Thailand and SE Asian lists during the period, either as new species as not yet described, or the reinstatement of names regarded as synonyms. Further work on the leucosphyrus group has indicated that little more may be expected in revision of that group unless areas as yet completely unsurveyed (such as the Lesser Sundas) yield additional forms. A local population on Con Son Island (Puolo Condore), Vietnam, appeared to differ morphologically from the form on the mainland, but a detailed examination of all stages indicates that it is merely a local race, not sufficiently different to warrent taxonomic status. Further examination of the Myzomyia group species in the collection indicates that the extension of some of the species in SE Asia may be far less than previously believed, due to confused identifications. Since several of the most important malaria vectors in SE Asia are in this group further studies are underway. The plates of Anopheles species for the Thailand publication have been revised to include the phallosome leaflets, and in some cases the pharyngeal apparatus where these structures may assist in identification. Some additional progress has been made on the text. Another species has been found from burrows of fresh water crabs in Thailand, Anopheles kyondawensis, described from fresh water pools from Burma over thirty years ago and never reported again. This habitat, which is very extensive in the tropics, appears to have been neglected in past surveys in SE Asia.

A revision of the keys to the Anopheles of Vietnam, primarily for use by military units in the field, is underway in cooperation with Dr. Quy of the Pasteur Institute, Saigon. Progress has also been made on an illustrated key to the larvae of the Thailand Anopheles, similar to the adult key published in 1967.

c. Heizmannia. Study of this genus has been completed by a consultant, Dr. P.F. Mattingly, British Museum. Illustrations have been completed and the publication will appear in a few months.

d. Aedes. Additional species of the subgenus Neomaclaya have been covered in a publication by Dr. Delfinado who left the project this year and is now at the University of Hawaii. The first in a series of papers on the subgenus Finlaya was published during the year by Dr. K. Knight, University of North Carolina, another contributor to the project. Dr. Huang has begun a detailed study of the Stegomyia species and has made a number of clarifications in these very important species. In particular, she has demonstrated the existance of a number of species in Thailand belonging to the albopictus group and it is hoped that this will assist in field studies

on control procedures this year. CPT Reinert has been examing the subgenus Diceromyia and has found that it is composed of two rather well defined groups of species, one from the Ethopian Region, the other from the Oriental Region. Six species are found within the project area, but all are poorly represented in the collections at present.

e. Orthopodomyia. A manuscript, covering all of the species in the SE Asian area, has been prepared by Dr. T. Zavortinck, consultant to the project, and will be published in late 1968 when the illustrations have been completed.

f. Uranotaenia. MSG Peyton has examined approximately 2,500 adults and 2,000 slides of these little known species in the USNM collection, primarily from Thailand. The last published compilation of Uranotaenia from Thailand listed 19 species, but at least 34 are now known, including at least 10 new to science. Other new species have been found from South Vietnam, Malaya and the Philippines. Most of the early work on the genus referred to the female stages only, particularly the color patterns. With access to large reared series from Thailand and Malaya it has been possible to depend on morphological features not as subject to variability as the color patterns.

g. Culex. Dr. Bram published several additional clarifying papers on the genus before his departure from the project. Dr. J. Klein, Pasteur Institute, Cambodia, has been furnished with all of the specimens of the subgenus Mocthogenes in the collection and he is revising that group.

h. Other genera. Other consultants are working on the genera Tripteroides, Topomyia, Zeugnomyia and Armigeres. All but a few rare species groups have now been assigned to members of the project from WRAIR or the Smithsonian Institution or collaborators, and detailed studies have been completed or are well underway on most. Additional specimens, particularly reared series, are still urgently needed from the more remote areas, including the outer islands of the Indonesian Archipelago, South China and East Pakistan, in order to determine the limits of distribution and variability of the various species.

Summary and Conclusions

Taxonomic studies were continued on the mosquito fauna of Southeast Asia. Discussions of a number of genera and species groups have been completed and are in various stages of publication. All of the important species groups have now been assigned to project personnel and various consultants and collaborators. It is believed that the entire project will be completed in approximately two additional years. Difficulty has been encountered in obtaining specimens from some of the remote areas of the project region, but plans are underway to overcome this difficulty.

Publications

1. Bram, R.A. 1967. Lectotype assignments for several species of the genus Culex in Southeast Asia (Diptera: Culicidae). Proc. Ent. Soc. Wash. 69(4):327-

2. Bram, R.A. 1968. A re[illegible]scription of Culex (Acallecomyia) obscurus (Leicester) (Diptera: Culicidae). Proc. Ent. Soc. Wash. 70(1):52-

RESEARCH AND TECHNOLOGY RESULTS

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26. (U) TECH OBJECTIVE - TO STUDY BY MEANS OF AN IN VITRO SYSTEM, THE METABOLIC ACTIVITIES OF THE MOSQUITO PHASE OF MALARIA PARASITES, PLASMODIUM GALLINACEUM AND P. BERGHEI YOELII, THE PHYSIOLOGY AND BIOCHEMISTRY OF THE INTERACTION BETWEEN THE INVERTEBRATE HOST AEDES AEGYPTI AND ANOPHELES STEPHENSI RESPECTIVELY AND PARASITE, AND THE FACTORS RESPONSIBLE FOR HOST SPECIFICITY OF THE PARASITES. MAINTENANCE AND DEVELOPMENT OF THE VARIOUS PARASITIC STAGES IN VITRO WOULD PROVIDE A SYSTEM FOR COLLECTING ANY ANTIGENS ELABORATED BY THE PARASITES OVER A FAIRLY EXTENSIVE PERIOD OF TIME. THE POSSIBILITY EXISTS THAT SUCH ANTIGENS COULD BE UTILIZED IN THE PRODUCTION OF ANTIMALARIAL VACCINES.

27. (U) APPROACH- DEVELOPMENT OF A CULTURE MEDIUM WHICH WILL PERMIT THE DIFFERENTIATION AND GROWTH IN VITRO OF THE INVERTEBRATE STAGES OF THE ABOVE-MENTIONED PARASITES. EVALUATION OF THE COMPETENCE OF VARIOUS ORGANS, TISSUES AND CELLS FROM THE APPROPRIATE MOSQUITO SPECIES TO PROVIDE THE PARASITES WITH A CELLULAR MILIEU IN VITRO COMPARABLE TO THAT FOUND IN VIVO. BIOCHEMICAL REQUIREMENTS OF THE PARASITES WILL BE DETERMINED PRIMARILY BY THE EMPLOYMENT OF CHROMATOGRAPHIC TECHNIQUES.

28. (U) PROGRESS - SEP 67 THRU MAY 68 CELL LINES OF AEDES AEGYPTI AND A. ALBOPICTUS, DEVELOPED BY DR. K. SINGH, HAVE BEEN OBTAINED. P. GALLINACEUM OOCYSTS OF VARIOUS AGES HAVE BEEN CULTURED IN T-FLASKS USING THESE CELL LINES AS SUPPORTING LAYERS. LITTLE OR NO FURTHER DEVELOPMENT OF THE OOCYSTS TAKES PLACE IN THE A. AEGYPTI LINE. PRELIMINARY RESULTS WITH THE ALBOPICTUS LINE ARE MORE ENCOURAGING. A RELIABLE METHOD FOR OBTAINING PRIMARY CULTURES OF ANOPHELES STEPHENSI EMBRYONIC CELLS HAS BEEN DEVELOPED. ATTEMPTS TO INITIATE CELL CULTURES FROM PUPAL OR ADULT TISSUES HAVE NOT BEEN SUCCESSFUL FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 126, Cultivation of mosquito tissues and malaria parasites *in vitro*

Investigators

Principal: Imogene Schneider, Ph.D., COL Robert T. Jensen, MC
Associate: 1LT David H. Chen, MSC

Description

These investigations are designed to develop methods for the production of large numbers of malarial parasites free or virtually free of host tissue for immunological, biochemical and physiological studies with particular emphasis on host specificity of the invertebrate phases. *In vitro* procedures are used for the cultivation of mosquito tissues, growth and differentiation of the sporogonous stages of Plasmodia and the attempted culture of the exoerythrocytic stages in mammalian liver cells. The technique of density gradient centrifugation is being explored as an alternative to the *in vitro* system for the mass isolation of sporozoites.

Progress

1. Cultivation of mosquito tissues and the sporogonous phase of malarial parasites

In a preliminary study, *P. gallinaceum* oocysts of various ages were excised from the midgut of the host, *A. aegypti*, placed in sitting drop cultures and their further development, or lack of it, observed over an interval of three or more days. The culture medium of Grace (Nature, 1962, 195:788-789), slightly modified with respect to the pH and osmotic pressure, was employed. With few exceptions, little or no development took place in oocysts which were 7 days old or less and such oocysts rarely survived more than 36 hours *in vitro*. The extent of development in eight day oocysts depended upon whether or not sporoblast formation with subsequent budding of the sporozoites had occurred before the oocysts were placed in culture. If incipient sporozoites were already present, oocysts in approximately 60 per cent of the cultures developed to the point of liberating sporozoites; if not, the percentage dropped to less than one per cent. Nine day oocysts usually ruptured within 12 hours and the released sporozoites remained viable at least 7 days. However, infectivity was lost if they remained in culture for more than 24 hours. Even within this short interval the sporozoites became somewhat attenuated. $5X10^4$ sporozoites were required to induce an infection with a prepatent period of 11 days which was double that of the controls.

Since the culture system employed was a relatively static one it seemed plausible that more substantial results would be obtained by the addition of actively growing mosquito cells to the cultures. During the earlier part of this study only one established cell strain of A. aegypti was in existence and a culture of this strain was obtained from Dr. T.D.C. Grace of Canberra, Australia.

The cell strain was grown in 30 ml T-flasks; the culture of oocysts plus cells was maintained in sitting drops or in Rose chambers. Dialysis tubing was occasionally employed in the Rose chambers to separate cells and oocysts. All cultures were maintained at 25 - 26°C. Parallel sets of experiments were carried out in which the oocysts were placed in culture with Grace's cell strain in Grace's original medium and secondly, in the modified medium to which the cell strain had been adapted over a period of approximately two months. Supplements to either medium consisted of a 10 per cent concentration of either chick or fetal bovine serum and a 0.5 per cent concentration of Átteva sp. (Lepidoptera: Yponomeulidae) hemolymph. A limited number of cultures contained approximately one per cent homologous hemolymph in place of the Átteva supplement. Regardless of the medium employed the results were essentially identical and are discussed together in the following paragraphs. Minor differences as recorded are shown in Table I.

Nine and eight day oocysts followed the same pattern of development as was seen in cultures without the cells. However, eight day oocysts when cultured with the cells showed a somewhat higher percentage and a faster rate of development as compared to oocysts cultured alone. On the adverse side there was a much greater tendency on the part of the oocysts not to rupture spontaneously in vitro once their development was complete or apparently complete. As many as one half of the oocysts in any one culture did not rupture even though apparently mature sporozoites maintained active movements within the oocysts for as long as fifteen days, provided the medium was renewed once or twice during this interval.

Younger oocysts showed varying behavior depending on the concentration of cells present in the culture. Concentrations equivalent to 10^5 cells/ml had no detectable effect. The oocysts survived for 24 to 48 hours but little or no further development was noted. If the concentration was raised to the equivalent of 10^6 cells/ml seven day oocysts continued to develop for the first 24 hours as evidenced by the visible partitioning of the oocyst cytoplasm. However, after this short period the cells began to overgrow the cultures. Once this occurred the oocysts rapidly degenerated and the cells themselves began to deteriorate after 72 hours. Attempts to maintain a fairly constant cell number by removing cells and partially renewing the medium every 24 to 48 hours were not successful. Placing the oocysts and cells, separated by dialysis tubing, in Rose chambers appeared to hold some promise but was eventually discontinued due to the difficulty of positioning the oocysts and keeping them so positioned for microscopic examination.

Since a certain initial concentration of cells seemed necessary for the oocysts to continue their development the problem of overcrowding by

the growing cells was circumvented by employing the feeder layer technique. Mitomycin C was selected to render the cells incapable of multiplication. Four different concentrations of the antibiotic were tested, namely, 50, 5, 2.5 and 0.5 γ/ml (see Table II). Cultures containing approximately 3×10^5 cells/ml were exposed to Mitomycin C for 6 hours, washed twice, counted and allowed to incubate in Mitomycin C-free medium prior to being recounted at 24, 48, 96 and 144 hours. The first two concentrations were toxic to the cells, the mortality being virtually 100 percent after two and three days, respectively. At 2.5 γ/ml almost one-half of the cells were dead after six days and multiplication of the rest was effectively suppressed. At a concentration of 0.5 γ/ml little cell death was recorded and the cells continued to multiply although at a reduced rate. Seven day oocysts placed in culture with cells which had survived the treatment with 2.5 γ/ml were not affected too much either way. Approximately one third of the oocysts developed to a slight extent, another third showed no change and the final third appeared granular after 24 hours. Somewhat better results were obtained with cells treated with 0.5 γ/ml. Almost one-half of the oocysts developed to the extent of containing immature sporozoites after a period of three or four days. However, this was balanced by an almost equal number which showed no change or deterioration in the same length of time.

Replacement of heterologous hemolymph by A. aegypti hemolymph had no apparent effect on either the survival time or development of the oocysts. Due to the difficulty of extracting it, the amount was very small and, consequently, it was used to supplement the medium only to the extent of one per cent in each of three cultures. Whether a higher concentration of hemolymph would have been beneficial is a moot question as the small size of the mosquito precluded obtaining sufficient amounts.

Recently, two more cell lines derived from mosquito tissues have been made available to investigators by Dr. K.P.R. Singh of Poona, India. The cell lines originated from first instar larvae of Aedes aegypti (L.) and Aedes albopictus (Skuse), respectively. The former contains cells which are predominantly diploid whereas the cells of the latter strain display various degrees of polyploidy in addition to diploid cells. At present, only a few attempts have been made to place oocysts in the Singh A. aegypti cells and results indicate that this line probably is no more effective insofar as supporting the growth and differentiation of P. gallinaceum oocysts than is the Grace A. aegypti cell line. As yet, oocysts have not been placed in culture with the A. albopictus cells.

In the successful establishment of any cell strain the process of adaptation inevitably leads to modification in the cells' morphology and properties. Such cells may, in many specific details, bear little resemblance to the cells of the original donor. Considering the host specificity of the malaria parasites, it seems quite plausible that such parasites have fairly stringent requirements for growth and development, requirements which the Grace cell strain and, in all probability, the Singh cell strains have lost the capacity to fulfill. Emphasis was, therefore, shifted toward the use of short term primary cell cultures. Attempts to obtain such cultures from adult A. aegypti ovarian tissue have failed. The methods used were

similar to those outlined by Kitamura (Kobe J. Med. Sci. 1966, 12:63-70) who claimed success for cultures involving ovaries from A. aegypti, A. albopictus and Culex pipiens. Cell migration was extensive for the first few weeks and the ovaries, either intact or cut in pieces, continued to contract for three or more weeks but cell division was not detected. Altogether, 47 cultures were initiated, having anywhere from 10 to 200 ovaries per culture. Attempts to culture first larval instar cells of A. aegypti were fairly successful but emphasis was then switched to the Anopheles stephensi - P. berghei yoelii system. Hence, efforts were made to culture anopheline cells. Primary cell cultures of larval A. stephensi can now be routinely made and one cell line, at least tentatively, has been established. This line is now being characterized on the basis of general morphology, karyology and growth characteristics. P. berghei yoelii oocysts will be introduced into the above cultures in the near future. Attempts to culture anopheline adult tissues have not been successful.

2. Cultivation of the exoerythrocytic stage of mammalian malaria in vitro

The first series of experiments were designed to study rodent liver cells in tissue culture chambers with variation in (1) type of rodent, e.g., mice, hamsters, rats, (2) nutrient media, (3) substrate, (4) age of animal and (5) methods in preparation of the tissue explant. When available, Plasmodium berghei yoelii sporozoites were inoculated into rodent liver cultures and P. cynomolgi into Rhesus monkey liver cultures.

The tissue explants were prepared as follows: a small section of the liver was surgically removed and transferred to a sterile petri dish. The section was flooded one or more times with Earle's balanced salt solution to remove as many blood cells as possible. Excess fluid was then withdrawn from the section by blotting with filter paper. Intact liver cells were separated by a gentle raking process. Once sufficient numbers of cells had accumulated on the edge of the rake they were transferred by brush onto a collagen substrate on the floor of a Sykes-Moore culture chamber and spread over the surface in a fairly uniform monolayer. This method, at least with respect to the monkey liver, has been more successful in isolating the cells than has the more conventional methods of dispersing cells by trypsin or versene.

The basic medium employed has been TC-199 supplemented with 20% inactivated calf serum and 5% chick embryo extract. The cultures were maintained at a temperature of 37 ± 1°C. Thus far, it has not been possible to maintain hepatic parenchymal cells for more than two weeks in the culture chambers. The great majority of hepatocytes appear to start degenerating almost from the time of culture. This degeneration is characterized by a loss of cytoplasmic detail and later by nuclear deterioration.

It has also been noted that the liver of an older animal has a greater number of fat bodies and glycogen granules than that of a juvenile

animal. Embryonic lines would send outgrowths of fresh hepatocytes in cords onto a substrate of collagen. It is doubtful, however, whether these cells are physiologically the same as mature liver cells.

It has been found that cells from monkey liver are easier to work with than rodent liver. The best size for the Rhesus monkey was at 5 to 6 pounds; when the monkeys were over eight pounds the liver was very fat; under five pounds there was less fat but the plasma membranes of the cells were more delicate and easily ruptured in raking and brushing the cells.

Efforts thus far to isolate liver cells in a regenerative cycle have been only partially successful. It has been noted that the liver cells in vivo seem to be in a cycle of regeneration and senescence. If the cells were isolated at the start of their growth cycle they appeared to have greater viability in vitro. The regenerative cells can be distinguished by their larger and denser appearing nuclei and by evidence of amitotic cell divisions.

Efforts to obtain penetration of the hepatic cells by sporozoites in vitro have not been successful. However, in one experiment it was noted that sporozoites if injected directly into an aberrant liver lobe (small appendage) would readily produce an infection. Also if this aberrant lobe was excised within an hour after injection it was possible to locate a few sporozoites in tissue culture and some of the sporozoites appeared to have attached to but not yet penetrated the hepatocytes.

It is planned to repeat this experiment in surgically produced liver appendages with variation in the time the sporozoites remain in vivo before excising for culture in vitro. In addition, tissue will be removed and prepared for electron microscopy of the very young cryptozoite stages.

Current experiments are designed to study the survival of sporozoites and rodent hepatic cells in vitro at different incubation temperatures.

3. Isolation of sporozoites by density gradient centrifugation

The method outlined below for the homogenization of infected adult female A. aegypti with subsequent filtration and centrifugation steps yields approximately 36 x 10^6 sporozoites per 1000 mosquitoes. Although the numbers of sporozoites collected are considerably greater than those obtained in the culture system, the preparations are not as clean as in the latter. Some cell debris as well as microorganisms is invariably present in the same fractions as the sporozoites and must be eliminated by further purification steps.

Approximately 1000 A. aegypti females (3.4 gm), heavily infected with P. gallinaceum sporozoites, are homogenized in 40 ml of Clement's buffered salt solution. The temperature at this and all subsequent steps is maintained at 4°C. The homogenate is gravity filtered through a 200 mesh bolting silk disc (47 mm diameter) mounted on a Millipore filter holder equipped with a stainless steel screen. Aliquot rinses are made under vacuum and the filtrate twice centrifuged at 100xg for 2 minutes in a Sorvall RC-2 refrigerated centrifuge equipped with a HB-4 swinging bucket rotor.

The supernatant, containing most of the sporozoites as well as some debris, is transferred to one or more clean tubes. The tubes are then spun at 2600xg to bring the sporozoites into a pellet. The pellet is resuspended and washed a second time. The preparation at the end of the differential centrifugation steps shows a 95 per cent reduction from the original mass, to approximately 170 mg.

A linear sucrose gradient is formed by mixing 13 ml each of a 31 per cent and a 43 per cent buffered sucrose solution into a 30 ml centrifuge tube. The mosquito preparation is resuspended in 2 ml buffered saline and layered on the gradient. After centrifugation for 60 minutes at 16,000xg, 26 one-ml-fractions are collected, diluted 1:4, spread on microscope slides and stained with Giemsa. Using oil immersion, the sporozoites are counted in a randomly selected area and for the width of the slide within that area. The numbers counted are then multiplied by a factor of 765 (determined by the length of the slide and the area of the grid used) to obtain a value for the entire slide.

The concentration of sporozoites per tube number and the respective percentages and densities within the sucrose gradient for a typical run are shown in Figure 1. Analysis of a typical peak fraction (tube #15) shows a sporozoite concentration of 9.7×10^6 per slide with a mean density of about 1.16 g/cc. Tubes 10 - 18 contained approximately 36×10^6 sporozoites or about 80 per cent of the total in the gradient. Considering all of the sporozoites in the gradient, the number of sporozoites recovered per mosquito was estimated at 40,000.

Further purification techniques are being explored, among the most promising being gel filtration, liquid-liquid polymer extraction and the formation of antibody-antigen complexes.

Summary and Conclusions

The extent of development in seven, eight and nine day *Plasmodium gallinaceum* oocysts cultured in the presence of the Grace cell strain of *Aedes aegypti* (L.) is described. Relatively little improvement was noted in the development of such oocysts when compared to their being cultured in medium alone. The failure of this cell strain to evoke a more promising response from the oocysts has been attributed to modifications in the cells' morphology and properties which inevitably accrue during the process of adaptation. More substantial results might be obtained by the use of short term primary cultures of adult mosquito tissue. Unfortunately, reliable techniques for obtaining such cultures are not yet available. It has been possible to obtain short term primary cultures from larval mosquito tissues. When provided with a larval milieu such primary cultures may stimulate the growth and development of the plasmodial oocysts to a far greater extent than was observed with the established cell lines from adult mosquitoes.

A method has been developed to obtain liver cells in a monolayer in Sykes-Moore culture chambers. The survival time of these cells is variable depending apparently on the phase of the natural regenerative cycle of the cells. Attempts thus far to induce penetration of these cells with sporozoites have not been successful.

Current efforts are directed at attempting to improve the survival of hepatocytes in culture by variation in temperature and by isolation of regenerative cells. Experiments planned are directed at the problem of surgical creation of small liver appendages for in vivo injection of sporozoites and phased time resection of these appendages for culture in vitro as well as for fine structure studies.

Density gradient centrifugation offers promise as a procedure for the separation of sporozoites from host tissue. Further modification of the technique is required to increase the yield and accelerate the collecting process.

Publications

Schneider, I. 1967. Insect Tissue Culture. In: Methods in Developmental Biology. Thomas Y. Crowell Company. New York. pp. 543-554.

TABLE 1

Response of 6, 7, 8 and 9 day old P. gallinaceum oocysts to conditions in vitro

Age of oocysts in days	Culture milieu	No. of cultures / No. of oocysts	Observations
6	Grace's A. aegypti cells in Grace medium	2/40	Survival for 24 hours; no differentiation
7		1/20	Survival for 24-48 hours; some visible partitioning of oocyst cytoplasm
8 (young)		2/41	Cytoplasmic cleavage in approx. 1/2 of oocysts
8 (mature)		4/90	Mature sporozoites within 24 hours; oocysts did not rupture
9		3/60	Mature sporozoites; 60% did not rupture
6	Grace's A. aegypti cells in modified Grace medium	2/44	Survival for 24-48 hours; no differentiation
7		2/38	Survival for 24-48 hours; partitioning of oocyst cytoplasm
8 (Young)		3/70	Cytoplasmic cleavage in majority of oocysts; immature sporozoites readily visible; oocysts did not rupture
8 (mature)		4/91	Apparently mature sporozoites within 24 hours but majority of oocysts did not rupture; infectivity of sporozoites was not tested
9		3/60	Mature sporozoites; 90% oocysts ruptured; infectivity of sporozoites not tested
6	Modified Grace medium	3/73	Majority of oocysts degenerated in 24 hours; no differentiation
7		4/60	Same as above
8 (young)		3/62	Cytoplasmic cleavage in 3 oocysts
8 (mature)		3/120	Cytoplasmic cleavage; immature sporozoites; none of oocysts ruptured
9		8/194	Sporozoites freed within 24 hours; one of three chicks injected with sporozoites developed parasitemia after 12 days

TABLE 1

Response of 6, 7, 8 and 9 day old P. gallinaceum oocysts to conditions in vitro (continued)

Age of oocysts in days	Culture milieu	No. of cultures / No. of oocysts	Observations
6	Modified Grace medium with A. aegypti ovaries and/or salivary glands	7/144	Survival for 24-48 hours; no differentiation
7		7/168	Survival for 24-72 hours; slight increase in size for some of oocysts; no further development
8 (young)		10/212	Cytoplasmic cleavage in 28 oocysts; relatively mature sporozoites in 2 oocysts but latter did not rupture
8 (mature)		25/587	Many oocysts with mature sporozoites; some oocysts ruptured in 20 cultures; sporozoites from 4 cultures injected into one chick; prepatent period was 12 days
9		23/565	Free sporozoites; five cultures with unruptured oocysts averaging 4 oocysts per culture. Two chicks injected with 2 and 4 cultures, respectively, developed parasitemias. Sporozoites freed longer than 24 hours noninfective.

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TABLE 2

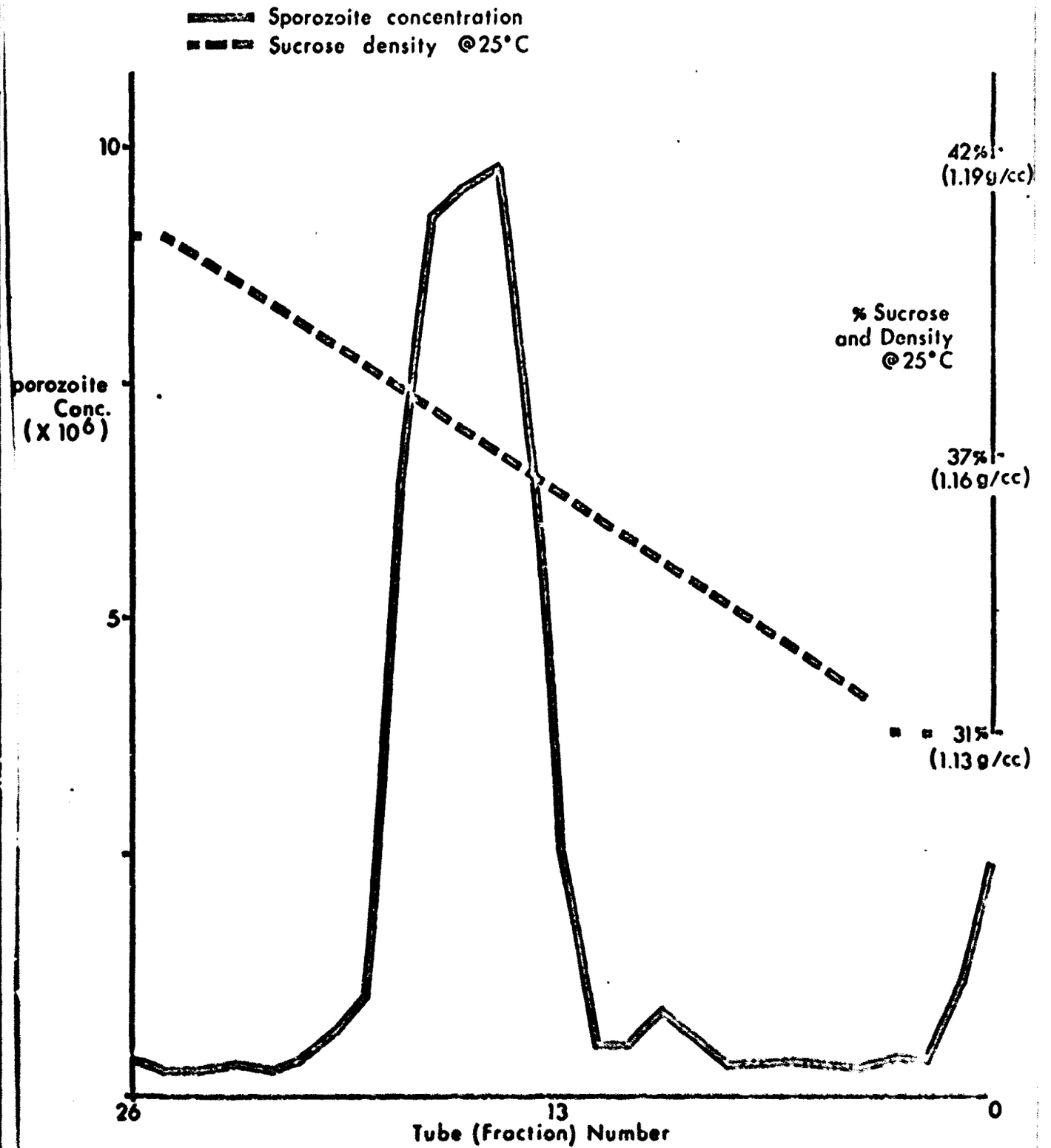
Exposure of Grace's Aedes aegypti cell strain to Mitomycin C

Cell line: MSQ 22/0.5% Atteva/10% FBS/ Modified Grace Medium

Conc. of Mitomycin C per ml	Number of viable cells per ml of medium					
	Initial count	Count after washing	Day 1	Day 2	Day 4	Day 6
50 µ	326 x 10^3	21 x 10^3	<10^3	0	0	0
5 µ	326 x 10^3	62 x 10^3	39 x 10^3	<10^3	<10^3	0
2.5 µ	375 x 10^3	71 x 10^3	53 x 10^3	59 x 10^3	40 x 10^3	37 x 10^3
0.5 µ	355 x 10^3	47 x 10^3	59 x 10^3	67 x 10^3	96 x 10^3	208 x 10^3
Control	300 x 10^3	131 x 10^3	141 x 10^3	226 x 10^3	576 x 10^3	1441 x 10^3

FIGURE 1.

DISTRIBUTION OF SPOROZOITES IN 31%-42% LINEAR SUCROSE DENSITY GRADIENT



RESEARCH AND TECHNOLOGY RESUME

				63 07 68	0600-168
67 07 68	D. 0000 30 11 67	U U	NA	DC	A. 10.8.1111

63161011 3M463018D829 00 127

(U) TEST SYSTEMS FOR PLASMODIUM FALCIPARUM 09

	07 69	04	OTHER DA	
	68	3	100	
C. 0000	69	3	100	

WALTER REED ARMY INST OF RES
WASHINGTON D C 20012

NA

NAME	WALTER REED ARMY INST OF RES
ADDRESS	DIV OF CD AND I WASHINGTON D C 20012
INVESTIGATORS PRINCIPAL	SADUN, E. H. SC.D.
ASSOCIATE	DOO, J. P. P. S.
TEL	202-576-3551 EXT DA

PERONEY, COL W. I.
202-576-3551

DRUG TEST SYSTEM

NA

MALARIA, CHIMPANZEE, CHLOROQUINE, IMMUNITY, ERYTHROCYTE, GAMMA GLOBULIN, ISOTOPE, SUSCEPTIBILITY, OWL MONKEY.

(U) TECH OBJECTIVE - SUSCEPTIBILITY OF CHIMPANZEES AND OTHER PRIMATES TO INFECTIONS OF HUMAN FALCIPARUM MALARIA. STUDY THE CHARACTERISTICS OF DRUG RESISTANT STRAINS, PROVIDE HIGH DENSITY OF PARASITES FOR PHARMACOLOGICAL AND BIOCHEMICAL STUDIES, CONDUCT PHYSIOLOGICAL AND IMMUNOLOGICAL STUDIES OF FALCIPARUM MALARIA AND PROVIDE TEST ANTIGENS FOR CHEMOTHERAPEUTIC AND IMMUNOLOGICAL INVESTIGATIONS.

(U) APPROACH- INFECT SPLENECTOMIZED, DRUG TREATED CHIMPANZEES, AND SAIMIRUS, WITH P. FALCIPARUM OF HUMAN ORIGIN (AFRICAN AND SOUTHEAST ASIAN STRAINS). OBSERVE THE EXTENT AND DURATION OF PARASITEMIAS, STUDY THE RESPONSE OF DIFFERENT STRAINS TO CHEMOTHERAPY, STUDY SUSCEPTIBILITY TO REINFECTION WITH HOMOLOGOUS AND HETEROLOGOUS STRAINS AND THE EFFECT OF IMMUNE GLOBULINS OBTAINED FROM HUMANS INFECTED WITH HOMOLOGOUS AND HETEROLOGOUS STRAINS. DETERMINE IODINE TAGGED HUMAN GAMMA GLOBULIN TURNOVER TIME AND CHROMIUM TAGGED LIFE SPAN OF COMPATIBLE AND INCOMPATIBLE HUMAN ERYTHROCYTES.

(U) PROGRESS - OCT 67 THRU JUN 68 CHLOROQUINE RESISTANT PLASMODIUM FALCIPARUM HAS BEEN MAINTAINED IN CHIMPANZEES THROUGH THE 14TH PASSAGE. PARASITEMIAS USUALLY REACH LEVELS OF AT LEAST 30 PERCENT. SIX PASSAGES OF P. FALCIPARUM HAVE BEEN MADE IN 15 SPLENECTOMIZED OWL MONKEYS. PREPATENT PERIOD HAS BEEN REDUCED FROM THE 40-60 DAY RANGE OF THE FIRST PASSAGE FROM CHIMPS TO 3-10 DAYS BY OWL MONKEY TO OWL MONKEY PASSAGE. GAMETOCYTES APPROACHING MATURITY HAVE BEEN SEEN BUT THEY HAVE INFECTED MOSQUITOES. FOUR PASSAGES OF PLASMODIUM VIVAX HAVE BEEN MADE IN OWL MONKEYS. PREPATENT PERIOD RANGED FROM 8-25 DAYS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

		63	1
NA		NA	

Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 127, Test Systems for Plasmodium falciparum

Investigators
Principal: Elvio H. Sadun, Sc.D.
Associate: CPT John C. Key, VC

Description.

The objective of this unit is to develope test systems which can be used to study the characteristics of drug resistant strains; to provide large numbers of parasites for morphological, physicochemical, and immunological studies, and to provide test animals for chemotherapeutic and immunological observations.

Progress.

1. Susceptibility of Aotus trivirgatus to infection with Plasmodium falciparum and Plasmodium vivax. Attempts were made to infect splenectomized owl monkeys (Aotus trivirgatus) with the Camp strain of Plasmodium falciparum and a strain of P. vivax obtained from a naturally infected human patient. Whole blood inocula containing approximately 10^8 parasitized red blood cells were used in each experiment. The inoculum was given by intravenous injection.

The first experiment was an attempt to infect three splenectomized owl monkeys (Nos. 3, 4, and 5) with blood infected with P. falciparum obtained from a chimpanzee with a high parasitemia. Since none of these owl monkeys had developed a patent parasitemia after 38 days, they were reinocula with blood from another chimpanzee.

The second P. falciparum experiment was an attempt to infect two nonsplenectomized owl monkeys (Nos. 6 and 7) with blood from an infected chimpanzee. Inoculations were made in the same manner as that used for the splenectomized owl monkeys.

The third experiment was done to determine the susceptibility of owl monkeys to P. falciparum subinoculated from a previously infected owl monkey (No. 3). Subinoculations were made to owl monkeys (Nos. 8 and 10) as the parasitemia neared the peak. Again about 10^8 parasites were given intravenously.

Blood obtained from a patient infected with P. vivax was used in an experiment to determine the susceptibility of the owl monkey to this species of parasite. Two splenectomized owl monkeys were inoculated with 10^8 parasites intravenously.

Examinations were made for patent parasitemia in each owl monkey three times weekly. After the first positive smear was found on an animal, slides were prepared and examined daily. Parasitemias were expressed as positive on the thick smear, the number of parasitized RBC's per 100 WBC's and the number of parasitized RBC's per 100 RBC's.

Results of the inoculations with P. falciparum are shown in the table. One owl monkey (No. 3) developed a patent parasitemia from the first inoculation of P. falciparum on the 48th day after inoculation. The parasitemia rose gradually and reached a peak of 11 1/2 percent on the 76th day after inoculation. This monkey died 48 days after inoculation. Another owl monkey (No. 5) developed a patent parasitemia 60 days after the second inoculation was made. The parasitemia in this monkey has not completed its course as the current parasitemia of 1 1/2 percent is the highest level that has been reached. The third owl monkey in the group has not developed a patent parasitemia.

Neither of the two nonsplenectomized owl monkeys (Nos. 6 and 7) developed a patent parasitemia even though they were splenectomized 80 days after the original inoculation.

Table 1

Plasmodium falciparum Infections in Owl Monkeys, Aotus trivirgatus

Monkey No.	Prepatent Period	Days Positive	Highest Parasitemia
3	48 days	36	11 1/2%
4	-	-	-
5	59 days	31	1 1/2%
6	-	-	-
7	-	-	-
8	4 days	44	1%
10	3 days	48	21/100WBC

Both of the animals subinoculated from monkey No. 3 developed patent parasitemias. The parasitemia of one of these (No. 8) showed a very slow rise and reached a peak of about 1.0% 33 days after inoculation. Although the parasitemia had dropped to less than 5 parasites per 100 WBC's, this monkey died 44 days after inoculation. At necropsy gross lesions were minimal. In an attempt to elucidate the cause of death

bacterial, viral, and histopathological studies are being done. The second monkey in this group also developed a patent parasitemia about 4 days after inoculation, but the parasitemia has remained very low, usually less than 4 parasites per 100 WBC's. Most of the parasites seen in this owl monkey have been gametocytes. Anopheles stephensi mosquitoes have been fed on all owl monkeys which produced gametocytes, but none of the mosquitoes have become infective.

One of the two animals (No. 2) inoculated with the P. vivax developed a patent parasitemia 35 days after inoculation, and by the 63rd day after inoculation the parasitemia had progressed to a high of 275 parasites per 100 WBC's or about 2% of the RBC's. Subsequently the parasitemia dropped sharply, although it has remained positive at a low level.

2. Chloroquine refractory Plasmodium falciparum in chimpanzees. Plasmodium falciparum has been carried for 14 blood passages in chimpanzees. In the last five passages, four of the five chimpanzees reached parasitemias of 40% or higher. Usually the animals are patent in 3-10 days. The chloroquine resistance was tested in April 1968 and confirmed by passage in May 1968. This chimpanzee had a parasitemia of 38 percent.

Summary and Conclusions.

1. Owl monkeys inoculated with Plasmodium falciparum-infected chimpanzee blood developed parasitemias in 48-59 days. Owl monkeys inoculated with infected owl monkey blood became patent in 3-4 days. One of two owl monkeys inoculated with P. vivax-parasitized human blood developed a patent parasitemia in 35 days.

2. After 14 serial blood passages in chimpanzees the Camp. strain of P. falciparum was still chloroquine refractory.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 046519	CSGRD-193

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 30 11 67	U U	NA	FC	A. WORK UNIT

10. CURRENT NUMBER / CODE
63150D11 3A635301D829 00 128

10a. PROJECT NUMBER / CODE

11. TITLE
(U) NATURAL AND ACQUIRED IMMUNITY IN RODENT MALARIA

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. EST. COMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY	07 65	NA	OTHER DA

16. PRIMARY METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	a. NUMBER NA b. TYPE NA c. DATE NA d. AMOUNT NA	PRIOR FY 68	3	100
		CURRENT FY 69	3	100

19. GOVT. LAB/INSTALLATION/ACTIVITY
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WASHINGTON D C 20012

INVESTIGATORS PRINCIPAL: SADUN, E. H. SC.D.
ASSOCIATE: POON, A. P. MS
TEL: 202-576-3308 TYPE: DA

21. TECHNOLOGY UTILIZATION
IMMUNOLOGY

22. COORDINATION
NA

KEYWORDS: MALARIA, RODENTS, SUSCEPTIBILITY, IMMUNITY, BIOCHEMISTRY, PLASMODIUM, SPLENECTOMY, ANTIBODY,

23.

(U) TECH OBJECTIVE - TO EVALUATE THE ROLE OF HUMORAL AND CELLULAR FACTORS IN DETERMINING SUSCEPTIBILITY OF HOSTS TO RODENT MALARIA, FOR THE MAINTENANCE OF THE COMPLETE LIFE CYCLE OF MALARIA IN THE LABORATORY, TO FIND A LABORATORY ANIMAL SUITABLE AND FOR THE PRODUCTION OF LARGE AMOUNTS OF INFECTED BLOOD FOR IMMUNOLOGICAL AND BIOCHEMICAL STUDIES.

(U) APPROACH- 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.

(U) PROGRESS - OCT 67 THRU JUN 68 IN ORDER TO FURTHER EXPLORE THE IMMUNITY TO P. BERGHEI DEMONSTRATED IN RATS AFTER AN INITIAL INFECTION. LYMPHOCYTE TRANSFER STUDIES WERE DONE USING THE INBRED LEWIS STRAIN RAT. LYMPHOCYTES OBTAINED FROM THE LYMPH NODES AND SPLEEN OF ANIMALS PREVIOUSLY INFECTED WITH P. BERGHEI ARE CAPABLE OF CONFERRING IMMUNITY TO RECIPIENT ANIMALS. A DOSE RESPONSE RELATIONSHIP CAN BE DEMONSTRATED IN THE RECIPIENTS. WHETHER THE IMMUNE RESPONSE IS MEDIATED BY ENHANCED ANTIBODY PRODUCTION OR TISSUE IMMUNITY IS BEING STUDIED. THE SPLEEN PLAYS THE MOST IMPORTANT ROLE IN THE REMOVAL OF 59FE TAGGED PARASITIZED CELLS. BOTH IMMUNE AND NON SPECIFICALLY STIMULATED SPLEENS SHOW GREATER ACTIVITY THAN NORMAL SPLEENS. NO INCREASED LIVER SEQUESTRATION OCCURS IN SPLENECTOMIZED ANIMALS. IMMUNIZATION WITH IRRADIATED BLOOD FORMS OF P. BERGHEI YOELI SUPPRESSES PARASITEMIA AFTER CHALLENGE WITH SPOROZOITES. CONCENTRATED GLOBULINS FROM HYPER-IMMUNE RAT SERUM SUPPRESS PARASITEMIAS TO A GREATER EXTENT THAN WHOLE SERUM. FRACTIONATION OF THESE GLOBULINS INDICATES THAT SUPPRESSION IS OBTAINED WITH G AND/OR A BUT NOT WITH M. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
a. COVER OF SECRET RELATED [ ] b. NOT RELATED [X]		BR	1

MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

REQUESTING AGENCY	34. SPECIAL EQUIPMENT
EST. FUNDS (In thousands)	35.

DD FORM 1 JAN 66 1498M REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A6353O1D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 128, Natural and Acquired Immunity in Rodent Malaria

Investigators:
Principal: Elvio H. Sadun, Sc.D.
Associate: Norman T. Briggs; CPT Robert M. Donati, MC;
1LT Charles A. Stancer, VC; CPT Daniel J. Stechschulte;
Bruce T. Wellde

Description.

The objective of this work unit is to maintain parasites in the laboratory in animal species suitable for producing large amounts of infected blood for immunological and physicochemical studies and to delineate the roles of humoral and cellular factors in the susceptibility and reaction of hosts to infection.

Progress.

1. Protective antibody in rats infected with Plasmodium berghei. The production of circulating protective antibody in rats infected with P. berghei has been reported. The type or types of antibody produced and their sequence of production following the initial infection are the subject of this report.

Immune serum was collected from rats 18 days after they had received a single intraperitoneal injection of 1×10^8 RBC's parasitized with P. berghei. Hyperimmune serum was obtained from rats given four inoculations of 1×10^8 parasitized RBC's and bled 8 days after the last infection. Globulin was removed from serum by precipitation in 50% saturated ammonium sulfate. The globulins were resuspended in phosphate buffered saline, pH 7.2, using one-third the original serum volume. Sephadex G-200 and DEAE Sephadex chromatography were then utilized to separate IgM, IgG, and IgA classes of immunoglobulin and the results were verified by immunoelectrophoresis. The protective capacity of sera was evaluated according to previously described methods. Mice injected with 2×10^7 parasitized RBC's were injected with globulin and globulin fractions from normal and infected rats. Parasitemias in untreated controls ranged from 30-40% on the third day of infection. Parasitemias were suppressed by globulin from infected rats, but they were not suppressed by globulin from normal rats. Maximum (+4) suppression was noted in groups treated with concentrated globulin from hyperimmune rats (Fig. 1); parasitemias were suppressed by 85% on the third day. Protection was noted in all fractions tested with the exception of the IgM from hyperimmune serum. Studies are in progress to separate and test IgG and IgA individually.

GRAPHIC NOT REPRODUCIBLE

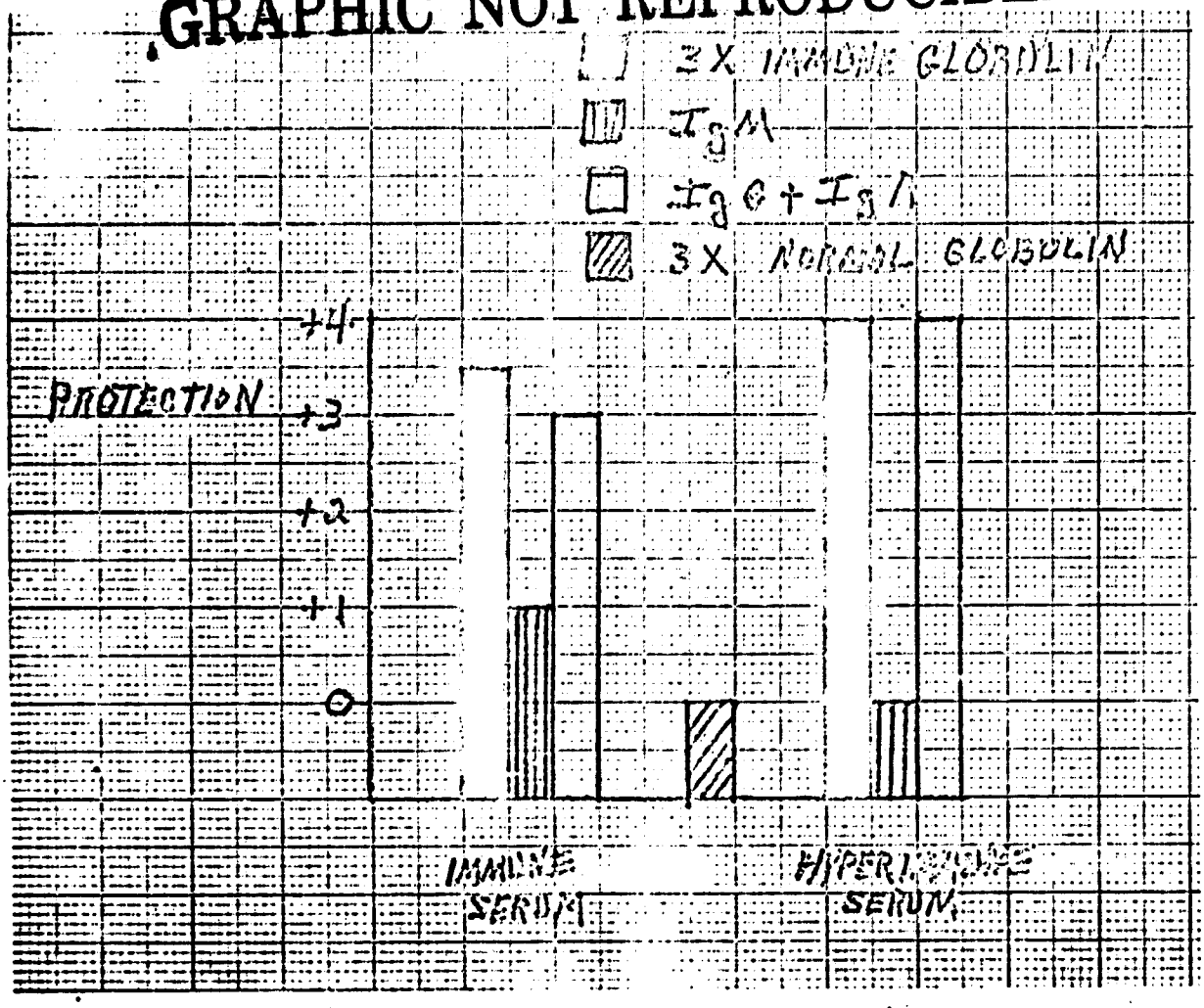

Fig. 1 Protective Capacity of Globulin and Globulin Fractions

2. Sequestration of ^{59}Fe tagged P. berghei parasitized reticulocytes. P. berghei infected young rats in which 90-95% of the reticulocytes were parasitized 5 days after inoculation were injected with 1 μc of ^{59}Fe and were bled 2 days later. ^{59}Fe Separation of the parasitized reticulocytes indicated that most of the ^{59}Fe was incorporated into the parasitized cells. Both normal and immune rats injected intravenously with the ^{59}Fe tagged parasitized cells were sacrificed at intervals of 5 minutes, 1, 3, 8 and 24 hours after injection and their blood and organs were assayed for radioactivity. Blood smears and radioactivity measurements showed that parasitized cells were cleared rapidly from the blood. In normal rats 35% of the injected dose had been cleared at 1 hour; 10-17% was recovered in the spleen. Immune rats removed 55% of the injected dose in 1 hour; 24-39% was recovered in the spleen. No differences in liver uptake between the two groups could be found through 3 hours (10-20%). Lung, kidney, and bone marrow did not appear to play a significant role in the clearance of parasitized cells.

Summary and Conclusions.

1. Parasitemias in Plasmodium berghei-infected mice injected with globulins and certain globulin fractions obtained from immunized rats were suppressed by 85% on the third day of infection. No protection was conferred by injected normal globulins.

2. Immunized rats removed a greater number of ^{59}Fe tagged P. berghei-parasitized reticulocytes from the circulating blood in one hour than normal rats. A greater percentage of the injected dose was recovered from the spleens of immune rats than normal rats.

Publications.

Wellde, B. T. and Sadun, E. H., 1967. Resistance produced in rats and mice by exposure to irradiated Plasmodium berghei. Exp. Parasit. 21:310-324.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. REPORT CONTROL SYMBOL
			DA 046520	CSCRD-103

5. DATE OF RESUME	6. KIND OF RESUME	7. SECURITY	8. REGRADING	9. RELEASE LIMITATION	10. LEVEL OF RESUME
01 07 69	D. CHANGE 30 11 67	U U	NA	DC	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 63153011 3A635301D829 00 129

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) HOST RESPONSES TO MALARIA 09

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY	07 65	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (IN THOUSANDS)
C. IN-HOUSE	a. DATE NA; b. NUMBER NA; c. TYPE NA; d. AMOUNT NA	PRIOR FY 69	3	100
		CURRENT FY 69	3.	100

19. GOV'T. LAB INSTALLATION/ACTIVITY

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INVESTIGATORS PRINCIPAL: SADUN, E. H. SC.D

ASSOCIATE: MOON, A. P. MS

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21. TECHNOLOGY UTILIZATION: PATHOLOGY, PHYSIOLOGY

22. COORDINATION: NA

23. KEYWORDS: MALARIA, GAMMA GLOBULIN, BIOCHEMISTRY, ANTIBODY, FLUORESCENT, ISOTOPE, METABOLISM.

(U) TECH OBJECTIVE - TO DETERMINE HOW ENERGY REQUIREMENTS (ATP) ARE MET WITHIN THE PARASITE.

(U) APPROACH- USE ULTRAMICRO TECHNIQUES TO DETERMINE CHANGES IN SERUM ENZYMES IN GERMFREE AND CONVENTIONAL MICE AND RATS. STUDY THE EFFECT OF INFECTION ON THE UPTAKE AND DISTRIBUTION OF RADIOISOTOPE-LABELLED AMINO ACIDS, STUDY THE LEVELS OF ENZYME ACTIVITY IN TISSUE EXTRACTS AND ALTERATIONS IN PROTEIN AND FREE AMINO ACID CONSTITUENTS OF BLOOD AND URINE, STUDY THE DEVELOPMENT OF RELAPSES, AND THE PATTERN OF PARASITEMIAS AND FLUORESCENT ANTIBODIES PRIOR TO, DURING, AND FOLLOWING THERAPY. INVESTIGATE THE USE OF IMMUNE GAMMA GLOBULINS AS AN ADJUVANT TO CHEMOTHERAPY IN HUMANS INFECTED WITH DRUG RESISTANT MALARIA.

(U) PROGRESS - OCT 67 THRU JUN 68 TECHNIC HAS BEEN ADVISED TO ELIMINATE HOST-BLOOD COMPONENTS FROM PREPARATIONS OF P. KNOWLESI. PRESENCE OR ABSENCE OF PASTEUR EFFECT IS BEING MEASURED BY ASSAY OF GLUCOSE UTILIZATION SPECTROPHOTOMETRICALLY UNDER GAS MEDIA. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

25. COMMUNICATIONS SECURITY	26.	27. DOD CODE	28. BUDGET CODE
a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

29. MISSION OBJECTIVE	30. PARTICIPATION
NA	NA

31. REQUESTING AGENCY	32. SPECIAL EQUIPMENT

33. FUNDS (IN THOUSANDS)	34.

FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 129, Host responses to malaria

Investigators
Principal: Elvio H. Sadun, Sc.D.
Associate: CPT Leonard W. Scheibel; Jane S. Winiarski

Description.

The object of this work unit is to study the physiological pathology of malaria including the enhancement of non-specific resistance to infection.

Progress.

1. Electron transport enzymes in Plasmodium knowlesi. The mechanisms of adenosine triphosphate generation and the nature of the terminal electron acceptor of Plasmodium knowlesi have not been defined. Their elucidation would indicate whether this protozoan depends primarily on an aerobic or anaerobic metabolism to meet its energy requirments. White blood cells were removed from the blood of monkeys infected with P. knowlesi. Parasites were then freed from the red blood cells by subjecting them to 2000 lbs/sq. in. pressure in a French press. After removing debris by washing, the parasite suspension was homogenized in .05M phosphate buffer, pH 7.4. Using the assay methods of Smith, cytochrome oxidase was found to be present. However, since blood platelets are known to have this enzyme, a method had to be developed for quantitatively eliminating platelets without a great loss of parasites. Yamanaka et al. showed that injection of adenosine diphosphate caused the platelet concentration in rabbit blood to be reduced 30 to 50 percent in 20 minutes, and O'Brien and Heywood found that up to 70 percent of platelets could be removed by passing cell suspensions through a glass bead column. After combining these two techniques microscopic examination showed that all platelets had been removed. These parasites when subjected to homogenization and freeze thawing in hypotonic media showed no significant levels of cytochrome oxidase activity, but when the plasmodia were subjected to 18,000 lbs/sq. in. in the French Pressure Cell, cytochrome oxidase activity was detected. However, the specific activity was approximately 37 times less than cytochrome oxidase isolated from guinea pig heart. All fractions of malaria preparations both soluble and particulate are stimulatory to mammalian cytochrome oxidase. This effect is resistant to boiling and dialysis. Fractionation through a Sephadex G-200 column is being carried out to determine purity of this enzyme enabling further kinetic studies to be undertaken.

Summary and Conclusions.

By combining two methods of removing platelets from parasitized blood, suitable preparations of Plasmodium knowlesi for the study of electron transport systems in the parasite have been obtained.

Publications.

Martin, L. K., Einheber, A., Sadun, E. H. and Wren, R. E., 1967. Effect of bacterial endotoxin on the course of Plasmodium berghei infection. Exper. Parasit. 20:186-199.

RESEARCH AND TECHNOLOGY WORK UNIT

		2 GOVT ACCESSION	3 DOD ACTIVITY CODE 61 CAC 23	4 PROJECT/TASK/WORK UNIT NO 6502D-1F3
5a START DATE 01 07 65	5b ESTIMATED COMPLETION 31 01 69	6 SECURITY U U	7 REG. AGENCY NA	8 PERFORMANCE METHOD IC
				9 LEVEL OF EFFORT A. WORK UNIT

10 KEY WORDS (P) 61502D11 3465401B829 00 130

11 TITLE

(U) LITERATURE OF MALARIAL TEST DATA 05

12a KIND OF FUNDING AGENCY 3460 BIOLOGY		13 START DATE 07 65	14 EST. COMPLETION DATE NA	15 FUNDING AGENCY OTHER DA
12b PROGRAM ELEMENT C. 1B-1L025F	12c CONTRACT/GRANT NA / NA	16 RESOURCES PROFESSIONAL 05	2	10
	12d PROJECT NO. NA	17 TOTAL 09	2	10

18a GOVT LAB INSTALLATION/ACTIVITY
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19a INVESTIGATORS PRINCIPAL ECKERMAN, LTC C. H.
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20 TECHNOLOGY UTILIZATION
INFORMATION RETRIEVAL

21 COORDINATION
NA

22 KEYWORDS
MALARIA, CHEMICAL, CHEMISTRY, PHARMACEUTICAL, LITERATURE.

23 (U) TECH OBJECTIVE - TO MAINTAIN IN MACHINEABLE FORM ALL BIOLOGICAL INFORMATION ASSOCIATED WITH THE TEST PROGRAMS SUPPORTED BY THE MALARIAL PROJECT.

24 (U) APPROACH- PRELIMINARY MACHINING OF APPROXIMATELY 90 PERCENT OF DATA IS ACCOMPLISHED AT THE SOURCE. THE REMAINDER OF PRELIMINARY MACHINING AND FINAL PROCESSING OF ALL DATA ARE DONE AT WALTER REED. THE CHEMICAL TYPEWRITERS ARE USED IN ASSOCIATION WITH CONVENTIONAL KEYPUNCH MACHINES. PROGRAMS HAVE BEEN WRITTEN TO ALLOW PROCESSING OF BOTH BIOLOGICAL AND CHEMICAL TO ONE OUTPUT.

25 (U) PROGRESS - JUL 67 THRU JUN 68 A TOTAL OF 145 PROGRAMS HAVE NOW BEEN WRITTEN TO HANDLE BIOLOGICAL DATA. PROCESSING OF DATA FROM THREE ADDITIONAL TEST SYSTEMS HAS STARTED DURING PERIOD JUL 67 - JAN 68 AND PROGRAMMING WAS BEGUN TO IMPLEMENT A MORE EFFICIENT INVENTORY AND SHIPPING SYSTEM. CURRENTLY 72 HOURS OF 1401 COMPUTER TIME AND 5 HOURS OF 7090 TIME ARE REQUIRED PER WEEK TO MAINTAIN EXISTING FILES AND PROCESS NEW DATA. RECORDS FOR 115,000 COMPOUNDS AND DATA FROM 600,000 BIOLOGICAL TESTS ARE STORED ON 19 TAPES. FILES ARE UPDATED WEEKLY WITH 1000 NEW COMPOUNDS AND 3000 BIOLOGICAL RECORDS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

26 PRINCIPAL INVESTIGATOR	27	28 DATE ENTERED 67	29 BUDGET CODE
30 DOD UNIT	31 RESPONSIBLE DOD ORGANIZATION		
32 LAB/INSTALLATION	33 APPROVING GOVERNMENT AUTHORITY		
34 COMMENTS	35		

FORM 1498, 1 JAN 65 REPLACES EDITION OF 1 JAN 63 WHICH MAY BE USED FROM THE RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

Project 3A635301D829

Task 01

Work Unit 130

Title: Malaria Prophylaxis

Malaria Investigations

Literature of Malarial Test Data

Investigators.
Principal: David P. Jacobus, M. D.
Associate: Edgar H. Eckermann, LTC, VC
David E. Davidson, MAJ, VC
Gen Jue, CPT, MSC
Alfred P. Feldman
June A. Schafer
John F. Waters
Daniel F. Boehle, 1LT, MSC

Project 3A63[illegible]D829 Title: Malaria Prophylaxis

Task 01 Malaria Investigations

Work Unit 130 Literature of Malarial Test Data

DESCRIPTION: Research and Inventory Control of Chemical Samples Accessioned for Biological Screening Program

PROGRESS:

1. General

An integrated computer based system for the storage and retrieval of chemical, biological, and inventory information generated in the drug development program for the Division of Medicinal Chemistry is currently being developed. Programming has been delivered to facilitate the control of the chemical inventory and to assist in the distribution of samples and compound information to the laboratories engaged in the research program. A record of every inventory transaction is maintained to permit central coordination of the testing programs and to assist in the selection of samples for test. For further control certain time elements have been written into these programs, that is, as compounds received at WRAIR proceed through the inventory program a record is kept of the time required to assign it an accession number, to ship it to a test center, to receive it at the test center and to return biological data to WRAIR. If a reasonable time limit is exceeded in any of these steps notification is sent to the proper individual so that action can be taken.

Computer programming has been written for the IBM 360 Mod 30 computer by the Computer Usage Development Corporation according to specifications and requirements developed by the Division of Medicinal Chemistry. Inventory programming is compatible and interfaces with biological and chemical programs written for the 1401 and 7090.

The steps involved are as follows: A standardized chemical data form is utilized to record all information available from documentation provided by the submitter of the compound. As much chemical, physical and biological data as possible is recorded. Numeric codes identifying the submitter are added and the compound is classified as to Open, Discreet, Purchased, Synthesized or Prep lab to facilitate later processing by the computer. The chemical sample, the chemical data sheet and an inventory accession record are then each assigned the same primary inventory accession number or bottle number. These bottle numbers are computer generated and contain a check digit so as to prevent errors through number transposition on keypunching.

The sample itself and the inventory accession card are forwarded to the inventory warehouse where they are shelved and the shelf location and quantity

of material is recorded on the inventory accession card. This card is then keypunched for input to an update of the master inventory tape file.

The completed chemical data sheet is forwarded to the information handling section where it is checked by supervisory personnel for completeness and correctness of information contained. The chemical structure is then typed on the Army chemical typewriter for input to the Chemical Information System. Once it is ascertained that this structure has been accepted on the master file of chemical structures the structure is then hand searched against the molecular formula file to determine uniqueness. If the compound is unique it is assigned a new Walter Reed accession number. Duplicate samples retain the number of the earlier samples. The now completed data sheet is reproduced in two copies for further processing. The original copy is retained on permanent file. One copy is forwarded for keypunching and verification of all recorded data. Cards coded "30" record the compound name and the compound classification. Cards coded "50" record the molecular weight and chemical data such as appearance, warning messages, and stability solubility information. Cards coded "00" record bottle number - Walter Reed number accession correspondence. These cards are used to generate a complete inventory accession record for each compound. They are also used to generate a letter of acknowledgement to the submitter and a new accession report to personnel at WRAIR. A special file of names and addresses is utilized to address acknowledgement letters automatically for distribution. The second copy of the data sheet is forwarded to those individuals responsible for the assignment of chemicals to the various testing laboratories engaged in the drug testing program. Each sheet is stamped or otherwise annotated with an indication of the test to be performed. The annotated sheets are used to generate keypunch forms which are in turn used to generate shipping orders. Shipping orders are processed by the computer against the master inventory file and master accession file. Shipments are written onto the output shipping list only if sufficient quantity of material is recorded on the master file. The amount to be shipped is subtracted from the amount in inventory after appropriate metric unit conversion. Each shipping order is checked against previous orders so as to prevent duplicate shipments to the same test system within thirty days. Shipment of commercially discreet samples is permitted only to authorized laboratories. Compound name and source identification is sent to the laboratory at the discretion of WRAIR personnel. The computer program permits only valid shipments to occur. With the use of proper override codes shipments ordinarily considered invalid can be made at the discretion of WRAIR personnel. When a shipping order is received at the laboratory facility and is filled and ready to mail a "70" card is submitted to inventory processing by the laboratory indicating that the order has been filled and has been sent to the proper test system. If the laboratory is not able to complete the entire shipping list this is also noted on the "70" card and the inventory records are changed by the computer. A return post card is inclosed with each shipment so that the laboratory receiving that shipment can notify inventory processing of the condition of receipt of that shipment. The master inventory file retains a permanent record of the date that a shipping order is written, the data that it is sent and the date that it is received at its destination.

Reports generated by inventory control include the new accession report in source sequence, bottle number - Walter Reed cross reference list, histories of shipping in bottle number sequence, Walter Reed sequence and source sequence. Over due reports are also generated when action on a compound has exceeded the time limit.

SUMMARY AND CONCLUSIONS

The IBM 360 Mod 30 computer is being utilized to supplement and facilitate administrative processing of chemical accession data and inventory control. Additional programming is planned to extend the usefulness of the information stored and to permit greater control of throughput and trouble shooting. A generalized inventory search program is being written to provide access to desired portions of the file by various parameters. A generalized conversion program is being written so that reports can be printed either on 1401 peripheral equipment or on the high speed printer which is owned by WRAIR and maintained at Herner and Company. Programming is being written to incorporate indicators of biological activity into the master inventory file record. It is hoped that existing programming can be used to develop inventory systems for the control and monitoring of areas other than compound shipment such as drug INDs and personnel control as well as for our existing supply of chemical compounds.

PUBLICATIONS

Feldman, Alfred P. "Computer Input of Forms" 1968 Spring Joint Computer Conference p. 323 - 331.

Literature of Antimalarial Data

Storage and retrieval of antimalarial drug screening data has been adapted to computer programming as an aid in guiding the antimalarial drug development program of the Division of Medicinal Chemistry.

This programming facilitates the rapid generation of reports for both external and internal distribution. Biological files can be interfaced with chemistry name files and chemistry structure files. Searches of files can be made based on activity, structure, periods of time, or submitter.

PROGRESS:

A. Antimalarial Data

1. Computer Programming

Advanced Computer Techniques has been contracted to assist in new programming and necessary modifications of existing programs for biological test systems. Service Bureau Corporation remains under contract but is now addressing their efforts primarily to the interface of biological and chemical systems. Data from current screening programs were added to the data processing system as they became operational. Existing programs were utilized with some modifications where possible. Programming for the primary mouse screen was modified to process data from the P. gallinaceum chick test put into operation by Dr. Leo Rane at the University of Miami. Existing programming was also modified to process data from the blood-induced P. gallinaceum - chick test, sporozoite induced P. gallinaceum - chick test, and sporozoite induced P. berghei - mouse test, operated at the Illinois Institute of Technology under the direction of Dr. Maurice King. Validation programs have been originated for all systems to assist in eliminating erroneous data. Complete programming was written to process data from the In Vitro screen operated by Dr. L. H. Saxe at the University of West Virginia. Effort is continuing on this system to automate the recording and calculation of raw data from this system which at present requires a major portion of the manual effort expended on this system. All files can now be updated and deletions and corrections made. Changes have been made in all biological systems to make them compatible with the new drug control and inventory system which itilizes a new format of compound identification number. In making this change consideration was given to the fact that a new computer system will be put into operation at WRAIR. All new files were designed to go into one general merge that has slight modifications to handle each system. A standard record length of 102 characters has been established for output merge with assigned fields for compound identification numbers, date, test system identification, submitter identification and a fixed field in which data may be entered in varying formats. Fields have been established in multiples of six characters to facilitate conversion of existing programming to the new machine system once details of that system are known.

2. Interface with Chemstructs

The 102 character record has been designed for quick and easy interface with chemstructs, requiring a minimum of reformat. A field for line control is added to this record to control the order and spacing of data for printing after the merge with Chemstructs. A file has been established to pick up the Chemstructs number on biology files when they are merged with chemistry name file. This technique will facilitate linking the biology data with the chemical structure. The time required for this linking process will be reduced because less machine time will be required.

3. Microfilm Storate of Raw Data Inputs

At present, 95% of the raw data (data sheets) has been put on microfilm. A computer system has been developed and is operational whereby the raw data is indexed for immediate retrieval from the microfilm.

SUMMARY AND CONCLUSIONS

Computer programming developed for retrieval of biological and chemical data has been expanded to include new test systems. All programming has been modified to interface with the new inventory system and to improve interfacing of biology and chemistry. All changes have been made to facilitate conversation to the third generation computer system when it becomes available. Data can presently be selected administratively or by chemical or biological search for generation of reports for internal and external distribution.

RESEARCH AND TECHNOLOGY WORK UNIT

01 07 69	30 11 67				

3A. CURRENT WORK UNIT CODE: 3A161611 AA830010829 00 137

11. TITLE: (U) CLINICAL STUDIES OF HUMAN MALARIA

12. SCIENTIFIC OR TECH AREA	13. START DATE	14. EST. COMPL. DATE	15. FUNDING AGENCY
006600 BIOLOGY	11 65	NA	OTHER DA

16. PROCURING METHOD	17. CONTRACT/GRANT			18. RESOURCES ESTIMATE	PROFESSIONAL MAN YEARS	TOTAL MAN YEARS
C. IN-HOUSE	a. NUMBER NA	c. DATE NA		FY 68	2	70
	b. TYPE NA	d. AMOUNT NA		CY 69	2	70

19. GOVT. LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
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PRIN. INVSTR: PERONEY, CCL M. F. TEL: 202-576-3551	INVESTIGATORS PRINCIPAL: TESCHAN, COL P. F. ASSOCIATE: CANFIELD, LTC C. J. TEL: 202-576-3369 TYPE: DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
CLINICAL STUDIES	NA

23. KEYWORDS: MALARIA, ANTIMALARIALS, PARASITE, RED BLOOD CELL.

24. (U) TECH OBJECTIVE - STUDY CLINICAL DISEASE OF ACUTE FALCIPARUM AND VIVAX MALARIA, ASSES VARIOUS MODES OF ANTIMALARIAL THERAPY WITH RESPECT TO CLINICAL RESPONSES AND RADICAL CURE, STUDY PATHOPHYSIOLOGY OF THE DISEASE.

(U) APPROACH-- DOCUMENT CLINICAL FEATURES OF ACUTE DISEASE, EVALUATE AVAILABLE THERAPEUTIC AGENTS WITH RESPECT TO CLINICAL RESPONSE AND RADICAL CURE, EVALUATE RENAL ERYTHROPOETIC, ADRENAL AND FLUID AND ELECTROLYTE CHANGES IN ACUTE DISEASE.

(U) PROGRESS - OCT 67 THRU MAY 68 RENAL STUDIES OF GLOMERULAR FILTRATION RATE AND RENAL PLASMA FLOW HAVE SHOWN NORMAL CLEARANCES IN UNCOMPLICATED FALCIPARUM MALARIA. THESE PATIENTS HAVE CONTINUED TO SHOW IN SOME INSTANCES MILD HYPOKALEMIA AND A FEW HAVE HAD A BLUNTED RESPONSE TO A WATER LOAD TEST. BODY SPACE STUDIES, HOWEVER, HAVE BEEN LARGELY NORMAL /TOTAL BODY WATER, EXTRACELLULAR FLUID, AND PLASMA VOLUME/. STUDIES OF ADRENAL STEROIDS HAVE SHOWN THAT THE DAILY EXCRETION OF 17-OHCS AND 17-KS IS DEPRESSED IN CHLOROQUINE-RESISTANT ACUTE FALCIPARUM MALARIA BUT ALDOSTERONE EXCRETION IS NORMAL OR INCREASED. IN CONTRAST, PLASMA 17-OHCS AND CORTISOL ARE NORMAL OR INCREASED. ACTH AND METOPIRONE ADMINISTRATION PRODUCED THE EXPECTED INCREASE OF PLASMA CORTISOL AND 11-DESOXYCORTISOL RESPECTIVELY AND INCREASED THE URINARY EXCRETION OF 17-OHCS IN ALL BUT ONE PATIENT. SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

25. PRIMARY MISSION & ACTIVITY	26.	26A. DOD CODE	26B. BUDGET CODE
a. SCIENCE OF [illegible] b. NOT RELATED		6R	1
26C. DOD OBJECTIVE: NA		26D. PARTICIPATION: NA	
27. PERFORMING AGENCY	28. SPECIAL EQUIPMENT		
29. FUNDS TRANSFERRED	30.		

DD FORM 1498M JAN 66 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 30 identical to NASA Form 1122)

Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigation

Work Unit 132, Clinical Studies of Human Malaria

Investigators.
Principal: COL Paul E. Teschan, MC
Associates: LTC Kevin G. Barry, MC
LTC Craig J. Canfield, MC
LTC William J. Cirksena, MC
LTC Paul F. Gilliland, MC
MAJ Jessie E. Hano, MC
CPT Marion Brooks, MC
CPT Seymour Rosen, MC
Dr. Joseph Bruton, Ph.D.

Description.

The objective of this work unit was to assess the clinical response of patients to acute falciparum and vivax malaria. Not only was the effectiveness of specific drug regimens being evaluated in terms of supression or cure, but also various aspects of the pathophysiology of the disease were studied. The particular fields of interest have been: therapeutics, fluid and electrolyte ("space studies"), erythropoetic, and endocrine.

Progress.

Due to a marked decrease in patients with malaria at Walter Reed General Hospital no clinical studies were undertaken this year.

Publications.

Miller, L. H., Makaranond, P., Sitprija, V., Suesanguan, C., and Canfield, C. J., Hyponatremia in Malaria, Ann Trop Med and Parasitol, 61:265, 1967.

[illegible]en, S., Roycroft, D. W., Hano, J. E., Barry, K. G. The Liver in Malaria: Electron Microscopic Observations on a Hepatic Biopsy Obtained 15 minutes Post Mortem. Arch Path 83:271--277, 1967.

Gilliland, P. F., Brooks, M. H., Cirksena, W. J., Malloy, J. P., Bruton, J., and Barry, K. G., Pituitary-adrenal function in acute falciparum malaria. Clin Res 16:330 (abstract).

Klainer, A. S., Gilliland, P. F., Cirksena, W. J., Bartelloni, P. J., and Beisel, W. R., Serum Glycoproteins in Naturally-acquired Malaria in Man, Am J Med (submitted for publication).

Brooks, M. H., Barry, K. G., Cirksena, W. J., Malloy, J., Bruton, J., and Gilliland, P. F.. Pathophysiology of Acute F. Malaria: Pituitary Adrenal Function. Submitted for publication.

Brooks, M. H. Acute Pulmonary Edema in F. Malaria: A Clinicopathologic Correlation. Submitted for publication.

Brooks, M. H., Malloy, J. P., Bartelloni, P. J., Sheehy, T. W. and Barry, K. G. Quinine, Pyrimethamine & Sulphorthodimethoxine: Clinical Response, Plasma Levels & Urinary Excretion During the Initial Attack of Naturally Acquired F. Malaria. Submitted for publication.

RESEARCH AND TECHNOLOGY WORK UNIT

01 07 63	01 07 67	W	0	DA 0[illegible]	C[illegible]

C3159611 3569520116429 00 132

(U) ACUTE RENAL INJURY AND FAILURE IN MALARIA

612960 PHYSIOLOGY	012600 PHARMACOLOGY		
C. IN-HOUSE	NA / NA	07 63 / NA / 64 / 65	3 / 3
			DA / DA / ICO / ICC

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202-576-3305 TYPE DA

TECHNOLOGY UTILIZATION: MEDICAL SCIENCE

22. COORDINATION: NA

KEYWORDS: MALARIA, KIDNEY, SHOCK, MANNITOL, RENAL FUNCTION, RENAL TUBULE, MALARIA ENDEMICS.

(U) TECH OBJECTIVE - TO ESTABLISH A RATIONAL APPROACH IN THE PREVENTION AND TREATMENT OF ACUTE RENAL FAILURE ASSOCIATED WITH MALARIA.

(U) APPROACH- ANIMAL MODELS WITH MALARIAL INFECTIONS ARE USED TO STUDY PHYSIOLOGIC ALTERATIONS WITH SPECIAL EMPHASIS ON THE PATHOPHYSIOLOGY OF ACUTE RENAL FAILURE.

(U) PROGRESS - JUL 67 THRU JUN 68 HEMODYNAMIC STUDIES HAVE BEEN PERFORMED ON RHESUS MONKEYS INFECTED WITH P. KNOWLESI AND P. COATNEYI INCLUDING MEASUREMENTS OF CARDIAC OUTPUT AND RENAL BLOOD FLOW. CARDIAC OUTPUT AND RENAL BLOOD FLOW AS MEASURED BY ELECTROMAGNETIC FLOW METERS ARE MAINTAINED IN INFECTED MONKEYS WHO DEVELOP PROGRESSIVE AZOTEMIA AND OLIGURIA. THESE INFECTIONS ARE ALSO CHARACTERIZED BY CONTAINMENT OF RENAL SODIUM AND WATER EXCRETION AND INCREASES IN HEART RATE IN ORDER TO MAINTAIN CARDIAC OUTPUT AND SYSTEMIC BLOOD PRESSURE. A CLINICAL-ANATOMICAL CORRELATIVE APPROACH TO THE PROBLEM HAS CONTINUED WITH ADDITIONAL OBSERVATIONS ON THE NORMAL RHESUS MONKEY AND THOSE WITH MALARIA NEPHROPATHY SUGGESTING ISCHEMIA OR IRREGULAR CORTICAL ISCHEMIA. PREVENTATIVE MEASURES INCLUDING VOLUME EXPANSION, MANNITOL, AND DIURETICS ARE CURRENTLY BEING EVALUATED IN ORDER TO FIND A SUITABLE MEANS OF ARRESTING THE RENAL FAILURE ASSOCIATED WITH MALARIA. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

[illegible]		EP	1
A1		NA	

DD FORM 1498 REPLACES EDITION OF 1 JAN 66 WHICH MAY BE USED [illegible]

Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigation

Work Unit 133, Acute renal injury and failure in malaria

Investigators.
Principal: COL Paul E. Teschan, MC
Associate: MAJ Jessie E. Hano, MC; CPT Seymour Rosen, MC; CPT Craig Tisher, MC; CPT Max Inman, MC; LTC Paul F. Gilliland, MC

Description.

An animal model that develops acute renal failure during malaria is utilized to study the pathophysiology sequence of acute renal injury. Clinical, laboratory, and morphological observations have been correlated in the Rhesus monkey infected with P. knowlesi malaria. The morphological baseline for these studies has been established by studies of normal monkey kidneys by means of light and electron microscopy and by histochemical technics.

Progress.

The normal morphology of the Rhesus monkey kidney has been further delineated by light and electron microscopy and the pertinent data have been included in the listed publications.

Publications.

1. Inman, M., Hano, J., Gilliland, P., Rosen, S., and Barry, K. G.: The Clinical Spectrum of Blackwater Fever. Clin. Res. 15: 307, 1967.

2. Rosen, S., Hano, J. and Barry, K.G.: Malarial Nephropathy in the Rhesus Monkey. Lab. Invest. 16: 636-7, 1967.

3. Hano, J. E. and Olsson, R.A.: Renal Hemodynamic and Metabolic Studies of Simian Malaria. American Society of Nephrology. 17 Oct 1967, Los Angeles, California.

4. Rosen, S., Hano, J. E., Inman, M., Gilliland, P. F., and Barry, K. G.: The Kidney in Blackwater Fever: Light and Electron Microscopic Observations. Am. J. Clin. Path. 49: 358-370, 1968.

5. Donadio, J. & Whelton, A.: Quinine Therapy & Peritoneal Clearance in Acute Renal Failure Complicating P. falciparum Malaria with Blackwater Fever. Lancet. 1: 375, Feb 24, 1968.

6. Donadio, J., Whelton, A., Gilliland, P. F., and Cirksena, W. J.: Peritoneal Dialysis in Quinine Intoxication, J. A. M. A., (Letter to the Editor) 204: 182, 15 April 1968.

7. Rosen, S., Hano, J. E. and Barry, K. G.: Malarial Nephropathy in the Rhesus Monkey. AMA Arch. Path. 85: 36-44, 1968

8. Canfield, C. J., Miller, L. H., Bartelloni, P. J., Eichler, P., & Barry, K. G.: Acute Renal Failure in Plasmodium falciparum Malaria: Treatment by Peritoneal Dialysis, Arch. Int. Med. (Accepted for Publication.)

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. REPORT CONTROL SYMBOL
			DA 046521	CSCRD-1[illegible]

5. DATE OF RESUME	6. KIND OF RESUME	7. SECURITY	8. REGRADING	9. RELEASE LIMITATION	10. LEVEL OF RESUME
01 07 68	D. CHANGE 30 11 67	U U	NA	DC	A. WORK UNIT

12. CURRENT NUMBER/CODE	13. PRIOR NUMBER/CODE
63150011 3A6353010829 00 134	

11. TITLE
(U) MALARIA SCREENING SYSTEMS

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. G. T. COMPL. DATE	15. FUNDING AGENCY
002300 BIOCHEMISTRY 008800 LIFE SUPPORT	10 66	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	19. PROFESSIONAL MAN-YEARS	20. FUNDS (IN THOUSANDS)
C. IN-HOUSE	a. NUMBER NA; b. DATE NA; c. TYPE NA; d. AMOUNT NA	PRIOR FY 68	5	150
		CURRENT FY 69	5	150

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	TEL.: 202-576-2211 TYPE: DA

23. TECHNOLOGY UTILIZATION	22. COORDINATION
MALARIA	NA

KEYWORDS
MALARIA PLASMOD SPP, MASS SCREENING TECHNIQUES, AUTOMATION.

(U) TECH OBJECTIVE - THE MEASUREMENT OF ANTI-MALARIAL DRUG EFFECTIVENESS AGAINST THE MALARIA PARASITE IN THE ERYTHROCYTIC PHASE IN VITRO.

(U) APPROACH- AUTOMATIC WET CHEMICAL ANALYSIS SUBDIVIDED INTO AN INCUBATION PHASE AND AN ANALYTICAL PHASE IS BEING EMPLOYED TO DEFINE THE ABILITY OF THE MALARIA PARASITE WITHIN THE RED CELL TO UTILIZE NUTRIENTS OR PRODUCE METABOLITES BEFORE AND AFTER INCUBATION WITH CHEMICAL COMPOUNDS UNDER TEST FOR ANTI-MALARIAL ACTIVITY.

(U) PROGRESS - OCT 67 THRU JUN 68 POTENTIAL ANTI-MALARIAL DRUGS ARE BEING SCREENED IN A SYSTEM DESIGNED TO EVALUATE SEVERAL METABOLIC PARAMETERS IN PLASMODIUM SPECIES. A TOTAL OF [illegible]81 COMPOUNDS EFFECTIVE IN SOME DEGREE AGAINST PLASMODIA HAVE BEEN PROCESSED. AMINO ACID REQUIREMENTS OF SEVERAL PLASMODIUM SPECIES ARE BEING EVALUATED BY COMPOSITE ELUTION CHROMATOGRAPHY. STUDIES OF EFFECTS OF IONIZING RADIATION ON METABOLISM AND INFECTIVITY OF PLASMODIUM CONTINUE, AND TECHNIQUES FOR MEASUREMENT OF NUCLEIC ACID SYNTHESIS IN P. BERGHEI ARE BEING DEVELOPED. FOR TECHNICAL REPORTS SEE WRAIR ANNUAL PROGRESS REPORT, 1 JULY 1967 - JUNE 1968.

TEXT NOT REPRODUCIBLE

COMMUNICATIONS SECURITY	24.	25. OSD CODE	26. BUDGET CODE
1. COMSEC OR COMSEC RELATED; X 2. NOT RELATED		AB	1

MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. COMPETING AGENCY	34. SPECIAL EQUIPMENT

FUNDS OF SUPPORT	35.

FORM 1498m JAN 64 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A635301D829, MALARIA PROPHYLAXIS

Task 01, Malaria Investigations

Work Unit 134, Malaria screening systems

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: LTC Dorsey T. Mahin, MC; CPT Robert M. Donati, MC; CPT Charles A. Stancer, VC; CPT Thomas McLeod, MSC; John I. Davis, BS; Nesbitt Brown, BS; Ann R. Berman, BS; Division of Medicinal Chemistry, David P. Jacobus, MD; LTC Edgar Eckermann, VC; Division of Medicine, LTC Craig Canfield, MC; Herman Polet, MD; Division of Communicable Disease and Immunology, Elvio Sadun, Ph.D.; CPT D. J. Stechshulte, MC; Bruce T. Wellde, BS; University of West Virginia, Leroy Saxe, Ph.D.; Richard Cenedella, Ph.D.; Knox Van Dyke, Ph.D.; Division of Biometrics, LTC Ernest O. Jones, MSC; Georgetown University, Martin Rubin, Ph.D.

Description.

The objective of this work unit is to provide automated or semi-automated screening systems to study the effects of chemical compounds on the metabolism of Plasmodium species, "in vitro." A broad spectrum of developmental studies provides basic developmental input to the work unit. This developmental effort is necessary to the successful development of automated methodology.

Progress.

This work unit is divided into four parts: screening activities, developmental studies, general malaria studies, and collaborative efforts.

1. Screening activities. (LTC Angel, Nesbitt Brown, John Davis, David Jacobus, LTC Eckermann, Leroy Saxe, Richard Cenedella, Knox Van Dyke, LTC Jones).

The screening system reported in last year's Annual Report has been materially improved and consolidated. The incubator has been enlarged in order that parasitized blood can be incubated for 120 and 240 minutes. The metering valves were redesigned and leakage problems eliminated. Four metabolic tests - glucose, lactic acid, carbon dioxide and alpha amino nitrogen - were consolidated on a single plattered manifold by means of a new type of autoanalyzer pump. The dialyzer unit was redesigned and deep-grooved plates were used instead of the ordinary dialyzer plates. As a result of the above improvements the system operates reproducibly without significant breakdown.

An information processing system has been added to the analytical system. This system accepts analog signals generated by the recorder pen, converts them to digits, and compares the digital value to a standard curve. The output of the system is simultaneously printed on typewriter paper and punched on paper tape.

Software (tapes, programming, etc.) development has been initiated, in collaboration with the Division of Biometrics, to process the output tapes from this system and a similar system at the University of West Virginia. The objective of the program is to provide machine-readable output to the Division of Medicinal Chemistry.

During the reporting period, a total of 781 compounds were tested. The effect on glucose uptake, lactic acid production, carbon dioxide production, and alpha amino nitrogen production in P. berghei parasitized mouse red blood cells was measured. Each of the compounds evaluated had demonstrated some efficacy against Plasmodium species. Of the compounds studied, 3% depressed glucose utilization, 23% reduced lactic acid production, 8% reduced carbon dioxide production, and 4% depressed alpha amino nitrogen production. These results demonstrate that drug effect on lactic acid production is most closely related to antimalarial activity and of the four measurements shows the most promise as a screening test. Several drugs that were tested in the system indicated increased rather than depressed metabolism. The significance of this type of response has yet to be established.

During the period of this report, intercomparative studies on five known antimalarial drugs have been evaluated repeatedly. Average response of the system for quinine, chloroquine, pyrimethamine, dapasone and primaquine is shown in Table I.

Replicate measurements during a single operating day did not vary more than two percent. Day to day variability does not exceed plus or minus ten percent.

TABLE I

Effects of Drugs on Metabolism in P. berghei

Percent Inhibition

Test	Quinine	Chloroquine	Pyrimethamine	Dapasone	Primaquine
Glucose	+12	+ 2	+ 0.6	+24	Interferes
Lactic acid	+26	+ 4	- 6	+52	+11
Carbon Dioxide	+53	+14	+52	+ 5	Interferes
Alpha amino Nitrogen	+ 2	- 2	+11	+ 6	Interferes

2. Developmental studies. (LTC Angel, LTC Mahin, John Davis, Nesbitt Brown).

a. Amino acids.

A composite gradient elution chromatographic system was established and standardized to evaluate the amino acid requirements of several Plasmodium species: P. berghei, P. knowlesi and P. fallax. The amino acid content of both whole cells and TCA-precipitated extracts of cells were examined in P. berghei infected mouse and rat cells, and in P. fallax infected turkey brain cells. The amino acids alanine, glycine, leucine, lysine and valine comprise the bulk of the amino acids produced by incubation of the parasitized cell system under study. P. fallax differs from the other in that taurine is produced in infected cells. No results have been obtained that indicate specific amino acid requirements for any of the malarial species studied.

The column system was utilized to determine the serum levels of cyclic glycine, a non-metabolically active analog of valine (WRAIR No. 14997E), after administration of a single oral dose of 1600 mg to a normal human volunteer. Blood samples were drawn and the serum separated from the cells and frozen immediately. One-ml samples of serum, taken before administration of the drug and 30 minutes, 1, 4, 8 and 24 hours after administration, were precipitated with 10% trichloroacetic acid and chromatographed. The concentrations of non-protein bound cyclic glycine and of valine, for which cyclic glycine is an antagonist, are shown in Table II.

TABLE II

Cyclic Glycine and Valine Levels Before and After Administration of Cyclic Glycine

Time Hours	Cyclic Glycine microgms/100 Ml	Valine milligms/100 Ml
0	Negative	2.13
0.5	620	2.49
1	750	2.34
4	360	2.05
8	460	3.19
24	550	2.14

Normal values for valine are listed in Harper's Review of Physiological Chemistry as ranging from 2.5-4.2 milligrams per 100 ml. Valine levels in animals infected with Plasmodium species have been consistently

elevated over uninfected animals. Although the results are distinctly limited at this time, cyclic glycine levels reached a maximum of 750 micrograms after 30 minutes, falling to a low level of 360 micrograms after four hours, and returning almost to the 30-minute level after 24 hours. The significance of this finding is unknown. Valine levels in the same serum samples were not significantly altered. This effort will continue because resin column chromatography is presently the only method for analysis of this type of test agent.

b. Effects of ionizing radiation on malaria parasites, "in vitro." (LTC Angel, LTC Mahin, CPT McLeod, John Davis).

It was previously reported (WRAIR Annual Report, 1967) that doses between 4500 and 9000 rads of ^{60}Co gamma rays reduced the infectivity of Plasmodium berghei. Radiation had limited effects on the ability of the parasite to utilize glucose and to produce lactic acid, carbon dioxide, and alpha amino nitrogen. This suggests that the metabolic activity of the parasite might be used to study the radioprotective effects of selected aminothiols and to test the hypothesis that in order to be effective against radiation injury, the thiol must be intracellular.

Samples of packed mouse red blood cells parasitized with P. berghei were incubated for 2 hours with two radioprotective compounds (WRAIR No. 638, 400 mg/kg, and WRAIR No. 2721, 600 mg/kg of mouse blood). One set of blood samples was irradiated in a ^{60}Co Gamma cell (Atomic Energy of Canada, Limited) at a dose rate of 12,500 rads per minute to a total dose of 200,000 rads. Measurements were made on each sample for glucose and lactic acid. The results of this study are shown in Table III.

TABLE III

Metabolic Effects of Radiation and Radioprotectants in P. berghei

	Glucose (mg/100 ml) Utilization	Lactic Acid (mg/100 ml) Production
0 rads	168	148
200,000 rads	195	148
0 rads plus 400 mg/kg WRAIR 638	188	186
200,000 rads plus WRAIR 638	125	98
0 rads plus 600 mg/kg WRAIR 2721	206	178
200,000 rads plus WRAIR 2721	140	136

The radiation alone slightly increased glucose utilization but did not change lactic acid production. The metabolic inhibition previously found was not confirmed at this large radiation dose. Addition of radioprotective compounds increased glucose consumption and lactic acid production in non-irradiated parasitized blood, but decreased them in irradiated samples. These findings are compatible with a hypothesis that radiation in lower doses inhibits glucose metabolism while larger doses are stimulating; thus, the addition of radioprotection might have reduced the effective radiation dose to parasitized cells into the range of metabolic inhibition. Further studies of this phenomenon are in progress.

c. Nucleic acid synthesis in Plasmodium berghei. (LTC Angel, LTC Mahin, John Davis, Nesbitt Brown).

A series of studies have been completed on nucleic acid measurements by conventional colorimetry. One of the most promising methods, using ethidium bromide, was evaluated and changes in nucleic acid synthesis in the Plasmodium could not be measured satisfactorily.

A second means to measure nucleic acid synthesis utilizes incorporation of a radioactive precursor. A satisfactory continuous flow scintillation cell has been developed for this purpose. Great difficulty was encountered in fabricating a cell which would accept the necessary aqueous flow rate of 1 ml/min without packing and generation of intolerable back pressure. A granular plastic scintillator was developed and proven to be satisfactory.

This flow cell is currently being used in amino acid analyses. The entire effluent of the resin column is passed through the flow cell and radioactivity is measured. The effluent stream is then utilized to measure total amino acid by conventional colorimetric means. This provides a semi-automated method to measure specific activities. It is being utilized in the studies of P. knowlesi metabolism conducted in collaboration with Dr. Polet (see below, paragraph 4, "Collaborative efforts," paragraph a, (2)).

The satisfactory application of the scintillation flow cell to automated measurement of nucleic acid synthesis requires that the incubation medium be significantly depleted of tagged precursor by plasmodial utilization during an incubation period of 2 hours. Orotic acid tagged with ^{14}C was added to heparin anticoagulated normal and P. berghei parasitized mouse blood and incubated with shaking at 34°C for 2 hours. The medium radioactivity was decreased by about 30% in normal blood, and there was a corresponding increase in cellular radioactivity. In parasitized blood however, there were no significant changes. Further biological studies are necessary before such methods can be adapted to automated antimalarial drug screening systems.

3. General malaria studies.

a. Studies on normal and parasitized mouse erythrocytes. (LTC Angel, CPT McLeod, Ann Berman).

Using a ^{51}Cr labeling technique and a standard exposure to 30 kilorads of ^{60}Co gamma radiation (12,500 rads per minute) the half-time survival of normal, normal irradiated, parasitized and irradiated parasitized P. berghei mouse cells has been initiated. Present results indicate that normal mouse-cell survival is materially shortened after exposure to the radiation dose. Studies with parasitized cells are in progress.

b. Distribution, sequestration and blood clearance of Plasmodium berghei in the rat. (CPT Donati, CPT Stancer, Bruce Wellde).

These studies represent a cooperative effort, with the Department of Medical Zoology, to characterize some physiological parameters of malaria in experimentally infected rodents and primates.

The distribution, sequestration and blood clearance of P. berghei in rats were studied. Radioactive labeling of the malarial parasite has been a serious technical problem in prior investigations, and no satisfactory technique is presently available. Because the P. berghei organism is known to grow primarily in reticulocytes, the labeling of the parasitized reticulocyte with Iron-59 was utilized. Young infected rats, in which 90-95% of the reticulocytes were parasitized five days following inoculation, were injected with 1 uCi ^{59}Fe and bled two days thereafter. Differential separation of the blood revealed that more than 90% of the radioactivity incorporated in the blood was present in the parasitized reticulocytes. Normal rats, rats which were made immune by prior infection, naturally immune rats, and rats immunized by serial prior injection of radiation-attenuated parasites were injected intravenously with labeled parasitized reticulocytes. These animals were sacrificed at intervals of 5 minutes, 1 hour, 3 hours, 8 hours and 24 hours following injection. The blood and organs of these animals were assayed for radioactivity. Blood smears and radioactivity measurements demonstrated a rapid clearance of parasitized cells from the blood. The normal non-immune rats cleared 35% and the immune rats cleared 55% of the injected dose from the blood in one hour. In immune rats 24-39% of the injected dose was recovered in the spleen, whereas only 10-17% of the injected dose was recovered in the spleen of normal animals. No differences in liver uptake between the two groups could be found through three hours. Lung, kidney and bone marrow did not appear to play a significant role in the clearance of parasitized cells. Further studies are currently in progress.

c. Primate studies. (CPT Donati, CPT Stancer, Elvio Sadun, CPT Stechshulte).

An intriguing observation in other studies of experimental malaria infection is that a higher degree of parasitemia and a more severe clinical course occurs in splenectomized primates infected with P. falciparnum, P. cynomolgi or P. coatneyi. The mechanism responsible is not known; therefore, a systematic evaluation of the spleen in experimental P. cynomolgi infection in Rhesus monkeys has been undertaken.

Initial studies were designed to determine whether unaltered splenic architecture is a prerequisite for the inhibitory effect of the spleen on malaria infection and to correlate antibody production with splenic integrity. Three pairs of Rhesus monkeys were studied. One pair was sham operated; the second pair was splenectomized; and the third pair was splenectomized after which the spleen was minced and reimplanted in the peritoneal cavity. The animals were allowed to recover from these surgical procedures for a ten-day period. A sample of red blood cells was then labeled, in vitro, with ^{51}Cr, heat damaged, and reinjected into the respective donor monkey. Blood-disappearance rates were measured and a scintillation scan of the monkey was performed. Maximum concentrations of radioactivity were found in the spleens of the intact animals and in the liver of the splenectomized animal. In the reimplanted animal, an intermediate distribution of activity between the liver and peritoneal area was observed. In order to test the relative ability of these groups of animals to make antibodies, the animals were inoculated with sheep red blood cells and the hemagglutinating titers were determined serially. Subsequently, the animals were infected with P. cynomolgi, after which serial determinations of the degree of parasitemia and the M and G antibodies to P. cynomolgi were determined by hemagglutination tests. This study is still in progress.

d. Nature of chloroquine-melanin interaction in conjunction with Dr. Martin Rubin. (LTC Swartz, Dr. Rubin)

We have continued to investigate the chemical basis of the known localization of chloroquine in melanin containing structure. One attractive hypothesis is based on the fact that melanin is a stable free radical and chloroquine reacts readily with free radicals. We have found, however, that there is no change in the quantity or quality of the ESR spectra of melanins before and after reacting with chloroquine. Further studies, reacting another stable free radical, DPPH (α,α'-diphenyl-β-picryl hydrazyl) with melanin, revealed that even in its reactions with another free radical melanin's unpaired electron does not interact. This nonreactivity of the unpaired electron of melanin strongly implies that it does not fulfill the biological roles that have been postulated for it.

4. Collaborative efforts.

The following studies, listed by title, are being conducted by the Division of Nuclear Medicine personnel and/or using Division of Nuclear Medicine facilities in collaboration with investigators from other divisions within the WRAIR. Reports of these studies are contained in the annual reports of the other divisions.

a. Division of Medicine:

1) Lactic acid measurements in the Plasmodium knowlesi culture system (LTC Canfield, MC).

2) Amino acid requirements of Plasmodium knowlesi (Dr. Herman Polet).

3) Amino acid patterns in patients infected with malaria (LTC Canfield, MC).

b. Division of Medical Zoology:

1) Immunoresponse of Plasmodium berghei to ^{60}Co gamma radiation (Dr. Sadun, Mr. Wellde).

2) Immunoassay (CPT Stechshulte, MC).

Summary and Conclusions.

The screening system was improved, consolidated and standardized. An information processing system was established and software programming has been initiated. Developmental studies centered on studies of amino acid metabolism and culture requirements of the malarial parasites and on development of radioassay procedures for nucleic acid synthesis. Studies are also being conducted on the interaction of P. berghei with ionizing radiation in relation to clinical aspects of malarial infections. Studies are being conducted using mice, rats and monkeys to observe the distribution, sequestration and blood clearance of Plasmodia in infected animals.

Publications.

1. Angel, C. R., J. I. Davis, K. T. Woodward and D. P. Jacobus, "The Effect of ^{60}Co Gamma Radiation on the Metabolic Activity of Blood Cells Parasitized with Plasmodium berghei," Radiation Research (abstract), 31:616 (1967).

2. Einheber, A., R. E. Wren, H. Rosen and L. K. Martin, "Ornithine Carbamoyl Transferase Activity in Plasma of Mice with Malaria as an Index of Liver Damage," Nature, 215:1489-1491 (1967).

3. Martin, L. K., A. Einheber, E. H. Sadun and R. E. Wren, "Effect of Bacterial Endotoxin on the Course of Plasmodium berghei Infection," Exper. Parasitol., 20:186-199 (1967).

4. Wellde, B. T., C. A. Stancer and R. M. Donati, "Sequestration of 59-Fe Tagged P. berghei Parasitized Reticulocytes," Parasitology, in press (1968).

RESEARCH AND TECHNOLOGY RESUME

1. AGENCY ACCESSION: DA 048934	2. REPORT CONTROL SYMBOL: CSGRD-193				
3. PERIOD COVERED: 01 07 67 TO: B. CHANGE 30 11 67	4. SECURITY: U / U	5. DOWNGRADING: GS	6. DISTRIBUTION LIMITATION: DC	7. LEVEL OF RESUME: A. WORK UNIT	

8. PROJECT NUMBER AND CODE: 63153611 3A835361A829 00 135

9. TITLE: (U) EXPERIMENTAL PATHOLOGY AND PERIPHERAL PERIPHERY IN MALARIA.

10. SCIENTIFIC OR TECH AREA: 52000 BIOLOGY	11. START (FY): 09 66	12. EST. COMPLETION: NA	13. FUNDING AGENCY: OTHER DA	
14. PERFORMING METHOD: C. IN-HOUSE	15. CONTRACT/GRANT: a. NUMBER NA b. TYPE NA c. DATE NA d. AMOUNT NA	16. RESOURCES: PROFESSIONAL MAN-YEARS: 69 / 2 / 30; FUNDING: 69 / 2 / 30		

17. GOVT LAB/INSTALLATION ACTIVITY:
a. NAME: WALTER REED ARMY INST OF RES
b. ADDRESS: WASHINGTON D C 20012

PERFORMING ORGANIZATION:
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19. TECHNOLOGY UTILIZATION: MEDICINE
20. COORDINATION: NA

21. KEY WORDS: PARASITOLOGY, PLASMODIUM, PATHOLOGY, BIOCHEMISTRY, CULTURE, HISTOCHEMISTRY, RETICULOENDOTHELIAL SYSTEM, MALARIA PIGMENT.

22. (U) TECH OBJECTIVE - TO TRACE THE FATE OF MALARIA PIGMENT AND THE PATHOGENESIS OF MORPHOLOGIC CHANGES OF THE LIVER IN MALARIA INFECTED RODENTS. TO STUDY METABOLIC PATHWAYS IN MALARIA PARASITES WITH REFERENCE TO THE EFFECT OF ANTIMALARIALS. TO DEVELOPE A SATISFACTORY METHOD OF ISOLATING, PURIFYING AND FRACTIONATING P. KNOWLESI. TO STUDY BIOCHEMICALLY THE SUBCELLULAR ORGANELLES OBTAINED.

23. (U) APPROACH- SEQUENTIAL MORPHOLOGIC STUDIES IN P. BERGHEI INFECTED HAMSTERS. ISOLATION, FRACTIONATION, PURIFICATION AND IDENTIFICATION OF SUBCELLULAR CONSTITUANTS OF P. KNOWLESI UTILIZING BIOPHYSICAL AND CHEMICAL TECHNIQUES IN CONJUNCTION WITH LIGHT AND ELECTRON MICROSCOPIC STUDIES. RADIO-ISOTOPE STUDIES IN CULTURED P. KNOWLESI.

24. (U) PROGRESS - JUL 67 THRU JUN 68 THE STUDY OF SEQUENTIAL HISTOLOGIC AND ENZYMATIC CHANGES IN LIVER OF HAMSTERS INFECTED WITH P. BERGHEI, A TIME-SEQUENCE STUDY OF THE APPEARANCE AGGREGATION AND DISAPPEARANCE OF MALARIA PIGMENT IN LIVER AND LUNGS AND OF THE FATE OF THE PIGMENT-BEARING MACROPHAGES, AND A STUDY OF RENAL PATHOLOGY IN THE SAME GROUP OF ANIMALS HAVE BEEN COMPLETED. A REACTION INVOLVING THE HEXOSEMONO PATHWAY, KNOWN TO BE PRESENT IN HUMAN LIVER CELLS COULD NOT BE DEMONSTRATED IN P. KNOWLESI INFECTED RED CELLS. THEREFORE, THE PENTOSE-PHOSPHATE PATHWAY OF THIS PARASITE WAS STUDIED. WORK IS IN PROGRESS ON FACTORS AND CONDITIONS INFLUENCING THE ISOLATION OF SUBCELLULAR CONSTITUENTS OF ERYTHROCYTIC STAGES OF P. KNOWLESI. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

25. PROJECT CATEGORY: B. BASIC	26.	27. OSD CODE: 88	28. BUDGET CODE: 1
29. LEVEL OF CONTROL: NA		30. PARTICIPATION: NA	
31. LABORATORY:	32. SPECIAL EQUIPMENT:		
33. PRINCIPAL INVESTIGATOR:	34.		

DD FORM 1498 REPLACES EDITION OF 1 JUN 67 WHICH MAY BE USED (Items 1 thru 24 are identical to AMSC Form 1222)

Project 3A635301D829, MALARIAL PROPHYLAXIS

Task 01 - Malaria Investigations

Work Unit 135, Experimental Pathology and Plasmodial Metabolism in Malaria

Investigators.

Principal: COL Helmuth Sprinz, MC
Associate: Helen R. Jervis, D. Sc.
CPT Donald MacCallum, MSC
CPT Marshall Barnes, MC
CPT Roger Ladda, MC
CPT Robert Cook, MC
Masamichi Aikawa, M. D.

Description.

A multidisciplinary approach was employed in the study of experimental malarial infection and of the metabolism of plasmodia in vivo and in vitro. We were particularly concerned with the effects of antimalarial drugs on parasite morphology and metabolism.

Progress.

A. Influence of antimalarial drugs on the carbohydrate metabolism of P. knowlesi in vitro. Studies have been completed in which the role of the hexose monophosphate shunt in the in vitro parasitized cells of P. knowlesi has been quantitated by isotopic tracer techniques. An alteration of the ratio of this pathway to the Embden-Meyerhoff pathway in total glucose utilization has been found, as a result of the inclusion of methylene blue in the in vitro culture medium in doses which also produce a schizonticidal effect. These findings are being prepared for publication.

B. Isolation, purification, and fractionation of P. knowlesi. In order to further our knowledge of the basic biology and biochemistry of the malarial parasite, it seems desirable to adopt the same approach that has yielded enormous gains in studying cells of all kinds - namely, fractionation into constituent parts and study of these individual parts. To this end the first phase of the present work consists of the development of an isolation technique for ridding the erythrocytic schizonts of P. knowlesi of all contaminating host cell membranes and hemoglobin. Satifactory lysis of the host cells has been obtained using hypotonic phosphate buffer at pH 8.0. Subsequently, centrifugation in a sucrose density gradient achieves removal of large quantities of host cell membranes. Work now in progress entails removal of the smaller remaining quantities of erythrocyte membranes.

After this has been achieved, the isolated parasites will be fractionated by a technique such as the French Press, or repeated

freezing and thawing. The homogenates will be separated by centrifugation in a linear gradient, probably sucrose. Each of the steps outlined in the procedure above is being monitored and studied by electron microscopy of the fractions isolated. Specifically, it is hoped that in this way, whole organelles of the parasite may be isolated, recognized, and studied chemically.

To date, considerable progress has been made in separating cellular contaminants from the parasitized erythrocytes. Fractionation studies are in progress.

C. A study was undertaken to clarify the relationship of pigment formation to chloroquine sensitivity. Young mice were inoculated with P. berghei yoeli, a naturally resistant strain which contains pigment, and P. berghei CR, a strain in which maximal resistance has been induced and which contains little pigment. The results were compared with the highly sensitive strain of P. berghei NYU-2 which forms large quantities of pigment, in regard to 1) actual ability of the parasites to grow over an extended period of time in the presence of chloroquine, 2) uptake of C^{14}-Chloroquine, and 3) presence or absence of pigment before, during, and after the experimental period. The study indicates that chloroquine resistance is probably independent of pigment formation. A manuscript is in preparation.

D. A study of chloroquine effect on the erythrocytic stages of P. gallinaceum showed similarities as well as differences from those reported by us in P. berghei. In both experimental models chloroquine exerts an almost immediate and primary effect on the food vacuoles. In the avian parasite they become enlarged, show subcompartmentalization and contain altered, crystalloid malarial pigment particles and fragments of the parasite cytoplasm. Next the parasite nucleus and nucleolus was affected. This sequence of events following chloroquine administration could be accounted for by an inhibition of DNA and messenger RNA synthesis. A report of this research by Aikawa and Beaudoin, entitled "Effects of Chloroquine on the Morphology of the Erythrocytic stages of Plasmodium gallinaceum," has been accepted for publication by the Am. J. Trop. Med. and Hygiene.

E. The effects of primaquine were studied in the exo-erythrocytic stages of P. fallax maintained in vitro. The principal effect consisted in a marked swelling of parasite mitochondria while those of the host cell remain unaffected. Pentaquine and Isopentaquine appear to act similar to primaquine. This study has been published by Beaudoin, R. L. and Aikawa, M. "Primaquine-induced changes in the morphology of the exo-erythrocytic stages of malaria." Science, 1968. 160:1233-1234.

F. Effects of pyrimethamine were studied in the erythrocytic stages of P. gallinaceum. Light and electron microscopic studies revealed a dissociation of nuclear division from cytokinesis. Nuclear division was

slowed or arrested while merozoite budding proceeded. The nuclear arrest frequently occurred in metaphase. Chromosome-like structures were noted, possibly made visible by the slowing of the mitotic process. A manuscript was prepared by Aikawa, M. and Beaudoin, R. L., entitled "Effects of Pyrimethamine on Nuclear Division of a Malarial Parasite."

G. Our studies on the pathology of experimental P. berghei infection in Syrian hamsters were brought to a successful completion. We studied the changes in kidney, liver, and lungs and the fate of the malarial pigment in the body. The results were incorporated in 5 papers. The first by Sesta et al. has appeared in the A. M. A. Arch. Path. (see Bibliography). The second by Jervis, MacCallum, and Sprinz entitled "Experimental Plasmodium berghei Infection in the Hamster: Its Effects on the Liver," and the third by MacCallum, entitled "Pulmonary Changes Resulting from Experimental Malaria Infection in Hamsters," have been accepted for publication by the A. M. A. Archives of Pathology. Manuscripts 4 and 5 entitled "Time Sequence Study on the Hepatic System of Macrophages in Malaria Infected Hamsters," and "A Study of Macrophage - Pulmonary Vascular Bed Interactions in Malaria Infected Hamsters," both prepared by CPT MacCallum are currently under editorial review.

Summary and Conclusions.

We wish to acknowledge the invaluable collaboration of Dr. H. Pollet and Dr. R. L. Beaudoin. The work produced in the period reported on reflects the training of the staff involved. Unfortunately, a major change-over in personnel is taking place while at the same time the goals of our research demand the application of ever-more sophisticated techniques. Training of personnel will be a very time-consuming process, particularly since one half of the professional and one hundred per cent of the technical staff have to be replaced.

Publications.

1. Macomber, P. B., Sprinz, H., and Tousimis, A. J. Morphological Effects of Chloroquine on Plasmodium berghei in Mice. Nature, 214:937-939, 1967.

2. Sesta, J. J., Rosen, S., and Sprinz, H. Malarial Nephropathy in the Golden Hamster. A. M. A. Arch. Path. 85:663-668, 1968.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6535	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY PRT	WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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10a. CURRENT NUMBER/CODE: 63153011 3A635301D829 00 136

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) METABOLIC AND ENZYMATIC STUDIES OF NORMAL AND MALARIA INFECTED RED CELLS

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY	12 66	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT		18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	a. NUMBER NA	e. DATE NA	PRIOR FY 68	1	30
	b. TYPE NA	d. AMOUNT NA	CURRENT FY 69	1	30

19. GOVT. LAB/INSTALLATION/ACTIVITY

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ASSOCIATE: CANFIELD, LTC C. J.
TEL.: 202-576-3365 TYPE: DA

21. TECHNOLOGY UTILIZATION: METABOLIC STUDIES

22. COORDINATION: NA

23. KEYWORDS: MALARIA, ANTIMALARIALS, PARASITE, RED BLOOD CELL.

(U) TECH OBJECTIVE - DOCUMENT METABOLIC ALTERATIONS OF HUMAN AND ANIMAL RED BLOOD CELLS WHEN INFECTED WITH MALARIA PARASITES AND TO ASSESS THE EFFECT OF ANTIMALARIALS ON THESE ALTERATIONS. TO CHARACTERIZE THE UPTAKE OF ANTIMALARIAL DRUGS ON P. KNOWLESI SCHIZOGONY.

(U) APPROACH- STUDY THE UPTAKE OF CERTAIN AMINO ACIDS BY INFECTED RED BLOOD CELLS, MEASURE FOLIC ACID REDUCTASE IN PARASITE SUSPENSIONS AND DETERMINE THE EFFECT OF PYRIMETHAMINE ON THIS ENZYME, TO MEASURE THE EFFECT OF ANTIMALARIAL DRUGS ON MORPHOLOGIC GROWTH, LACTATE PRODUCTION AND 14-C METHIONINE INCORPORATION IN IN VITRO SCHIZOGONY, AND TO STUDY RED CELL LUX OF SODIUM.

(U) PROGRESS - JAN 67 THRU MAY 68 P. KNOWLESI INFECTED MONKEY RBCS SHOW ELEVATED DISTRIBUTION RATIOS FOR ISOLEUCINE AND METHIONINE, BUT APPARENTLY NORMAL VALUES FOR CYSTINE, LEUCINE, AND HISTIDINE, INCREASES WERE FOUND IN INCORPORATION INTO PROTEIN OF ALL THESE AMINO ACIDS. THE INCORPORATION VALUES WERE HIGHER FOR THE AMINO ACIDS WITH HIGHER DISTRIBUTION RATIOS. PRELIMINARY STUDIES SUGGEST THAT P. KNOWLESI PARASITES ARE UNABLE TO REDUCE FOLIC ACID TO TETRAHYDROFOLIC ACID WHEN SEPARATE FROM HOST RBC. SODIUM INFLUX IS INCREASED IN ERYTHROCYTES FROM MALARIOUS MONKEYS AND COMBINED WITH THE KNOWN SODIUM EFFLUX DEFECT ACCOUNTS FOR THE ELEVATED INTRACELLULAR SODIUM. INCUBATION OF NORMAL CELLS WITH MALARIAL PLASMA INDUCES A SODIUM EFFLUX INHIBITION. THERAPY OF INFECTED MONKEYS WITH CHLOROQUINE REVERSES THESE CATION ABNORMALITIES OVER THE COURSE OF ONE WEEK. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

24. COMMUNICATIONS SECURITY	25.	26. OSD CODE	27. BUDGET CODE
1. COMBAT OR COMBAT RELATED / 2. NOT RELATED		AR	1

28. MISSION OBJECTIVE	29. PARTICIPATION
NA	NA

30. REQUESTING AGENCY	31. SPECIAL EQUIPMENT

32. EST. FUNDS (In thousands)	33.

FORM D JAN 66 1490m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A635301D829 MALARIA PROPHYLAXIS

Task 01, Malaria Investigation

Work Unit 136, Metabolic and Enzymatic Studies of Normal and Malaria Infected Red Blood Cells

Investigators:
Principal: COL Paul E. Teschan
Associates: LTC Craig J. Canfield
CPT Michael J. Dunn
Gerald J. McCormick, Ph.D.
Esther P. Jorolan, Ph.D.

Description.

The objective of this work unit is to study the pathophysiologic alterations induced at a cellular level by malaria infection. The specific areas of study at the present include: 1. Red cell sodium flux; 2. Parasite and red blood cell folic acid reductase; and 3. Amino acid uptake and incorporation by infected red blood cells.

Progress.

1. Cellular function as measured by Cellular Sodium Kinetics

Previous studies have suggested that malarial infection causes changes in RBC membrane permeability and susceptibility to osmotic lysis. In the present study RBC membrane transport of the Na was investigated, in vitro, using cells from rhesus monkeys infected with Plasmodium knowlesi, at low levels of parasitemia (10%). Bi-directional (efflux and influx) movement of radioactive Na was measured using paired control and experimental RBC's. Active Na transport (efflux) was defined by ouabain and ethacrynic acid inhibition. RBC Na concentration (Na_c) was found to be significantly elevated in 37 parasitized animals (21.8 $\pm$ 1.2 mM; mean $\pm$ SEM) as compared to 23 control monkeys (10.0 $\pm$ 0.38 mM) $P < .001$. The Na_c increased with the density of parasitemia and the severity of the infection and reached levels of 40 mM. Parasitized and non-parasitized RBC's from infected monkeys were studied separately after differential centrifugation and these separate fractions showed similar elevations of Na_c. Active Na efflux rate constants were depressed in 8 animals with malaria (.202 $\pm$ .012) as compared to 8 paired controls (.325 $\pm$ .027) P .01. Passive Na influx rate constants were higher in 5 infected monkeys (.037 $\pm$ .012) than in 5 controls (.021 $\pm$ .005) P .01.

Six cross incubation studies, in which normal monkey RBC's were incubated with malarial plasma for 24 hours, showed that malarial plasma induces a 25% inhibition of active sodium efflux. No changes

were observed in passive sodium influx. These experiments gave additional support to the concept of a circulating toxic substance in malaria.

Therapy of P. knowlesi infected monkeys, with chloroquine, causes a prompt increase of RBC potassium within 48 hours and a more gradual decrease of RBC sodium over 96 hours. Spontaneous remissions have also been studied and it has been shown that the return to normalcy of the intracellular environment is a function of the disappearance of the parasites and cessation of the malarial infection.

Experiments with P. falciparum in chimpanzees and P. coatneyi in rhesus monkeys show similar alterations of erythrocytic cation concentration. P. berghei infections in rats and hamsters show lesser changes in these parameters.

Studies are in progress to study the effect of raising the RBC sodium in normal simian RBC's. Preliminary results show a response similar to human cells in which an increase of Na_c causes an increase of total active Na efflux. These results confirm the abnormal situation found in malaria since the elevated Na_c, in this disease, does not stimulate Na efflux. Other experiments are underway to compare the effects of sulfhydryl group inhibition in the erythrocyte to the effect of malaria. It is concluded that the elevated Na_c is the net result of lowered Na efflux and increased Na influx. Malarial infection seems to alter RBC Na transport in all RBC's, parasitized and non-parasitized alike. The plasmodium organism may produce a circulating cellular toxin which adversely affects RBC cation transport.

2. Folic Acid Reductase Studies

A radioassay for folic acid reductase (FAR) using ^{14}C-folic acid as substrate has been carried out. The change to ^{14}C-folic acid from ^{3}H-folic acid was prompted by increased stability of this radiochemical, and increased counting efficiency.

The enzymatic assay used is as follows: to a reaction mixture containing labelled folic acid, TPNH, and 2-mercapto-ethanol were added varying amounts of enzyme in citrate buffer (pH 5.6). The mixture was incubated at 37° C and the reaction was stopped at varying intervals ranging from 0-30 minutes with $Zn\ SO_4$ solution followed by stable folic acid. This precipitated any unreacted folic acid (labelled and unlabelled) thereby leaving the product, tetrahydrofolic acid (THF), in solution. Separation was done by centrifugation at 10000 x g for 10 minutes. An aliquot of the supernatant was digested at 70--80° C with a perchloric acid-hydrogen peroxide mixture and radioactivity was measured by counting in a liquid scintillation mixture containing 7% PPO in toluene and 2-ethoxy ethanol.

The enzyme extracts were prepared as follows: blood from Rhesus monkeys, normal and parasitemic (infected with P. knowlesi), was collected in ice with heparin. Separation of the erythrocytes and the malaria parasites was carried out in the cold (4°C) by a series of centrifugation and washing procedures. The cells (both erythrocytes and parasites) were ruptured by means of a French pressure cell followed by centrifugation. The enzyme fractions were kept cold until ready for assay. All assays were done on the same day the blood samples were drawn.

Results thus far indicate that there is FAR activity in hemolysates of both "normal and parasitized erythrocytes." However, thus far parasite extracts have failed to reduce the labeled folic acid to tetrahydrofolate. Kinetic studies are being done using normal and parasitized fractions to try to obtain the respective Mechaelis constants (Km) and the velocity constants (Vm). Inhibition studies using pyrimethamine (a folic acid antagonist) as inhibitor will also be done on both normal and infected erythrocytes.

3. Red Blood Cell Uptake of Amino Acids:

The uptake from plasma and incorporation into protein of several amino acids by parasitized (P. knowlesi) red blood cells from Rhesus monkeys has been compared to that of normal monkey blood. The amino acids used were isoleucine, methionine, leucine, cystine and histidine. Uptake was measured by the amount of ^{14}C-labeled amino acid leaving the plasma during 30, 60, 90, and 120 minute incubations and expressed as "Distribution Ratio," the ratio of the specific activity in intracellular water to the activity in extracellular water. A ratio greater than unity indicates active transport into the cell, of unity indicates passive diffusion, and less than unity indicates active transport out of the cell. Incorporation into protein was measured as the radioactivity in non-lipid material precipitated by trichloroacetic acid and expressed as specific activity in intracellular water. Activity was found in both the red blood cell hemolysate and the remaining stroma.

Changes in distribution ratios were observed. The greatest change occurred with isoleucine, from unity in the normal to values approximating nine in malarial blood. Methionine showed an increase from 1.3 to three. Cystine changed from 0.5 to approximately unity. Leucine and histidine did not change appreciably from approximately unity.

Incorporation into protein by malarial blood was greatest for isoleucine (150 times normal), followed by methionine (30 times normal), leucine and histidine (16 times normal) and cystine (4 times normal) after two hours of incubation.

4. Antimalarial Drug Screening:

A system for study of antimalarial drugs using in vitro cultures of intraerythrocytic forms of P. knowlesi has been developed and is actively being used for secondary screening. Young ring forms of the parasite introduced into this system will predictably develop into mature schizonts. During this 18 hour growth period, lactic acid is produced and labeled; amino acids are incorporated into trichloracetic-acid-precipitable parasite protein. The effects of drugs on morphologic maturation, lactic acid production and the incorporation of these amino acids have been observed. Dose response curves have been obtained with known antimalarials which correlate well with clinical experience.

Publications.

Dunn, M. J.: Alteration of Red Blood Cell Sodium Transport During Malarial Infection. Clin Res, Jan 68.

Dunn, M. J.: Alteration of Red Blood Cell Sodium Transport During Malarial Infection. Clin Res, Apr 68.

PROJECT 3A025601A830
BIOSENSOR SYSTEMS

Task 01
Biosensor Systems

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. PROJ. CONTROL SYMBOL
			BA 0[illegible]641	CSCRR-[illegible]

5. DATE OF RESUME	6. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 09 67	U U	NA	GA	A. WORK UNIT

10. CURRENT NUMBER CODE: 62156C11 3A025601A830 00 055

10b. PRIOR NUMBER CODE:

11. TITLE: (U) DEVELOPMENT AND EVALUATION OF IMPROVED BIOLOGICAL SENSOR SYSTEMS

12. SCIENTIFIC OR TECH AREA	13. START DATE	14. EXIT COMPL. DATE	15. FUNDING AGENCY
01700 ANIMAL HUSBANDRY 011600 OPERATIONS	09 67	07 72	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST	19. PROFESSIONAL MAN-YEARS	20. FUNDING (Thousands)
C. IN-HOUSE	a. NUMBER NA; b. TYPE NA; c. DATE NA; d. AMOUNT NA	PRIOR FY 68: 3; CURRENT FY 69: 4		200; 350

21. GOVT. LAB INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
NAME: WALTER REED ARMY INST OF RES	NAME: WALTER REED ARMY INST OF RES
ADDRESS: WASHINGTON D C 20012	ADDRESS: DIV OF VET MED, WASHINGTON D C 20012
SP. INDIV.: MERONEY, COL W. H.	INVESTIGATORS PRINCIPAL: CASTLEBERRY, COL M.
TEL.: 202-576-3551	ASSOCIATE: MORRIS, LTC J. H.
	TEL.: 202-576-5375 TYPE: DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
CANINE GENETICS BREEDING	NA

23. KEYWORDS: DOGS, GENETICS, SELECTION.

(U) TECH OBJECTIVE - TO DEVELOP A MORE INTELLIGENT AND SENSUALLY ACUTE DOG WHICH IS PHYSICALLY AND TEMPERMENTALLY BETTER SUITED FOR MILITARY PURPOSES THAN IS NOW GENERALLY AVAILABLE.

(U) 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 WILL BE UTILIZED TO ACCOMPLISH THE OBJECTIVE.

(U) PROGRESS - SEP 67 THRU JUN 68 ALL NECESSARY EQUIPMENT AND VETERINARY MEDICAL SUPPLIES HAVE BEEN ACQUIRED OR ARE ON ORDER. AN OPERATIONAL SITE AT EDGEWOOD ARSENAL HAS BEEN MADE AVAILABLE. ENGINEERING PLANS FOR REQUIRED CONSTRUCTION WERE COMPLETED AND SUBMITTED TO DA FOR ALLOCATION OF URGENT MINOR CONSTRUCTION FUNDS. ALTHOUGH DISAPPROVED FOR FY 68, RESUBMISSION AND ALLOCATION OF FUNDS IS EXPECTED EARLY IN FY 69. REGISTERED BREEDING STOCK WHICH INCLUDES THE GERMAN SHEPHERD, LABRADOR RETRIEVER, STANDARD POODLE, AND THE AIRDALE HAVE BEEN PROCURED. TWENTY-FIVE PUREBRED PUPPIES HAVE BEEN BORN DURING THE PERIOD OF THIS REPORT. THESE ARE NOW UNDERGOING EVALUATION. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

24. COMMUNICATIONS SECURITY	25.	26. OSD CODE	27. BUDGET CODE
☐ a. CONSEL OR COMMAND RELATED ☑ b. NOT RELATED		AR	1

28. MISSION OBJECTIVE	29. PARTICIPATION
CDEG	NA

30. REQUESTING AGENCY	31. SPECIAL EQUIPMENT

32. CST FUNDS	33.

FORM 1 JAN 66 1490m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 25 inclusive to NASA form 1122)

435

ANNUAL RESEARCH PROGRESS REPORT
for FY 1968

Project 3A025601A830, BIOSENSOR SYSTEMS

Task 01, Biosensor Systems

Work Unit 001, Development and evaluation of improved biological sensor systems (Vet Med)

Investigators.

Principle: COL M. W. Castleberry, VC

Associate: LTC John Morris, VC; CPT Jeffrey M. Linn, VC; 1LT Stuart J. Dearing, MSC.

Description.

Studies of behavioral patterns of several breeds of dogs and application of appropriate genetic principles are being made in order to produce a dog better able to detect the presence of the enemy.

Progress.

During the past year, the Biological Sensor Research Team has established a small genetically-sound foundation stock for its selective breeding program. Breeds purchased included the German shepherd (20 F: 6 M), Labrador Retriever (11 F: 3 M), Standard Poodle (10 F: 3 M), Airedale (3F: 1 M), and English Pointer (7 F: 2 M). Individual foundation animals were carefully screened and selected. Purchases were made on the basis of physical requirements, including radiologically-determined freedom from hip dysplasia, and champion line pedigrees. American Kennel Club registrations were required to assure maximum genetic information for the breeding program. Preparation has been made to assure AKC registration of progeny selected for military service or breeding-stock purposes. Several matings within the foundation stock breeds were accomplished, and evaluation of the offspring will begin shortly.

A review of the literature was performed in the area of canine genetics and behavior. In addition, contacts have been made with several professional investigators currently involved in canine behavior and evaluation techniques in order to keep the team abreast of the latest observations and discoveries. Mr. L. Wilson Davis, one of the world's outstanding dog-trainers, is being retained as a consultant.

The scout dog training center at Ft. Benning, Ga., was visited in order to observe training techniques and to determine those areas of deficient performance amenable to improvement.

Standard operating procedures have been established for the puppy testing and evaluation. Plans have been devised for the subsequent care and reinforcement training of puppies required prior to transfer to an appropriate military purpose.

A permanent site for the activities of the Biological Sensor Research Team has been established at Edgewood Arsenal. Three trailers have been purchased for use as temporary operating facilities. Acquisition has been made of necessary medical equipment and drugs. Required kennel runs and dog houses have been procured.

Summary and Conclusions.

The Biological Sensor Research Team has acquired foundation breeding stock and has formulated progeny testing and evaluating procedures. Necessary facilities and equipment have been obtained. Foundation stock matings have been accomplished.

Publications.

None.

PROJECT RD 41-51
NUCLEAR WEAPONS EFFECTS RESEARCH

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CA7473	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	U. CHANGE 01 07 67	U U	NA	CA	A. WORK UNIT

10. CURRENT NUMBER/CODE
NMRR-440 DASA 03.008

10A. PRIOR NUMBER/CODE

11. TITLE (U) DURABILITY OF BEHAVIOR FOLLOWING LETHAL RADIATION EXPOSURE (S)

12. SCIENTIFIC OR TECH AREA	13. START DATE	14. EST. COMPL. DATE	15. FUNDING AGENCY
017100 WEAPONS EFFECTS 013400 PSYCHOLOGY 014100 RADIOBIOLOGY	07 62	NA	NMRR OD

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
C. IN-HOUSE	NA a. NUMBER NA b. TYPE c. DATE NA d. AMOUNT	PRIOR FY 68	1	25
		CURRENT FY 69	1	25

19. GOVT. LAB/INSTALLATION/ACTIVITY
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INVESTIGATORS PRINCIPAL: SHARP, MAJ J. C.
ASSOCIATE: HAWKINS, C. T.
TEL: 202-576-3252 TYPE: DA

21. TECHNOLOGY UTILIZATION
X-RAY EFFECTS

22. COORDINATION
NA

23. KEYWORDS
RADIATION, INCAPACTIATION, BEHAVIOR, PERFORMANCE, PSYCHOLOGY, PRIMATE.

24.

(U) TECH OBJECTIVE - ULTIMATE AIM OF THIS RESEARCH IS THE SPECIFICATION AND TIME COURSE OF THOSE BEHAVIORAL PATTERNS WHICH DETERIORATE FOLLOWING MASSIVE AND LETHAL DOSES OF IONIZING RADIATIONS AND HOW THOSE BEHAVIORS ARE BEST MAINTAINED. THESE ARE NECESSARY AND CRITICAL STEPS FOR AN ANALYSIS OF RADIATION EFFECTS, THE LACK OF SUCH INFORMATION IS IMPEDING ACCURATE MILITARY AND CIVIL DEFENSE PLANNING.

(U) APPROACH- BEFORE CONSIDERING THE EFFECTS OF THE RADIATIONS ON COMPLEX BEHAVIOR PATTERNS IT WAS NECESSARY FOR THIS LABORATORY TO DETERMINE THE PRECISE TIME COURSE OF GENERAL BEHAVIORAL INCAPACITATION THROUGH UTILIZATION OF A HIGHLY MOTIVATED, SHOCK AVOIDANCE TASK. PROCEEDING FROM THIS BASIC DATA, CURRENT WORK HAS FOCUSED ON DEVELOPMENT OF MORE COMPLEX BEHAVIORAL TASKS CALLING FOR A SERIES OF CLOSELY-SPACED, DISCRETE DECISIONS ON VISUAL PATTERN DISCRIMINATION PROBLEMS. MEASURES SUCH AS REACTION TIME, ACCURACY, NATURE OF ERRORS, VISUAL ACUITY, AND DURATION OF EFFECTIVE PERFORMANCE ARE VARIABLES WHICH ARE BEING INVESTIGATED.

(U) PROGRESS - JUL 67 THRU JUN 68 A SERIES OF EXPERIMENTS TO INVESTIGATE AN IMPORTANT MOTIVATIONAL PARAMETER WHICH HERETOFORE HAS NEVER BEEN STUDIED HAS BEEN INITIATED AND COMPLETED. THIS WAS A STUDY TO CIRCUMVENT THE USUAL PROBLEMS ASSOCIATED WITH USING FOOD OR WATER MOTIVATED BEHAVIORS IN RADIATION EXPERIMENTS AND YET USE SOME FORM OF POSITIVE REINFORCEMENT. IN THESE EXPERIMENTS ELECTRICAL SELF-STIMULATION OF THE BRAIN WAS USED TO GENERATE BEHAVIORAL BASELINES WHICH COULD THEN BE COMPARED TO ANIMALS WORKING ON A SIMILAR BEHAVIORAL SCHEDULE BUT MOTIVATED BY SHOCK AVOIDANCE. RESULTS OF THESE EXPERIMENTS SHOW THAT ELECTRICAL SELF-STIMULATION CAN SERVE TO MAINTAIN POST-IRRADIATION BEHAVIORS FAR MORE EFFECTIVELY THAN FOOD OR WATER MOTIVATED BEHAVIORS. HOWEVER, PERFORMANCE MAINTAINED ON SHOCK AVOIDANCE IS MAINTAINED LONGER AND AT HIGHER RATES THAN PERFORMANCE MAINTAINED ON ELECTRICAL SELF-STIMULATION. THE OBVIOUS IMPORTANCE OF MOTIVATIONAL VARIABLES WOULD SEEM TO DICTATE A CAREFUL ANALYSIS OF THE SIMILARITIES AND DIFFERENCES BETWEEN BEHAVIORS MAINTAINED BY SELF-STIMULATION AND THOSE MAINTAINED BY SHOCK AVOIDANCE. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

25. COMMUNICATION SECURITY	26.	27. DOD CODE	28. BUDGET CODE
☐ EXEMPTED ☒ UNCLASSIFIED		AR	1

29. REMARKS (Optional)
CDCG ANNEX I PARA 1

30. PARTICIPATION
NA

31. RESEARCH AGENCY

32. SPECIAL EQUIPMENT

33. PROJECT NUMBER

34.

FORM DA 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to AAFA Form 1129)

Project RD 41-51 NUCLEAR WEAPONS EFFECTS RESEARCH

Work Unit 03.0081 Durability of behavior following lethal radiation exposure

Investigators.

Principal: MAJ Joseph C. Sharp, MSC
Associate: Daryl T. Hawkins, B.S.; John Schrot, M.S.

Description.

The intent of the present series of experiments is a systematic analysis of complex behavioral repetoires following exposure of monkeys to ionizing radiations delivered at very high rates. There are three behavioral experiments being conducted at present; 1) analysis of radiation effects upon a task requiring elements of visual pattern discrimination, vigilance, reaction time, and attention; 2) investigations into the acquisition of a new behavioral task following radiation; and 3) a study designed to determine the efficacy of both positive and negative reinforcers in relation to radiation effects. In general these experiments are designed to use a mixed spectrum source of ionizing radiation delivered in a single pulse to simulate the primary radiation spectrum generated by a nuclear weapon detonation. To date, the general emphasis of work done in this laboratory has been to establish the precise time course of general behavioral incapacitation following exposure to massive doses of ionizing radiation.

Progress.

Those cases where data were derived from the accidental exposure of personnel and from animal experimentation are considered insufficient for forming medical and military opinions of the degree and type of incapacitation to be expected at various times following exposure to lethal amounts of radiation. During the progress of this investigation, facilities for the simultaneous housing and training of ten primates have been constructed at the Behavioral Radiology Laboratory, Forest Glen Section, WRAIR. A close working relationship has been established with the radiation facilities at the Armed Forces Radiobiological Research Institute (AFRRI) in Bethesda, Maryland. Equipment includes automatic timers, counters, programming devices, recorders, and other electronic apparatus for the presentation of environmental events, reinforcement contingencies, and the objective recording of behavioral data.

A previous report from this laboratory (WRAIR Technical Report #3, 1964) described the results from animals exposed to 10,000, 20,000, and 40,000 r (gamma) at 2,000 r/minute. The animals were monitored for behavioral degradation during and following the exposure. It was found the 10K animals performed normally, on the average, for 58 hours, the 20K for 3 hours, and the 40K for 0.10 hours. In a more recent study utilizing the facilities at AFRRI, animals trained in an identical avoidance task as in the above mentioned experiment were exposed to a pulsed, mixed source (60% gamma and 40% neutron). It was found that the 10K group performed normally for an average of 5.17 hours, the 20K group for 2.49 hours, and the 40K group for 0.55 hours (WRAIR Technical Report #4, 1965).

The differential effects obtained raised questions about the mechanism of radiation effects and suggested another experiment. This was an attempt to isolate the radiation variables (i.e., dose rate and radiation spectrum) using the mixed source at 2000 r/minute. Thirty monkeys were thoroughly trained and then exposed at the AFRRI facility. The results of this experiment will be reported in detail in Technical Report #5 which is in preparation. In brief, the results of these experiments indicate that animals exposed to a mixed spectrum perform and live a shorter time than those exposed to only gamma radiations. This was true for animals exposed to a "pulse" or a square wave (i.e., 2,000 rad/minute).

An investigation was conducted in collaboration with the Department of Medicinal Chemistry to assess the effectiveness on primates of a radio-protective compound, n-Decylaminoethanethiosulfuric acid, which had been developed in rodents -- and at supralethal doses in contrast to the LD50/30 criterion used in development. This drug, when injected 30 minutes prior to exposure to 10,000 rads or greater, was effective in the following three ways. 1) It increased survival time, 2) increased the total duration of adequate performance, and 3) completely prevented the period of temporary incapacitation which is of central interest to military planners.

To further assess the effects of ionizing radiation upon complex behaviors, 16 monkeys were trained to perform a matching-to-sample paradigm. Four of these monkeys have been exposed, and the results seem to suggest the vigilance and/or attention is quite resistant to the effects of the radiation. However, reaction times become markedly increased as radiation illness progresses.

A series of experiments utilizing rats to assess the efficacy of motivational sign in relation to radiation exposure has been completed. The results of these experiments indicate that post-radiation performance persists somewhat longer when maintained by shock avoidance than by positively rewarding brain stimulation. However, self-stimulation of the brain maintains post-irradiation performance far longer than food- or water-maintained behavior.

Summary and Conclusions.

The task of developing an objective behavioral program which will enable an assessment of the time course of the debilitating effects of massive doses of ionizing radiations is well under way. Much information has been gleaned from completed studies utilizing a relatively simple behavioral requirement. The present phase has and will continue to generate data on more complex psychological and central nervous system functions. These will include information on vigilance, attention, reaction time, visual and auditory discriminations, motivational variables, and acquisition of new tasks. It was expected at the outset of this phase of experimentation that positive motivation would maintain performance as well as shock avoidance, if the problems of appetitive degradation could be overcome. To a considerable extent this proved to be the case.

Publications.

Sharp, J.C. Critical flicker frequency in albino rats following prenatal X-irradiation. Radiation Res., 1968, 33, 22-29.

Sharp, J.C., Kelly, D.D., and Brady, J.V. The radio-attenuating effects of n-decylaminoethanethiosulfuric acid in the rhesus monkey. Proc. of Symposium of the Use of Subhuman Primates in Drug Evaluation, Univ. of Texas Press, Austin: (In Press).

Kelly, D.D., Sharp, J.C. and Brady, J.V. Manipulating post-irradiation survival times with controlled behavioral environments. Proc. of the IIIrd International Congress of Radiation Research. Cortinal D'Ampezzo, Italy, June-July 1966. G. Silini Ed. North-Holland, Amsterdam; Interscience (Wiley), New York, 1967.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6474	CSCRD-10[illegible]

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 09 67	C. TERMINATED 01 07 67	U U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: NMHR-A4E DASA 03.074

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) BIOLOGICAL RESPONSES TO TOTAL AND PARTIAL BODY IRRADIATION

12. SCIENTIFIC OR TECH. AREA: 014100 RADIOBIOLOGY; 016200 STRESS PHYSIOLOGY; 011500 NUCLEAR WEAPONS E

13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
07 62	NA	MIPR DD

16. PROCURE. METHOD: C. IN-HOUSE

17. CONTRACT/GRANT: a. NUMBER NA; b. TYPE NA; c. DATE NA; d. AMOUNT NA

18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (IN THOUSANDS)
PRIOR FY 67	2	55
CURRENT FY 68	1	25

19. GOV'T. LAB/INSTALLATION/ACTIVITY

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ASSOCIATE: ANGEL, LTC C. R.

TEL.: 202-576-2211 TYPE: DA

21. TECHNOLOGY UTILIZATION: RADIATION BIOLOGY, CIVIL DEFENSE

22. COORDINATION: NA

IRRADIATION, BIOCHEMISTRY, RADIATION INJURY, RADIATION GENETICS, PHYSIOLOGY, RADIATION, PATHOGENESIS.

24.

(U) TECH OBJECTIVE - THE TECHNICAL OBJECTIVE OF THIS WORK UNIT IS THE QUANTITATION OF RADIATION INJURY, ADAPTATION AND RECOVERY.

(U) APPROACH- THE OBJECTIVE WILL BE PURSUED THROUGH THE INTERPLAY OF BIOCHEMISTRY, PHYSIOLOGY AND PATHOLOGY. MULTIPLE PARAMETERS WILL BE USED TO STUDY THE BIOLOGICAL EFFECTS OF RADIATION EXPOSURE.

25. (U) PROGRESS - JUL 66 THRU JUN 67 PROJECT FUNDING TERMINATED 1 SEP 67. RESEARCH WORK UNDER THIS WORK UNIT TRANSFERRED TO PROJECT 3A014501871P, ACCESSION NO. DA 0A6533.

TEXT NOT REPRODUCIBLE

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. REMARKS OR COMMENTS RELATED ☒ b. NOT RELATED		AR	1

31. MISSION OBJECTIVE: CDCG ANNEX I PARA 1

32. PARTICIPATION: NA

33. REQUESTING AGENCY:

34. SPECIAL EQUIPMENT:

35. EST. FUNDS (IN THOUSANDS):

36.

FORM 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 29 identical to NASA Form 1122)

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alter Reed Army Institute of Research
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2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

REPORT TITLE RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES, INCLUDING IOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY, INTERNAL MEDICINE, UCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY EDICINE (U)

DESCRIPTIVE NOTES *(Type of report and inclusive dates)*
nnual Progress Report, 1 July 1967 - 30 June 1968

AUTHOR(S) *(First name, middle initial, last name)*
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b. PROJECT NO.	
c.	9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)*
d.	

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

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U. S. Army Medical Research and Development Command Washington, D. C. 20315

13. ABSTRACT

The various subjects covered in this report are listed in the able of Contents. Abstracts of the individual investigations are ncluded on the DD Form 1498 introducing each work unit report.

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

Unclassified

Security Classification

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